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Cloning of a Pectate Lyase Gene from *Xanthomonas campestris* pv. *malvacearum* and Comparison of Its Sequence Relationship with *pel* Genes of Soft-Rot *Erwinia* and *Pseudomonas*

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The cotton blight pathogen, *Xanthomonas campestris* pv. *malvacearum* strain B414, produces an extracellular pectate lyase (Pel) with an estimated M_r of 41,000 and pI of 9.7. The gene coding for this enzyme initially identified in a 1.8-kb *Pst*I genomic DNA fragment was cloned. The nucleotide sequences of this 1.8-kb fragment and two *pel* genes previously cloned from *Pseudomonas fluorescens* and *P. viridiflava* were determined. These *pel* genes encoded pre-Pel proteins consisting of 377 to 380 amino acids (a.a.). A signal peptide consisting of 26 to 29 a.a. was present at the amino-terminus of each pre-Pel. Multiple sequence analysis revealed that Pel proteins of non-*Erwinia* phytopathogens including *Xanthomonas, Pseudomonas*, and *Bacillus* constituted a distinct cluster, which showed 20 to 43% a.a. identity to the four established Pel families of *Erwinia*. Homologous *pel* sequences were detected in various pathovars or strains of *X. campestris*. All of these xanthomonads produced an alkaline Pel and were capable of causing soft-rot in potato tuber slices and green pepper fruits.

Pectate lyase (Pel) degrades polygalacturonates and other pectic components in plant cell walls and is believed to be the principal pathogenicity factor responsible for tissue maceration caused by most strains of soft-rot bacteria. Pel enzymes produced by soft-rot *Erwinia* have been extensively studied (Collmer and Keen 1986; Kotoujansky 1987). Based on sequence homologies, the *Erwinia* Pels are divided into four families (Barras et al. 1994; Chatterjee et al. 1995; Heffron et al. 1995), which include (i) extracellular PelADE, (ii) extracellular PelBC, (iii) periplasmic Pels, and (iv) *E. carotovora* Pel3. Unlike the complex pectic enzyme system of *Erwinia*, the Pel system of other phytopathogens is in general much simpler. For example, *Pseudomonas viridiflava* (Liao et al. 1988), *Pseudomonas fluorescens* (Liao 1991), *Xanthomonas campestris* pv. *vesicatoria* (Beaulieu et al. 1991), *Bacillus subtilis* (Nasser et al. 1990) and *P. syringae* pv. *lachrymans* (Collmer et al. 1990) have been shown to produce only a single Pel.

Recently, *pel* genes have been cloned from a number of non-*Erwinia* phytopathogens including *P. viridiflava* (Liao et al. 1992), *P. fluorescens* (Liao 1991), *P. syringae* pv. *lachrymans* (Collmer et al. 1990), *X. c.* pv. *campestris* (Dow et al. 1989) and *X. c.* pv. *vesicatoria* (Beaulieu et al. 1991). However, the nucleotide sequences of *pel* genes from non-*Erwinia* phytopathogens are largely unknown. Thus far, only two *pel* sequences originating from non-*Erwinia* phytopathogens have been reported; one from *P. marginalis* (Nikaidou et al. 1993) and the other from *B. subtilis* (Nasser et al. 1993). Due to the limited availability of sequence information, the structural relationship of *pel* genes of non-*Erwinia* phytopathogens has not been thoroughly examined.

Production of pectolytic enzymes has been detected in a number of *X. campestris* pv. *malvacearum* strains previously examined (Dye 1960; Abo-El-Dahab 1964; Venere et al. 1984). However, the type of pectic enzyme produced by these strains has not been definitely determined. Here, we report the cloning and sequence of a *pel* gene from the cotton blight pathogen *X. c.* pv. *malvacearum* and nucleotide sequences of two *pel* genes previously cloned from *P. viridiflava* and *P. fluorescens* in our laboratory. We show that Pel proteins of non-*Erwinia* phytopathogens form a distinct cluster, which exhibits 20 to 40% identity in amino acid (a.a.) sequence to the *Erwinia* Pel families. We also found that a vast majority of the xanthomonad strains that were tested in this study produced an alkaline Pel and were capable of causing soft rot in potato tuber slices and green pepper fruits.

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Nucleotide and/or amino acid sequence data has been submitted to GenBank as accession numbers L38574, L41673, and L38573,

Table 1. Bacterial strains and plasmids used in the study

Designation	Description	Reference or source
Xanthomonas campestris pv. *malvacearum*		
B414	Isolated from cotton by R. N. Goodman (University of Missouri)	C. J. Chang
C	Isolated from cotton in Africa by J.-C. Collin	W. F. Fett
D	Race 2, Isolated from cotton in Texas by L. S. Bird	Gabriel et al. 1986
H	Race 4-2, isolated from cotton in Oklahoma by M. Essenberg	Gabriel et al. 1986
X. c. pv. *campestris*		
Xc-10	Isolated from cabbage in Georgia by R. Gitaitis, original designation GC	W. F. Fett
Xc-11	Isolated from cabbage in Georgia by C. J. Chang, original designation B-31	C. J. Chang
X. c. pv. *vesicatoria*		
T-1	Race 1, isolated from tomato, original designation 75-3	Beaulieu et al. 1991
T-2	Race 2, isolated from tomato, original designation XV56	Beaulieu et al. 1991
X. c. pv. *glycines*		
Xc-7	Isolated from soybean, original designation 42	W. F. Fett
X. c. pv. *phaseoli*		
Xc-6	Isolated from bean, original designation B-496	C. J. Chang
Soft-rotting xanthomonads		
Xc-1, Xc-4	Isolated from rotted tomato and cucumber, original designations TJ071 and CJ092	Liao and Wells 1987
P. viridiflava MI-4	Rif^r Pel$^-$ Mutant of strain SF312	Liao et al. 1988
E. coli DH5α	Subcloning host	Life Technol.
Plasmids		
pLAFR3	IncP Tc^r Cos$^+$ rlx$^+$, cloning vector	Staskawicz et al. 1987
pRK2013	Helper plasmid used for triparental mating	Ditta et al. 1980
pUC19	Ap^r, subcloning vector	Life Technol.
pXCM17 and 18	Primary clones containing the *Pel* gene of *X. c.* pv. *malvacearum*	This study
pXCM189	1.8-kb *Pst*I pel fragment from pXCM17 subcloned into pUC19	This study

RESULTS AND DISCUSSION

Characterization of the *X. c.* pv. *malvacearum* Pel enzyme.

All four strains of *X. c.* pv. *malvacearum* (B414, C, D, and H) examined in the study (Table 1) were pectolytic and capable of causing depression of different degrees in the semisolid pectate (SSP) medium (Liao 1991). In order to study Pel induction in cultures, these strains were grown in the minimal medium MY (Liao et al. 1988) containing polygalacturonate (PGA), glucose, or glycerol (0.25% wt/vol). At the stationary phase, the extracellular Pel activities (= activities in culture supernatants) of strains B414, C, D, and H grown in medium containing PGA were determined to be 15, 13, 2, and 3 units (U) ml^{-1}, respectively. Less than 15% of Pel activity was detected in the periplasmic or cytoplasmic fraction, indicating that the majority of Pel was excreted out of the cells immediately after the synthesis. Very low levels of activities (0.3 U ml^{-1} or lower) were produced when bacteria were grown in media containing glucose or glycerol, indicating that Pel production was inducible by PGA.

For further characterization of the enzyme, the Pel of *X. c.* pv. *malvacearum* was purified from culture supernatants of the highest-yielding strain B414 as previously described (Liao et al. 1988), except that the cellulose phosphate (Whatman P11) column was used to replace DEAE cellulose and the Pel was eluted by phosphate buffer containing 0.25 M NaCl. Purified Pel samples were then analyzed by electrophoresis using pre-made sodium dodecyl sulfate (SDS)–polyacrylamide (12%) gels (Bio-Rad Laboratories, Richmond, CA) and isoelectric focusing (IEF) polyacrylamide gels (PAG plates, pH 3.5-9.5, Pharmacia Biotech Inc., Piscataway, NJ) Results shown in Figure 1A and B indicated that the *X. c.* pv. *malvacearum* B414 Pel was purified to near homogeneity. The M_r and pI were estimated to be 41,000 and 9.7, respectively. The purified Pel readily macerated potato tuber tissue at 20°C; a maceration zone of 10 to 15 mm was observed 1 day after the addition of 0.5 to 1.0 unit of Pel.

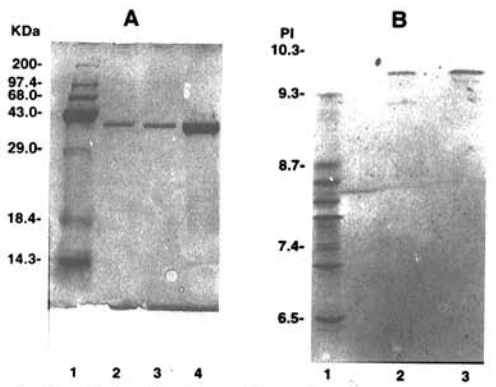

Fig. 1. A, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of purified Pel samples from *Pseudomonas viridiflava* PJ-08-6A (lane 2), *Xanthomonas campestris* pv. *malvacearum* B414 (lane 3), and *Pseudomonas fluorescens* CY091 (lane 4). **B,** Isoelectric focusing gel electrophoresis of purified Pel samples from *X. c.* pv. *malvacearum* B414 (lane 2) and *P. viridiflava* PJ-08-6A (lane 3).

Cloning and expression of the *X. c.* pv. *malvacearum pel* gene.

A genomic library of *X. c.* pv. *malvacearum* B414 was constructed in a cosmid vector pLAFR3 as previously described (Staskawicz et al. 1987). About 1,500 *Escherichia coli* clones were screened for pectolytic activities in SSP medium. None of these clones exhibited visible pectolytic activity in this medium, indicating that the *X. c.* pv. *malvacearum pel* gene in the primary clone may be poorly expressed in *E. coli*. A similar result was reported by Collmer et al. (1990),

who found that the cosmid clone containing the *P. syringae* pv. *lachrymans pel* gene also expressed very poorly in *E. coli*. The poor expression in *E. coli* of the cosmid clones containing the *pel* gene is possibly due to the large size (approximately 18 to 20 kb) of the genomic insert and low copy number of the vector plasmid. Despite this, pLAFR3 derivatives containing the *X. c.* pv. *malvacearum pel* gene appeared to express more efficiently in *P. viridiflava* than in *E. coli*. When the *X. c.* pv. *malvacearum* genomic library was conjugated en mass into the Pel⁻ mutant MI-5 of *P. viridiflava* SF312 (Liao et al 1988), two recombinant clones pXCM17 and pXCM18 that were capable of directing the synthesis of high levels of Pel in mutant MI-5 were isolated. When grown in minimal liquid medium at 28°C for 2 days, mutant MI-5 carrying one of these two clones produced approximately 5 U ml^{-1} of Pel in the culture medium. These two clones were later digested with various restriction enzymes and probed with the cloned *P. viridiflava pel* gene (Liao et al. 1992), the *X. c.* pv. *malvacearum pel* gene was located in a 1.8-kb *Pst*I fragment in pXCM17 and in pXCM18. The 1.8-kb *Pst*I *pel* fragment was then subcloned into pUC19 to yield pXCM189. *Escherichia coli* cells carrying pXCM189 caused deep depression in SSP

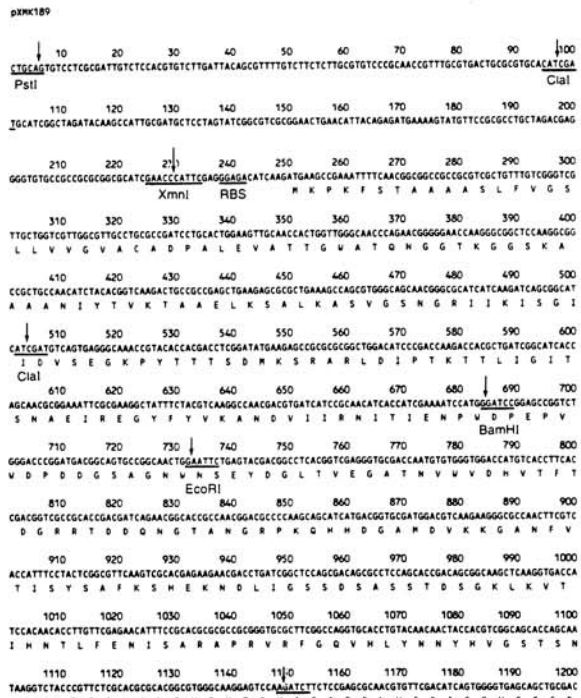

Fig. 2. Nucleotide sequence of the DNA fragment (1,500 base pair region) containing the *Xanthomonas campestris* pv. *malvacearum* B414 *pel* gene and deduced amino acid sequence of the protein product. Restriction sites identified initially during the clonings and subclonings are underlined and indicated by the arrows. RBS = putative ribosome binding site. Two opposing arrows spanning across base no. 1,396 and 1,418 represent the potential transcriptional termination sequence.

medium and produced approximately 7 U ml^{-1} of Pel activity in the minimal liquid medium. When analyzed by SDS-polyacrylamide gel electrophoresis, IEF gel electrophoresis, and enzyme overlay-activity stain, the Pel produced by *E. coli* was found to be similar or identical in M_r and pI to that produced by *X. c.* pv. *malvacearum* (data not shown).

Nucleotide sequence determination.

Nucleotide sequence of the *X. c.* pv. *malvacearum* 1.8-kb *pel* fragment was determined by the dideoxy chain termination method. Analysis of this sequence with the PC/GENE software programs (Intelligenetics Co., Mountain View, CA) revealed an open reading frame (ORF) consisting of 1,131 nucleotides (base no. 250 to 1,380) (Fig. 2). At the 5′ noncoding region two inverted repeats, one from base no. 98 to 109 (CGATGCATCG) and the other from base no. 209 to 220 (CGCCGCGCGGCG), were identified. A potential ribosome binding site (GGAGA, base no. 238 to 242) was located 8 bases upstream of the translational start codon ATG (base no. 250 to 252). The translational stop codon TAA (base no. 1,381 to 1,383) was followed by a Rho-independent transcriptional termination sequence (base no. 1,396 to 1,418). This ORF was predicted to encode a pre-Pel consisting of 377 a.a. A signal peptidase cleavage site located between a.a. no. 26 and 27 was identified by protein sequence analysis program PSIGNAL (PC/GENE) and confirmed by chemical determination (automated Edman degradation) of the leading a.a. at the NH_2-terminus of the mature protein. Nucleotide sequences of two *pel* genes previously cloned from *P. fluores-*

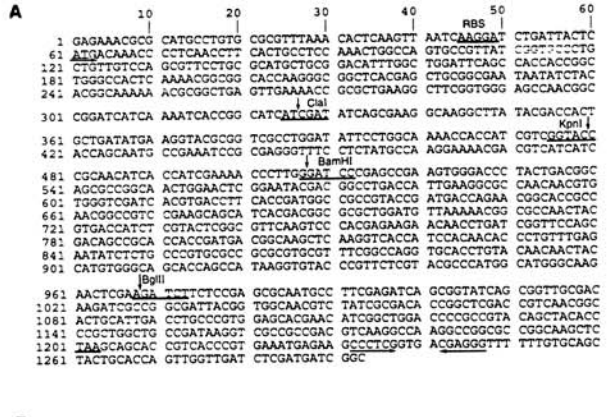

Fig. 3. Nucleotide sequences of two *pel* genes previously cloned from *Pseudomonas viridiflava* SJ074 (Liao et al. 1992) and *Pseudomonas fluorescens* CY091 (Liao 1991). **A,** *P. fluorescens* CY091 *pel*; **B,** *P. viridiflava* PJ-08-6A *pel*. RBS = putative ribosome binding site.

cens and *P. viridiflava* in our laboratory were also determined. A 1.7-kb *Xho*I-*Sal*I DNA fragment containing the *P. fluorescens pel* gene (Liao et al. 1991) was sequenced. The first 1,293 bases from the *Xho*I end of the fragment are shown in Figure 3A. This sequence was predicted to encode a pre-Pel protein consisting of 380 a.a. A signal peptide consisting of 29 a.a was identified initially by computer sequence analysis (PSIGNAL) and later confirmed by direct sequencing (automated Edman degradation) of the leading a.a. at the NH_2-terminus of the mature protein. A potential ribosome binding site (base no. 45 to 49) and a transcriptional termination sequence (base no. 1,232 to 1,247) were also identified (Fig. 3A). Similarly, the nucleotide sequence of a 1.2-kb *Pst*I-*Bgl*II fragment containing the *P. viridiflava pel* gene (Liao et al. 1992) was determined (Fig. 3B). This sequence was found to contain an incomplete ORF (base no. 107 to the end of the fragment) and predicted to encode a functional preprotein consisting of 377 a.a. The M_r of this cloned Pel is close or identical to that of the native Pel of *P. viridiflava* (Liao et al. 1992). This result in combination with the observation obtained from protein sequence alignment (Fig. 4) indicates that the nearly complete *pel* gene of *P. viridiflava* SJ074 is contained within the 1.2-kb fragment. Nucleotide sequences of *pel* genes from *P. viridiflava* SJ074, *P. fluorescens* CY091 and *X. c.* pv. *malvacearum* B414 have been entered into the GenBank, where the accession numbers are L38574, L41673, and L38573, respectively. Deduced a.a. sequences of these three pre-Pel proteins are shown in Figure 4.

Sequence homologies.

Multiple sequence alignment program CLUSTAL (PC/GENE, Intelligenetics Co.) was used to determine the sequence homologies of four pre-Pel proteins from *E. chrysanthemi* (PELE_ERWCH, Keen and Tamaki 1986), *P. fluorescens* (PELF_PSEFL), *P. viridiflava* (PELV_PSEVI), and *X. c.* pv. *malvacearum* (PELX_XANMA) (Fig. 4). Putative signal peptidase cleavage sites at the NH_2-termini of pre-Pel proteins are indicated by the arrows. Over 80% identity in a.a. was observed between Pels originating from members of *Pseudomonas* and *Xanthomonas*. Only 40 to 43% identity in a.a. was observed between PelE of *E. chrysanthemi* and the Pel of *Pseudomonas* or *Xanthomonas*. Four conserved regions suspected of being involved in Ca^{+2} binding, catalytic activities, and protein-export function (Tamaki et al. 1988; Hinton et al. 1989; Barras et al. 1994; Heffron et al. 1995) were also located in the Pels of *Pseudomonas* and *Xanthomonas* (Fig. 4). These four conserved patterns are AxDiKxGxxxVTxS (Region I), vxxRxPxxRxGxxHxxxN (Region II), vWiDH (Region III) and GyatxxxxTxGG (Region IV). In addition, 27 conserved a.a. residues identified previously based on three-dimensional structure of PelC and PelE of *E. chrysanthemi* Ec16 (Henrissat et al. 1995) were also found in Pels of *Pseudomonas* and *Xanthomonas*.

To further compare the sequence relationship of the Pel proteins of non-*Erwinia* phytopathogens with four established Pel families of *Erwinias* (Chatterjee et al. 1995; Heffron et al. 1995; Barras et al. 1994), sequence alignments were extended to include 12 additional Pels of other origins. An alignment dendrogram as depicted in Figure 5 shows that Pels of non-*Erwinia* phytopathogens including *Pseudomonas, Xanthomonas* and *Bacillus* constitute a distinct cluster. This non-*Erwinia* Pel cluster exhibits 35 to 43% a.a. identity to the PelADE (Family I), and 18 to 20% a.a. identity to the PelBC (Family II) and the other two Pel families (Families III and IV). These results indicate that Family I can be further divided into two subfamilies, Family Ia including the PelADE of *E. chrysanthemi* and Family Ib the Pels of non-*Erwinia* phytopathogens (Fig. 5).

Analysis of Pels from other pathovars of *X. campestris*.

Production of pectolytic enzymes is common among pathovars of *X. campestris* (Dye 1960). Although Pel has been assumed to be the principal enzyme produced by most strains of xanthomonads (Starr and Nasuno 1967), only the Pels produced by *X. c.* pv. *campestris* (Dow et al. 1989), *X. c.* pv. *vesicatoria* (Beaulieu et al. 1991) and *X. c.* pv. *malvacearum* (discussed above) have been determined biochemically. To evaluate whether Pel is a pectolytic enzyme coded for by other pathovars (or strains) of *X. campestris,* the cloned 1.8-kb *Pst*I genomic fragment or the 0.5-kb *pel*-specific *Bam*HI-*Bgl*II internal subfragment was used to probe *Pst*I genomic digests prepared from 12 strains of phytopathogenic xanthomonads. *pel* homologs were detected in all 12 strains of xanthomonads included in the study, including four strains of *X. c.* pv. *malvacearum* (B414, C, D, and H), two strains each of *X. c.* pv. *vesicatoria* (T1 and T2) and *X. c.* pv. *campestris* (Xc-10 and Xc-11), one strain each of *X. c.* pv. *gly-*

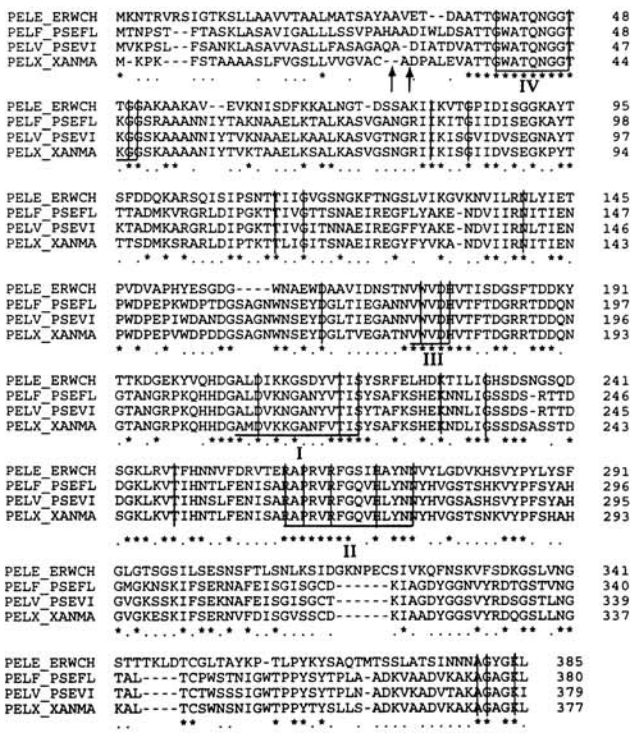

Fig. 4. Multiple sequence alignment of four precursor pectate lyase (Pel) proteins from *Erwinia, Pseudomonas,* and *Xanthomonas*. PELE_ERWCH = *E. chrysanthemi* PelE (Keen and Tamaki 1986), PELF_PSEFL = *P. fluorescens* CY091 PelF (GeneBank accession no. L41673, this study), PELV_PSEVI = *P. viridiflava* SJ074 PelV(GenBank accession no. L38574, this study), and PELX_XANMA = *X. c.* pv. *malvacearum* B414 PelX (GenBank accession no. L38573, this study) * = identical amino acid (a.a.); . = conserved residue. Underlines denote the conserved a.a. regions and vertical lines indicate the conserved a.a. residues. Putative signal peptidase cleavage sites are indicated by the arrows.

cines (Xc-7) and *X. c.* pv. *phaseoli* (Xc-6), and two strains of soft-rotting xanthomonads (Xc-1 and Xc-4) (Fig. 6). A single hybridization band of about the same intensity was detected in the *Pst*I-generated genomic digest of each strain, indicating that *pel* genes are well conserved in all xanthomonads. To determine IEF profiles of Pels produced by different pathovars of *X. campestris*, concentrated culture supernatants were prepared from eight representative strains (or pathovars) and analyzed by IEF gel electrophoresis and overlay enzyme-activity stain (Liao et al. 1988). Figure 7 shows that all eight strains (or pathovars) included in this gel run including *X. c.* pv. *campestris* strain Xc-10 produce a single alkaline Pel of about the same pI. Another *X. c.* pv. *campestris* strain, Xc-11, included in this study was also found to produce a single Pel activity band in the overlay gel (data not shown). Previously, it has been reported that *X. c.* pv. *campestris* strains produced two or more Pel isozymes (Dow et al. 1989; Beaulieu et al. 1991). It is presently unclear if the IEF profiles of Pels produced by *X. c.* pv. *campestris* are varied with the strains. Results presented here and elsewhere by Beaulieu et al. (1991), however, indicate that production of a single Pel appears to be a more common feature among members of *X. c.* pv. *malvacearum* and *X. c.* pv. *vesicatoria*.

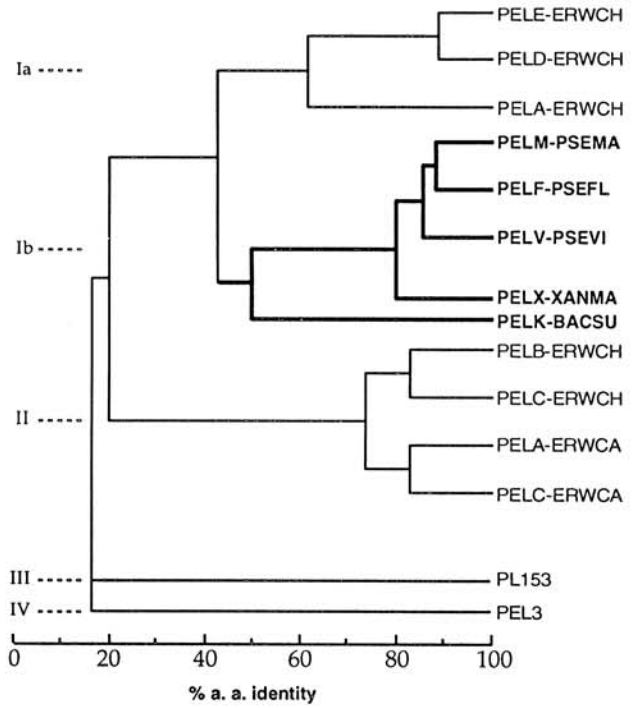

Fig. 5. Sequence relationship of pre-Pel proteins from *Erwinia* and non-*Erwinia* phytopathogens bacteria including *E. chrysanthemi* (ERWCH), *E. carotovora* (ERWCA), *Pseudomonas marginalis* (PSEMA), *P. fluorescens* (PSEFL), *P. viridiflava* (PSEVI), *Xanthomonas campestris* pv. *malvacearum* (XANMA), and *Bacillus subtilis* (BACSU). The scale represents the percent identity in amino acid sequence. The roman numerals shown on the left indicate the designations of gene families. The Pel species examined include PelABCDE of *E. chrysanthemi* (Tamaki et al. 1988; Keen and Tamaki 1986; van Gijsegem 1989), PelA of *E. carotovora* subsp. *carotovora* (Lei et al. 1988), PelC of *E. carotovora* subsp. *carotovora* (Hinton et al. 1989), PelK of *B. subtilis* (Nasser et al. 1993), PL153 of *E. carotovora* subsp. *carotovara* (Trollinger et al. 1989), Pel3 of *E. carotovara* subsp. *carotovara* (Liu et al. 1994), PelM of *P. marginalis* (Nikaidou et al. 1993), and PelF, PelV, and PelX of *P. fluorescens, P. viridiflava,* and *X. c.* pv. *malvacearum* described in this report.

The role of Pel in disease development.

Production of Pels by phytopathogenic xanthomonads usually is not reflected in the disease symptoms caused by these bacteria in the field. Two recent studies have shown that production of Pel by xanthomonads is not essential for the disease development in growing plants. Beaulieu et al. (1991) demonstrated that both pectolytic and nonpectolytic strains of *X. c.* pv. *vesicatoria* were present within this species and that the nonpectolytic activity did not seem to affect its ability to evoke disease symptoms or to induce hypersensitive reactions in nonhost plants. Dow et al. (1989) reported that *X. c.* pv. *campestris* strain 8004 produced three Pel isozymes and that inactivation of one of these isozymes did not alter the black rot development in turnip plants. As described above, four strains of *X. c.* pv. *malvacearum* included in this study produced different levels of Pel activities (2 to 15 U ml^{-1}) in culture media. When the secondary leaves of the susceptible cotton cultivar (Ac44E) were inoculated with these four strains using the methods previously described (Cason et al. 1977), no significant difference in the numbers of disease lesions or the index of disease severity was observed with four different strains (data not shown). Production of high levels of Pel in vitro is therefore not absolutely required for symptom development in growing plants. It should be noted, however, that those strains that did not produce high levels of Pel culture media might do so when grown in plants. Despite extensive efforts, we have been unable to construct nonpectolytic mutants of *X. c.* pv. *malvacearum* by using *Tn5*-mediated marker exchange mutagenesis. It is presently unclear if the loss of pectolytic activity in a highly pectolytic strain of *X. c.* pv. *malvacearum* (such as strain B414) would affect the growth rate of the bacteria or the timing of the symptom development in cotton leaves.

Although phytopathogenic xanthomonads usually do not cause typical soft-rot symptoms in growing plants in the field, it has not been determined if pectolytic strains of xantho-

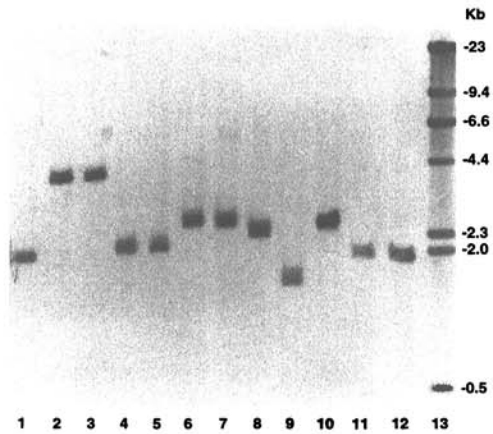

Fig. 6. Detection of *pel* homologs in various pathovars or strains of *Xanthomonas campestris*. *Pst*I-generated genomic digests were hybridized with the cloned 1.8-kb *pel* fragment of *X. c.* pv. *malvacearum*. Lane 1, *X. c.* pv. *phaseoli* (Xc-6); lanes 2 to 3, *X. c.* pv. *campestris* strains Xc-10 and Xc-11; lane 4, *X. c.* pv. *glycines* (Xc-7); lanes 5 to 8, *X. c.* pv. *malvacearum* strains B414, H, D, and C; lanes 9 to 10, *X. c.* pv. *vesicatoria* strains T2 and T1; lanes 11 to 12, soft-rotting strains Xc-4 and Xc-1; and lane 13, digoxigenin-labeled λ *Hin*dIII DNA molecular weight markers.

monads cause soft rot in harvested fruits and vegetables. To evaluate the potential of pectolytic xanthomonads as postharvest pathogens, we determined the tissue-macerating (soft-rotting) ability of these bacteria in potato tuber slices and green pepper fruits using methods previously described (Liao and Wells 1987). Seven strains of xanthomonads including two strains each of *X. c.* pv. *malvacearum* (B414 and D) and *X. c.* pv. *vesicatoria* (T1 and T2), and one strain each of *X. c.* pv. *campestris* (Xc-10), *X. c.* pv. *glycines* (Xc-7), and *X. c.* pv. *phaseoli* (Xc-6) were used. A known soft-rotting strain (Xc-1) isolated previously (Liao and Wells 1987) was also included as a reference. These strains were grown in NYGA medium (Dow et al. 1989) and the cell masses at the late log-phase were suspended in sterile distilled water to make cell densities of approximately 5×10^7 CFU ml^{-1}. Potato tuber slices and bell pepper fruits were inoculated with the cell suspension as previously described (Liao and Wells 1987) and degrees of tissue-macerating ability were determined after 72 h incubation at 20°C. Results summarized in Table 2 indicate that the ability of an *X. campestris* strain to produce Pel is closely associated with its ability to induce soft rot in potato tuber slices or pepper fruits. Pectolytic strains of *X. c.* pv. *malvacearum* and *X. c.* pv. *vesicatoria* (strains B414 and T2) were capable of causing maceration in potato tuber slices and in pepper fruits. However, strains D and T1, which produced very little or no Pel in culture media, were unable to cause soft rot in potato tuber slices or bell pepper fruits. The inability of *X. c.* pv. *phaseoli* to cause maceration was presumably due to its special nutritional requirements for growth in vitro and in planta (Starr and Nasuno 1967). Although *X. campestris* pathovars normally do not cause soft-rot symptoms in growing plants, the results presented here suggest that they do have the potential to act as opportunistic postharvest pathogens causing soft rot in nonhost plants in storage or during transit.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions.

Bacterial strains and plasmids used in the study are listed in Table 1. Luria broth (Life Technologies, Gaithersburg, MD) were used for routine cultivation of both *E. coli* and *Xanthomonas* and *Pseudomonas* strains. When a solid medium was required, Luria agar (Life Technologies), *Pseudomonas* agar F (Difco Lab., Detroit, MI), and peptone–yeast extract–glycerol agar (NYGA) medium (Dow et al. 1989) were used for *E. coli, Pseudomonas* sp., and *Xanthomonas* sp., respectively. For detection of pectolytic activity, bacteria were spotted on a semi-solid pectate (SSP) medium (Liao 1991) and positive reactions was indicated by the formation of pits surrounding the bacterial growth. The minimal medium MY needed for the study of Pel induction was prepared as previously described (Liao et al. 1988). When required, antibiotics were added at the concentrations as previously reported (Liao et al. 1991).

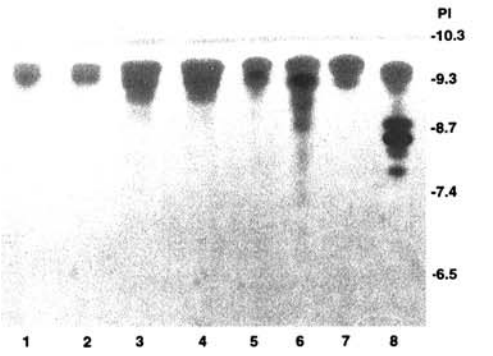

Fig. 7. Isoelectric focusing profiles of Pels produced by various pathovars of *Xanthomonas campestris* and soft-rotting bacteria as determined by the overlay enzyme-activity stain. Lane 1, *X. c.* pv. *glycines* (Xc-7); lane 2, *X. c.* pv. *vesicatoria* (T1); lane 3, *X. c.* pv. *malvacearum* (B414); lane 4, soft-rotting strain Xc-1; lane 5, *X. c.* pv. *campestris* (Xc-10); lane 6, *P. fluorescens* CY091; lane 7, *Pseudomonas viridiflava* PJ-08-6; and lane 8, *Erwinia chrysanthemi* EC16.

Enzyme assays.

Pel activity was assayed in a 0.5-ml volume containing 100 mM Tris-HCl (pH 8.0), 1 mM $CaCl_2$, 0.2% (wt/vol) PGA, and enzyme sample. One unit of activity was defined as the amount of enzyme that caused an increase of 1.0 absorbance unit per min at 232 nm and 20°C. An increase of 1.73 absorbance unit was considered to generate 1 μmole of unsaturated uronide (Liao et al. 1988). Activities in the subcellular fractions were determined in accordance with the methods previously described (Liao 1991). The protein concentrarion was assayed based on the Bradford's procedures included in the Bio-Rad protein assay kit. The enzyme was purified from the culture supernatant following the procedures previously described (Liao et al. 1988), except that DEAE cellulose was replaced by cellulose phosphate (Whatman P11) and the pel was eluted using the 0.1 M phosphate buffer (pH 7.2) containing 0.25 M NaCl (L. J. C. Wong, manuscript in preparation).

Gel electrophoresis and overlay enzyme-activity stain.

SDS-polyacrylamide gel electrophoresis, ultrathin-layer IEF gel electrophoresis, and overlay enzyme-activity stain

Table 2. Comparison of pectolytic and tissue-macerating abilities in various strains or pathovars of *Xanthomonas campestris*[a]

Strains	Pel activity (unit ml^{-1})[b]	Maceration on potato tuber[c]	Maceration on pepper fruit[d]
X. c. pv. *malvacearum*			
B414	14.1	5.0	15
D	1.0	ND[e]	ND
X. c. pv. *vesicatoria*			
T2	12.2	4.0	2
T1	0.2	ND	ND
X. c. pv. *campestris*			
Xc-10	13.7	5.0	17
X. c. pv. *glycines*			
Xc-7	3.2	3.5	10
X. c. pv. *phaseoli*			
Xc-6	4.1	ND	ND
Soft-rotting xanthomonad			
Xc-1	14.1	5.0	13

[a] The value represents an average of two experiments, two duplicates an experiment.
[b] Pel activities in culture supernatants. One unit of activity is defined as the amount of the enzyme that causes an increase of 1.0 absorbance at 232 nm at 20°C per min.
[c] Maceration index was judged on an arbitrary scale of 0 to 5 representing 0, 20, 40, 60, 80, and 100% degree of maceration (Liao and Wells 1987).
[d] Maceration zone (or lesion) was measured in mm diameter.
[e] Not detected

were performed according to the previously described procedures (Liao 1989). The enzyme sample containing 4 to 6 μg of protein was added to each well. After electrophoresis, protein bands were stained with Coomassie Brilliant Blue R250 and their molecular weights estimated by the molecular weight standards (Life Technologies) included in the run. For analysis of IEF profiles of Pels produced by various strains or pathovars of *X. campestris*, 3 to 10 μl of concentrated culture supernatants containing 0.3 to 1.5 U of Pel activity was added to the gel. Following electrophoresis, the IEF gel was overlaid onto the agarose-pectate gel and incubated at 28°C for 2 h. After that, the agarose-pectate gel was submerged in 1% mixed alkyltrimethly ammonium bromide and activity band visualized by the formation of a clear zone.

Pathogenicity assays.

The ability of bacterial strains to macerate plant tissue was tested on potato tuber slices and detached bell pepper fruits as previously described (Liao and Wells 1987). The cotton plants susceptible to various races of *X. c.* pv. *malvacearum* were grown in 20-cm-diameter clay pots containing sterile soil/vermiculite/peat (3:1:1) mixture in a controlled environment chamber. For virulence assays, bacterial inocula were prepared (Venere et al. 1984) and secondary leaves were inoculated with bacterial inocula in accordance with the procedures previously reported (Cason et al. 1977).

Recombinant DNA techniques.

Standard procedures (Sambrook et al. 1989) were used for isolation of chromosome and plasmid DNA, preparation of genomic library, cloning, subcloning, and restriction analysis of cloned genomic DNA fragments. Conjugational gene transfer and triparental matings were conducted according to the procedures previously described (Liao et al. 1994). DNAs were labeled and detected nonradioactively using the Genius DNA Labeling and Detection Kit purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Southern hybridization analyses were performed according to the published procedures (Sambrook et al. 1989).

DNA and protein sequence analysis.

Plasmids pSJB720, pROTM2, and pXCM189 containing the *pel* gene of *P. viridiflava* (Liao et al. 1992), *P. fluorescens* (Liao 1991), and *X. c.* pv. *malvacearum* (this study), respectively, were constructed as described here or earlier. Sequencing was performed by the dideoxy chain termination methods on double-stranded plasmid templates using Sequenase version II of United States Biochem. Corp. (Cleveland, OH). DNA and protein sequence data were analyzed using the PC/GENE DNA and protein sequence analysis programs (release 6.0, Intelligenetics Inc.).

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