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Structure and Organization of the *pel* Genes from *Erwinia chrysanthemi* EC16

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The *pelA* and *pelC* genes from *Erwinia chrysanthemi* EC16 were sequenced and overexpressed in *Escherichia coli* cells. These genes and two others from the same strain that were characterized previously encode catalytically related pectate lyase proteins that are involved with the maceration and soft-rotting of plant tissue. The *pel* genes of strain EC16 were organized as two loosely linked clusters, with two structurally homologous genes in each. The *pelA/E* cluster also contained the remains of an additional *pel* gene, the 5′ portion of which had been removed by a prior deletion event. Each of the four functional *pel* genes but not the deleted one contained an efficient rho-independent transcriptional terminator after the translational stop. These and other data indicate that the *pel* genes are all independently regulated despite their structural homology and tandem clustered organization. Two of the genes, *pelA* and *pelE*, encoded proteins that differed greatly in their isoelectric points and ability to macerate plant tissue. A recombinant gene constructed with the 5′ portion of *pelE* and the 3′ portion of *pelA* yielded a chimeric protein with high pectate lyase activity but relatively low maceration activity. This result raised the possibility that the poor maceration ability of the *pelA* gene product may involve other properties in addition to its low isoelectric point.

Substantial evidence (4, 5, 13) has established that the production of several pectate lyase (PL) proteins is causally involved in the soft-rotting disease of plant tissue caused by *Erwinia chrysanthemi*. Furthermore, high-level production of the *E. chrysanthemi* PLe protein enabled *Escherichia coli* cells to efficiently macerate potato tuber tissue (12, 19). *Erwinia* spp. secrete several additional enzymes that attack higher-plant cell walls or membranes. These include xylanase, cellulase, protease, phospholipase, pectin lyase, and pectin esterase, but their role in pathogenicity has not yet been established (13).

Most strains of *E. chrysanthemi* studied to date produce five different PLs encoded by unique *pel* genes (14), but Barras et al. (2) demonstrated that strain EC16 produces only four different PL proteins. The *pel* genes encoding these proteins have been found to occur in two clusters on the *E. chrysanthemi* EC16 chromosome (1, 2, 11, 12). We initially isolated two different cosmid clones, pPEL3 and pPEL7, which encoded different PL proteins (11). It was subsequently shown that cosmid clone pPEL3 encoded two different PL proteins, but a 6.6-kilobase (kb) subclone (pPEL34) contained only one gene (2; Thurn and Chatterjee, personal communication; Lei and Wilcox, personal communication). We previously sequenced one of these genes (*pelB*) as well as the 3′ portion of a closely linked gene, assumed to be *pelC* (12). In this paper we report the full sequence of the *pelC* gene.

Plasmid pPEL74, a subclone of cosmid pPEL7 (11), was also found to contain two different *pel* genes (2; Collmer, personal communication). We previously sequenced one of these genes, *pelE*, and overexpressed it in *Escherichia coli* (12). In this study we subcloned and sequenced the *pelA* gene, which is closely linked to *pelE* in pPEL74. Despite considerable homology, the isoelectric points of the protein products encoded by these genes are considerably different (pH 4.6 for PLa and pH 9.8 for PLe). The *pelA* protein is also ca. 1,000 times less efficient in maceration of plant tissue than the *pelE* protein. In order to determine whether this difference was due to the isoelectric point or to some other feature of the proteins, we constructed recombinant genes and tested the resultant proteins for maceration activity in plant tissue.

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MATERIALS AND METHODS

Recombinant DNA methods. The *E. coli* strains, phages, and plasmids used are shown in Table 1. Plasmid constructs were generally made by the soft agarose cloning method of Crouse et al. (6). DNA-modifying enzymes were purchased from New England Biolabs, Bethesda Research Laboratories, or Boehringer Mannheim. Transformation of *E. coli* cells and miniboil plasmid extractions were done as described previously (12). Large-scale plasmid isolations were generally done by the alkaline lysis method (16).

DNA sequencing. A series of exonuclease III (ExoIII)/S1 nuclease deletions were made on both orientations of DNA fragments cloned in pUC118 or pUC119 by the method of Henikoff (8). Following plasmid religation, deletions were transformed into strain DH5α. Appropriate deletions were selected and transformed into *E. coli* MV1193, which was then transfected with lambda M13K07, and templates of single-stranded plasmid DNA were isolated (27). These templates were sequenced by the dideoxy method as described previously (12). Data were analyzed by the BIONET system (supplied through Intelligenetics Corp., Mountain View, Calif.) or by the programs of Pustell and Kafatos (20).

Cell culture and plant maceration assays. *E. coli* cells carrying various plasmids were grown to the stationary phase at 28°C in 15 ml of LB medium with the appropriate antibiotics and additives. Cells were recovered by centrifugation, and periplasmic fractions were prepared as described previously (11). Plant tissue maceration assays were performed with cucumber fruit mesocarp tissue as described previously (24).

Analytical techniques. Sodium dodecyl sulfate (SDS)-polyacrylamide gels of whole *E. coli* cells were run as described previously (12). Thin-layer electrofocusing of PLs from *E. coli* periplasmic fractions and polygalacturonate overlay as-

TABLE 1. Bacterial strains, phage, and plasmids

Strain, phage, or plasmid	Description	Source or reference
E. coli		
DH5α	*endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1* λ^- *recA1 gyrA relA1* φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)*U169*	Bethesda Research Laboratories
MV1193	Δ(*lac-proAB*) *thi rpsL endA sbcB15 hspR4* Δ(*srl-recA*)*306*::Tn*10* (Tet^r) [F'::*traD36 proAB lacI*^{q}ZΔ*M15*]	Messing, unpublished
HB101	F$^-$ *hsdS20* (*hsdR hsdM*) *recA13 ara-14 proA2 lacY1 galK2 rpsL20* (Str^r) *xyl-5 mtl-1 supE44* λ^-	16
D1210λ	HB101 (*lacI*^{q} y$^+$) [λ *xis* (Δ*Sal*I-*Xho*I)*Kil cI857*]	7
BMH71-18	(Δ[*lac pro*] F' *lacI*^{q}Z*ΔM15 pro*$^+$)	23
Phage lambda M13K07		27
Plasmids		
pBR322	Cloning plasmid	16
pUC18 and pUC19	Cloning plasmid	29
pUC118 and pUC119	Plasmid for production of single-stranded DNA	27
pBluescript KS	Cloning plasmid	Stratagene
pJRD184	Cloning plasmid	9
pINK1	Expression vector	12
pNH8a	Expression vector	7
pUR290	Plasmid containing *lacZ* with a polylinker site at the 3' end of the gene for construction of gene fusions	23
pPEL3	pHC79 cosmid clone containing *pelB* and *pelC*	11
pPEL401	8.2-kb *Cla*I fragment from pPEL3 cloned into pBR322 and containing the *pelB* and *pelC* genes	This paper
pPEL402	3.2-kb *Cla*I-*Ava*I fragment from pPEL401 cloned into the same sites of pBR322 and containing only *pelC*	This paper
pPEL403	3.2-kb *Cla*I-*Xho*I fragment from pPEL401, containing the *pelC* gene, cloned into the *Cla*I-*Xho*I sites of pBluescript KS (anti orientation to the vector *lac* promoter) (PL positive)	This paper
pPEL405	1.4-kb *Sac*I-*Ava*I fragment of pPEL401 inserted (after end-filling both termini with Klenow fragment) into the *Hin*cII site of pUC119 (anti orientation to the vector *lac* promoter) (PL positive)	This paper
pPEL406	1.4-kb *Hin*dIII-*Eco*RI insert of pPEL405 inserted into pUC119 in the opposite orientation (in the correct orientation behind the vector promoter) (PL positive)	This paper
pPEL407	1.0-kb *Pst*I fragment of pPEL405 cloned into pUC118 (PL negative)	This paper
pPEL410	*pelC* expression plasmid containing a 1.2-kb fragment obtained from pPEL406; an ExoIII sequencing deletion including 41 bp of DNA 5' to the translational start codon and extending to the filled *Ava*I terminus was removed from the pPEL406 deletion clone with *Hin*dIII and *Eco*RI and ligated into the same sites of pINK1 (PL positive)	This paper
pPEL413	*pelC* expression plasmid; a 1.4-kb *Bam*HI-*Sph*I fragment from pPEL406 was cloned into the same sites of pNH8a (in the correct orientation following vector promoter inversion)	This paper
pPEL74	8.2-kb *Pst*I fragment containing *pelA* and *pelE*, cloned in pBR329	11
pPEL7421	1.2-kb *Eco*RI-*Sal*I fragment carrying *pelE* only, cloned in the orientation downstream of the promoter in pUC8	12
pPEL7422	Same construction as pPEL7421, except cloned in the opposite orientation to the promoter in pUC19	This paper
pPEL712	pPEL 7421 with deletion of a 360-bp internal *Eco*RV fragment (PL negative)	12
pPEL743	2.0-kb *Hin*dIII-*Sal*I fragment from PEL74 cloned into pUC19; contains *pelE* and 5' DNA	12
pPEL748	Translational fusion containing the *pelE* gene from pPEL7421 fused to pINIII	12
pPEL760	1.2-kb *Eco*RI-*Sal*I insert fragment from pPEL7421 blunted with S1 nuclease and recloned into the *Sam*I site of pUC119, in the orientation downstream from the vector promoter (PL positive)	This paper

Continued on following page

TABLE 1—*Continued*

Strain, phage, or plasmid	Description	Source or reference
pPEL770	372-bp internal *Eco*RV fragment from pPEL810 inserted into the *Eco*RV site of pPEL712 in the correct orientation to maintain reading frame integrity (PL negative)	This paper
pPEL780	Recombinant *pelEA* gene consisting of the 5′ region of *pelE* to the *Spl*I site and a portion of the *pelA* gene 3′ to this site; constructed by removing a 380-bp *Spl*I-*Sst*I fragment from pPEL743 and replacing it with the corresponding 620-bp fragment from pPEL810 (PL positive)	This paper
pPEL781	Expression construct of the recombinant *pelEA* gene in pPEL780; a ca. 1.4-kb *Eco*RI-*Hin*dIII fragment from pPEL780 was cloned into the same sites of pINK1	This paper
pPEL785	*Pst*I-*Spl*I fragment from pPEL760, encoding the 5′ end of *pelE*, cloned into the same sites of pPEL810 (PL positive)	This paper
pPEL801	3.2-kb *Mlu*I fragment from pPEL74 containing *pelA*, cloned into the *Mlu*I site of pJRD184	This paper
pPEL802	Reverse orientation of pPEL801	This paper
pPEL803	1.2-kb *Hin*dIII fragment from pPEL801 cloned in the orientation downstream from the vector promoter in pUC119 (PL positive)	This paper
pPEL804	Reverse orientation of pPEL803 (anti-promoter) (PL negative)	This paper
pPEL810	1.4-kb *Hin*cII-*Bgl*II fragment from pPEL802 cloned into the *Hin*cII-*Bam*HI sites of pUC19 (in the orientation downstream from the vector promoter)	This paper
pPEL811	Same construction as pPEL810, except cloned into pUC18	This paper
pPEL812	*pelA* expression plasmid; a 1.4-kb *Hin*cII-*Sst*I fragment from pPEL810 cloned into the *Sma*I-*Sst*I sites of pINK1 (in the orientation downstream from the vector *lac* promoters)	This paper
pPEL819	pPEL810 with a 372-bp *Eco*RV internal fragment deleted (PL negative)	This paper
pPEL820	360-bp internal *Eco*RV fragment from pPEL7421 inserted into the unique *Eco*RV site of pPEL819 in the correct orientation to maintain reading frame integrity (weakly PL positive)	This paper
pPEL822	Recombinant gene including 5′ DNA of *pelA* to the *Spl*I site and 3′ DNA of *pelE* after this site; constructed by removing a ca. 620-bp *Spl*I-*Sst*I fragment from pPEL810 and replacing it with the corresponding ca. 380-bp fragment from pPEL743 (PL negative)	This paper
pPEL824	*Pst*I-*Spl*I fragment from pPEL810, containing the 5′ end of *pelA*, inserted into the same sites of pPEL760, containing the 3′ end of *pelE* (PL negative)	This paper
pPEL841	*Hin*dIII fragment from pPEL810 cloned in the correct orientation in the *Hin*dIII site of pUR290 to generate a *lacZ-pelA* fusion protein (PL positive)	This paper
pPEL843	*Hin*dIII fragment from pPEL822 cloned in the correct orientation in the *Hin*dIII site of pUR290 to generate a *lacZ-pelA-pelE* fusion protein (PL negative)	This paper

says were done essentially by the methods of Ried and Collmer (22).

RESULTS

Subcloning of *pelC*. Previous sequence data suggested that *pelC* occurred 5′ to *pelB* in the original cosmid clone pPEL3 (12) but that the *Pst*I site used to construct subclone *pelB* in pPEL34 occurred within *pelC*. The *pelC* gene was therefore subcloned from pPEL3. Initially, an 8.2-kb *Cla*I fragment from pPEL3 was subcloned into pBR322 to yield pPEL401 (Table 1). This clone contained both *pelB* and *pelC*. A 3.2-kb *Cla*I-*Ava*I fragment gave only PLc activity in *E. coli* when subcloned into the same sites of pBR322 (pPEL402). The *pelC* gene was further subcloned as a 1.4-kb *Sac*I-*Ava*I fragment that was PL positive in both orientations when subcloned into pUC119 to generate pPEL405 and pPEL406.

TABLE 2. PL produced in the periplasmic space of *E. coli* cells carrying various *pel* gene constructs

Plasmid	Gene	PL activity[a] (U/g [fresh wt] of cells)	
		Uninduced	Induced
pPEL401	*pelC*	50	136
pPEL402	*pelC*	170	150
pPEL403	*pelC*	1,200	1,040
pPEL410	*pelC*	1,490	3,500
pPEL413	*pelC*	20	2,400
pPEL801	*pelA*	3	4
pPEL803	*pelA*	13	22
pPEL804	*pelA*	<0.1	<0.1
pPEL810	*pelA*	210	290
pPEL812	*pelA*	1,470	2,925
pPEL748	*pelE*	1,770	—[b]
pPEL781	*pelEA*	1,430	5,600
pPEL822	*pelAE*	<0.1	<0.1
pPEL841	*lacZ-pelA*	—	2,150[c]
pPEL843	*lacZ-pelAE*	—	<0.1[c]

[a] DH5α cells were used in all experiments with the following exceptions: pPEL413, strain D1210λ; pPEL748, HB101; and pPEL841 and pPEL843, strain BMH71-18. All cultures were grown at 28°C for ca. 20 h before spheroplasting was done to obtain the periplasmic fraction (12). IPTG was used for induction and added to 1 mM at culture initiation; IPTG was added to all cultures containing pPEL413, and induction in this case was achieved by incubation at 42°C for 15 min when cultures had attained an A_{600} of ca. 0.7. Plating of cells from these cultures on pectate plates showed that >95% were induced to produce high levels of PL activity. PL activities were assayed in the culture medium and cellular fractions as done previously (12). Since these fractions each contained 10% or less of the total PL activity in all cases, only data for the periplasmic fractions are reported.

[b] —, Not determined.

[c] Whole cells were lysed by sonication and used for the assay in these cases.

Overexpression of the *pelC* gene in *E. coli*. In order to overexpress the *pelC* gene, various expression constructs were made. As noted in Table 2, *E. coli* cells carrying pPEL401 or pPEL402 produced only low levels of PL activity, but a 3.2-kb subclone, pPEL403, produced higher levels. This plasmid was constructed by cloning the insert DNA in the opposite orientation from the *lac* promoter of pBluescript KS (Table 1). This result suggested that the *pelC* promoter was active in *E. coli* because, as expected, cells carrying pPEL403 did not exhibit increased PL production in the presence of IPTG (isopropyl-β-D-thiogalactopyranoside) (Table 2). pPEL410, an expression construct in pINK1, contained only 41 base pairs (bp) of DNA 5′ to *pelC* and resulted in high PLc production in the presence of IPTG. However, as previously observed with other constructs in the pINK1 expression plasmid (12), the uninduced level was also quite high (Table 2). Construct pPEL413 was constructed with a reversible promoter vector, pNH8a. Expression of *pelC* by pPEL413 in the uninduced state was considerably lower than that by the pINK construct, pPL410 (Table 2). The induced level of PLc in cells containing pPEL413 was nearly as high, however, as in those containing pPEL410. Thus, pNH8a resulted in approximately 100-fold induction. PL activity was efficiently secreted to the periplasmic space in all cases (Table 2).

Subcloning of *pelA*. Subcloning disclosed that an additional *pel* gene occurred 5′ to *pelE* on pPL74. This gene, presumed to be *pelA*, was initially subcloned as a 3.2-kb *Mlu*I fragment which expressed PL activity in both orientations when cloned into the *Mlu*I site of pJRD184 to yield pPEL801 (Tables 1 and 2). Further subcloning showed that a 1.2-kb *Hin*dIII fragment from pPL801 gave low PL activity when cloned downstream from the promoter in pUC19 (pPEL803) but yielded no detectable activity when cloned in the opposite orientation (pPEL804; Table 2). This was later found to be due to the construction of a translational fusion in pPEL803, since the 5′ *Hin*dIII site occurs inside the ATG start codon (see Fig. 4).

High-level expression of the *pelA* gene in *E. coli*. A convenient *Hin*cII site just 5′ to the Shine-Dalgarno box of *pelA* was located from sequencing data (see below) and used to construct pPEL810 in pUC19. This construct produced moderate PL activity in *E. coli* (Table 2). The insert was also transferred to pINK1 to yield pPEL812. This construct led to high PLa activity in the periplasmic space of *E. coli* cells when IPTG was supplied (Table 2). Significant expression (ca. 50% of the induced level) occurred in the absence of IPTG.

Sequencing of *pelC*. The 1.4-kb insert in pPEL405 and pPEL406 was sequenced to identify the *pelC* gene (Fig. 1). As expected, a single open reading frame (ORF) was identified that exhibited considerable homology with the *pelB* gene. As noted previously (12), *pelC* contains a sequence after the translational stop which would be expected to function as an efficient transcriptional terminator. The putative signal peptide sequence of *pelC* also showed considerable homology with that of *pelB* (Fig. 2), but the *pelC* cleavage site has not been definitely assigned by N-terminal amino acid sequencing of the mature protein. Significant overall homology did not occur between the 5′ noncoding DNA of *pelC* and that of *pelB*, but possible promoter elements were identified in both genes (Fig. 3). The *pelC* sequence, however, exhibited less similarity to the *E. coli* consensus promoter. Unlike *pelB* (12), no identifiable catabolite repressor-binding site was present in the 5′ DNA of *pelC*. The predicted molecular weight of the PLc preprotein was 39,923 and that of the mature protein was 37,676. This corresponds to values of 40,213 and 37,922, respectively, for PLb, determined previously (12). The proteins encoded by *pelB* and *pelC* had 84% amino acid identity, with two compensatory single amino acid deletions occurring in what were otherwise colinear reading frames (Fig. 2).

Sequencing of *pelA* and detection of a deleted *pel* gene. The 3.2-kb insert of pPEL801 contained a single long ORF corresponding to *pelA*, located ca. 900 bp 5′ to the start of the *pelE* gene (Fig. 4). No additional ORFs of significant length were observed in either strand of the ca. 1 kb of DNA 5′ to *pelA*. A Shine-Dalgarno box occurred just before the assumed start codon of the *pelA* gene at position 1099. The 5′ ends of the *pelA* and *pelE* coding regions were dissimilar, and the *pelA* gene product had a longer putative signal peptide sequence than that of *pelE* (Fig. 5). However, the predicted cleavage site of the PLa preprotein has not yet been confirmed by N-terminal amino acid sequencing of the mature protein. The preprotein encoded by *pelA* had 393 amino acids and a calculated molecular weight of 42,077. The putative mature protein contained 361 amino acids with a calculated weight of 38,756. The *pelA* gene product also had more amino acids at the amino-terminal end of the mature protein than PLe (Fig. 5). Although several short compensatory deletions occurred in the remainder of the coding regions of *pelA* and *pelE*, the mature proteins had 62% amino acid identity and read colinearly (Fig. 4 and 5). Both genes terminated with TAA stop codons. There was a relatively large amount of intergenic DNA between the ORFs constituting *pelA* and *pelE* (ca. 900 bp), and the 5′ untranslated ends of both genes had unusually long stretches of AT-rich DNA (e.g., base 830 to the translational start at

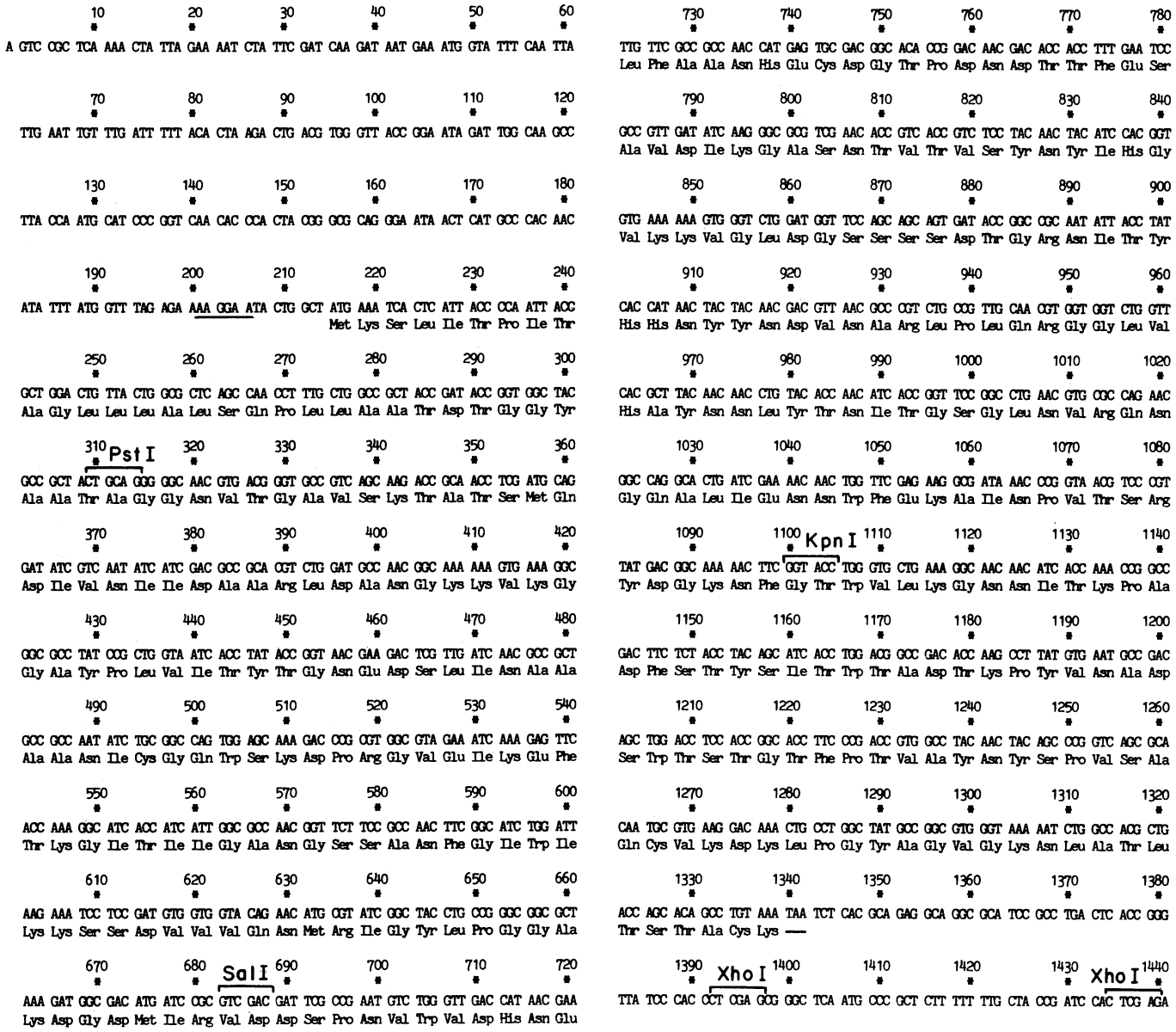


FIG. 1. Sequence of the *pelC* gene and flanking DNA in pPEL405; the sequence 3′ to the *Kpn*I site was also determined previously (12). The Shine-Dalgarno sequence preceding *pelC* is underscored, and selected restriction sites are shown.

base 1099 in *pelA* and position 2860 to the translational start of *pelE* at base 3198 [Fig. 4]; a particularly AT-rich stretch occurred 5′ to *pelE* between positions 2871 and 2931). No significant homology was noted between the AT-rich regions preceding *pelA* and *pelE*. Similar to *pelB*, *pelC*, and *pelE*, a GC-rich palindromic sequence followed by a T repeat occurred after the translational stop of *pelA* at positions 2295 to 2320 (Fig. 4).

The DNA sequence data (Fig. 4) disclosed the presence of a short open reading frame beginning at ca. base 2325, immediately following the putative *pelA* transcriptional terminator. This ORF had considerable DNA and amino acid homology with *pelA* and *pelE* (Fig. 5), but it encoded only the carboxy-terminal 132 amino acids of a putative PL protein. The observed high homology and the similarity of arrangement of this ORF and *pelA* and *pelE* with the *pelADE* clusters of other *E. chrysanthemi* strains (21) established that it represents the remains of a *pel* gene, provisionally called *pelD*, in which about 2/3 of the 5′ end has been deleted, in strain EC16. Interestingly, a GC-rich palindromic sequence occurred at bases 2737 to 2766, following the translational stop of *pelD*, but it was not followed by a string of T or AT residues.

Construction of recombinant genes between *pelA* and *pelE*. Despite their considerable amino acid homology, the proteins encoded by *pelA* and *pelE* had widely different isoelectric points, 4.6 and 9.8, respectively. The *pelA* gene product was also ca. 1,000-fold less efficient in maceration of potato tuber tissue than the *pelE* gene product (Table 3). In order to investigate whether this considerable difference in biologic activity was due only to the isoelectric point disparity of the two proteins or to other reasons, recombinant *pelA/E* genes were constructed. In the first constructs, a conserved internal *Eco*RV fragment (Fig. 4 and 7) was interchanged, gener-

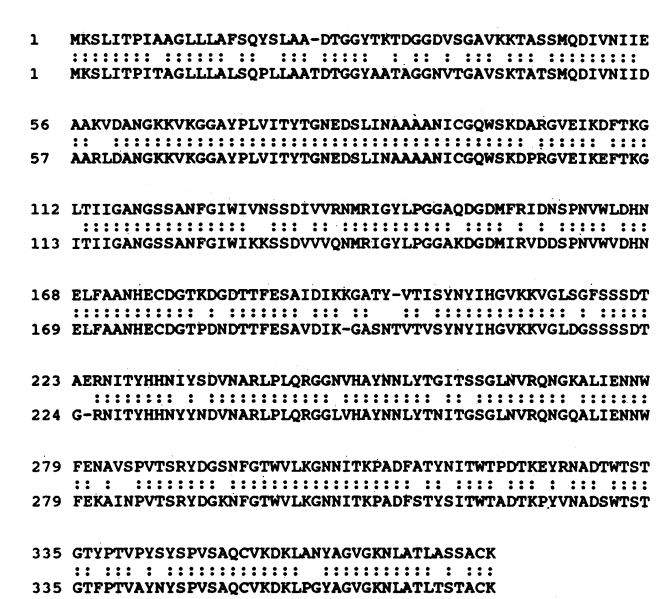


FIG. 2. Homology of the protein products of the *pelB* (top line) and *pelC* (bottom line) genes. Identical amino acids are connected by two dots.

ating pPEL770 and pPEL820 (Table 1). Transformants carrying the former construct, however, did not produce any detectable PL activity in pectate plate tests, and cells with pPEL820 produced only very weak activity. These constructs were therefore not investigated further. The *pelA* and *pelE* genes also contained conserved, unique *Spl*I restriction sites (Fig. 4 and 7), and constructions were therefore made in which the DNAs 3′ to this site were interchanged. One of the resulting constructs, pPEL822, gave no detectable PL activity in *E. coli* (Table 2), but the reciprocal construct (pPEL780) containing the 5′ portion of *pelE* and the 3′ end of *pelA* was active. *E. coli* cells containing pPEL780 gave high PL activity (data not shown), as did cells containing the recombinant gene cloned in the expression plasmid pINK1 (pPEL781, Table 2).

In order to ensure that a mutation was not inadvertently introduced during the construction of the PL-negative pPEL822, the reciprocal construct was made by using pPEL810 (containing *pelA*) and pPEL760 (containing *pelE*). The resulting chimeric genes in pPEL785 and pPEL824 (Table 1) gave the same results in *E. coli* as the previous constructs; pPEL785 led to high PL activity, while pPEL824 gave no detectable activity (data not shown). Since the proteins encoded by pPEL822 and pPEL824 may not be stable in *E. coli*, the chimeric gene in pPEL822 was fused at the *Hin*dIII site of the *pelA* portion at position 1109 (Fig. 4) to the 3′ end of *lacZ* in pUR290 to generate pPEL843. *E. coli* transformants produced a strong band on SDS-polyacrylamide gels at ca. 155 kilodaltons (kDa), the predicted weight of the *lacZ* fusion protein (data not shown). However, these

FIG. 3. Homology of possible promoter elements occurring 5′ to the coding regions of *pelB* and *pelC* along with the consensus *E. coli* promoter sequence (below). Numbering begins with bases 511 (12) and 60 (Fig. 1) for *pelB* and *pelC*, respectively.

TABLE 3. Maceration of cucumber mesocarp tissue by periplasmic fractions of *E. coli* cells containing overexpressed *pel* genes or a recombinant *pelEA* gene

Plasmid	Gene	Minimal PL concn (U/ml) required for maceration[a]
pPEL812	*pelA*	45.1
pPEL781	*pelEA*	0.3
pPEL748	*pelE*	0.007
pPEL344	*pelB*	0.04
pPEL410	*pelC*	0.05
pPEL841	*lacZ-pelA*	38.4

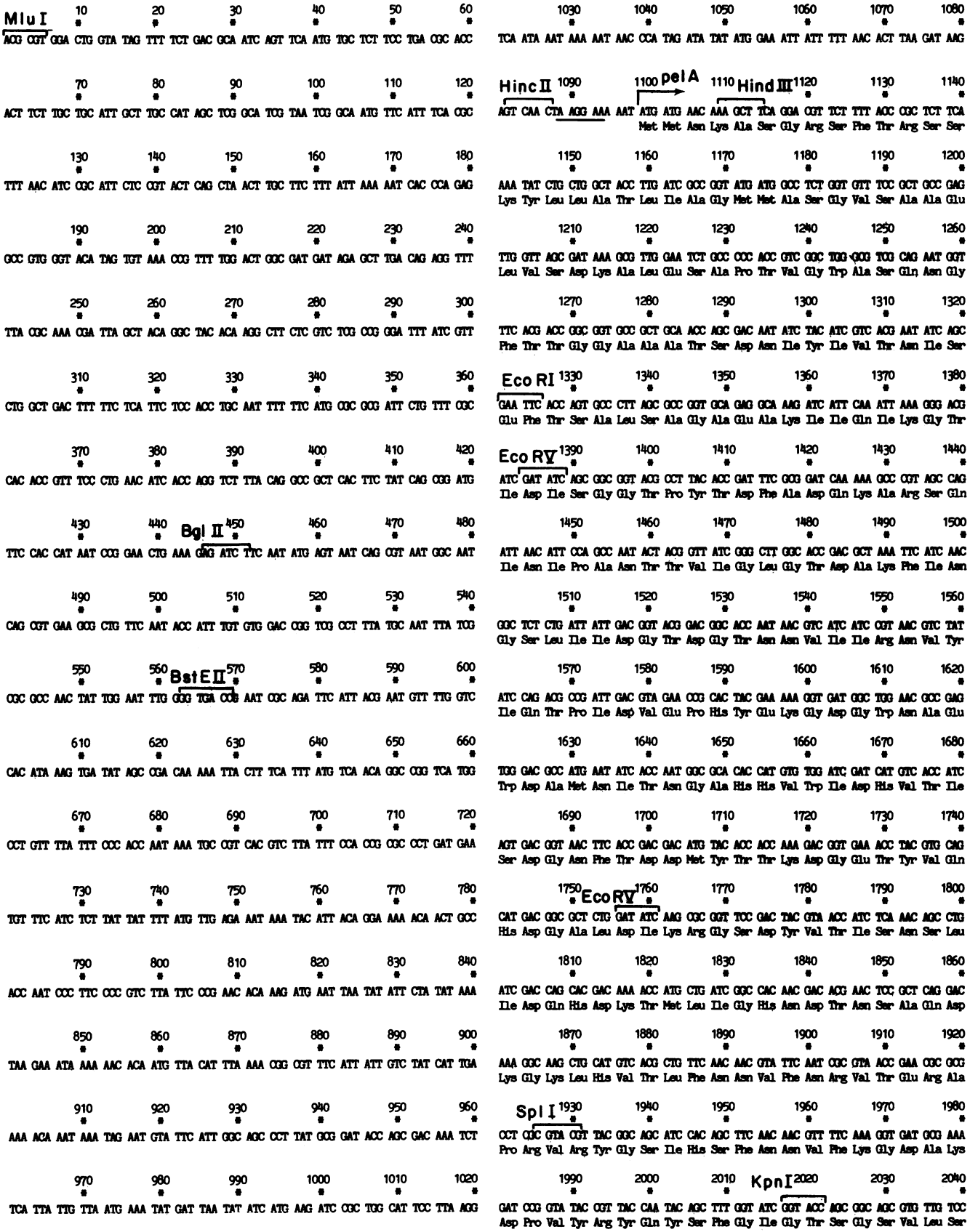
[a] Maceration was determined by incubating serial dilutions of periplasmic fractions with cucumber mesocarp slices for 60 min at 37°C as described previously (24). All assays used a total volume of 1 ml, and all solutions were brought to 0.02 M Tris hydrochloride, pH 8.0. Five cucumber slices were used per assay tube. Dilution series of the various enzymes were prepared, and the minimal amounts of PL which caused detectable cell separation during the experiment are shown.

bacteria contained no detectable PL activity (Table 2). The corresponding *Hin*dIII fusion between *lacZ* of pUR290 and the wild-type *pelA* gene (pPEL841) exhibited high PL activity (Table 2). It therefore appeared that the chimeric *pelA-pelE* protein lacked PL activity but the recombinant *pelE-pelA* (*pelEA*) product (PLea) was fully active.

Characterization of the gene products of *pelA*, *pelB*, *pelC*, *pelE*, and *pelEA*. Periplasmic fractions were collected from *E. coli* cells containing high-expression plasmid constructs of the four *pel* genes as well as the recombinant *pelEA* gene. Electrofocusing of these preparations on thin-layer gels disclosed that all of the constructs produced PL proteins with the expected isoelectric points, as determined by Coomassie blue staining, and these also gave PL activity following substrate overlays (data not shown). *pelA*, contained in pPEL812, produced the PLa protein with a pI of 4.6; *pelB*, carried by pPEL344, produced the PLb protein with a pI of 8.8; *pelC*, carried by pPEL410, produced the PLc protein with a pI of 9.0; and *pelE*, contained in pPEL748 (12), produced the PLe protein with an isoelectric point of ca. 9.8; the recombinant PLea protein encoded by pPEL781 gave a pI of 7.05 on the electrofocusing gel.

SDS gel electrophoresis of *E. coli* cells carrying various expression plasmids showed that the *pelC* gene product ran as a 39-kDa protein, very close to the value found by DNA sequencing. This value was also close to that of the *pelB* protein, as determined previously (12). The *pelA* gene product as well as the recombinant PLea encoded by pPEL781 migrated more slowly in SDS gels (estimated weight, 45 kDa; data not shown) than the ca. 39 kDa determined for the mature proteins from DNA sequence data. Thus, like PLe (12), PLa and PLea migrate anomalously slowly on SDS gels. This behavior with PLe was previously suspected to be due to its relatively high pI (12), but pI can clearly not account for the behavior of PLa and PLea.

Maceration of plant tissue. Periplasmic fractions were prepared from *E. coli* cells containing expression plasmids for various PL proteins and assayed for PL activity and maceration activity on cucumber tissue slices. Several experiments with purified and dialyzed PLa and PLe showed that ca. 1,000 times more units of PLa were required to produce the same maceration as PLe (data not shown). In tests with periplasmic fractions from *E. coli* cells carrying expression plasmids, it was observed that PLb and PLc gave similar maceration activity (Table 3), which was less than that of PLe, as noted previously (12). The recombinant PLea


MluI
BglII
BstEII
HincII
pelA
HindIII
EcoRI
EcoRV
EcoRV
SplI
KpnI

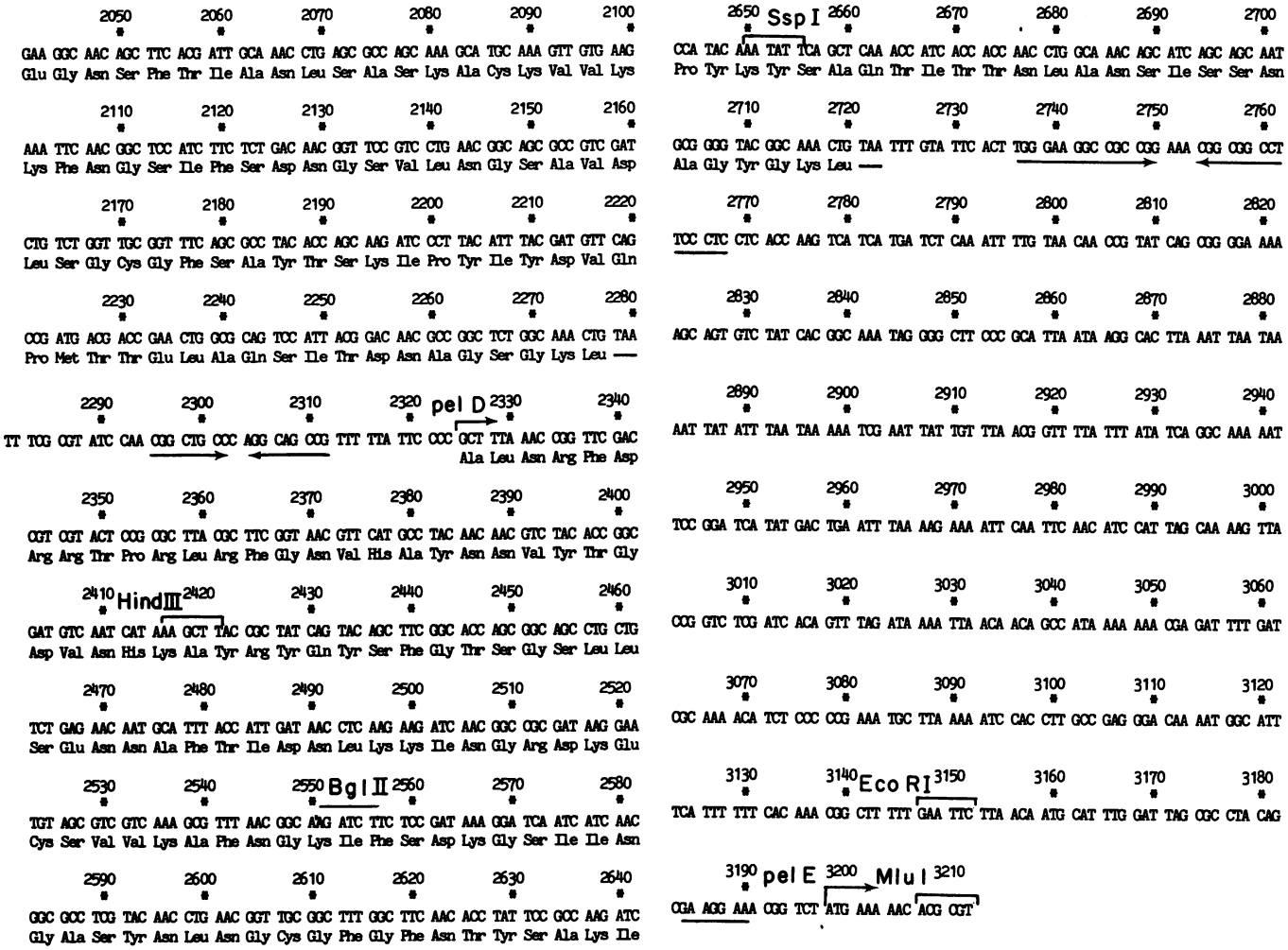


FIG. 4. Sequence of the *pelA* gene and associated 5′ DNA, the 3′ end of a deleted gene presumed to be *pelD*, and intergenic DNA between the 3′ end of *pelD* and the previously sequenced *pelE* gene (12). Sequence data shown are for the 3.2-kb insert of pPEL801. Selected restriction sites are shown. Palindromic sequences are indicated by arrows, and Shine-Dalgarno boxes are underscored. The start of the truncated *pelD* gene at base 2325 was determined by homology with corresponding regions of *pelA* and *pelE*.

protein encoded by pPEL781 (*pelEA*) macerated tissue much less efficiently than PLb, PLc, or PLe but was more active than PLa (Table 3). The *lacZ* fusion protein with PLa from pPEL841 produced maceration similar to that of the wild-type PLa. However, it is not clear whether degradation of the fusion protein might have occurred in the cucumber tissue during the maceration assay. Thus, it cannot be concluded with assurance that the much larger fusion protein in fact has maceration properties similar to those of PLa.

DISCUSSION

In conjunction with our past work (12), the sequence data presented here for the *pelA* and *pelC* genes from *E. chrysanthemi* EC16 reveal the structure and organization of all four known genes encoding endo-PLs in this strain. In addition, the sequence data detected a deleted *pel* gene occurring between *pelA* and *pelE*. The *pel* genes are organized in two chromosomal clusters. The *pelB* and *pelC* genes are linked in tandem with ca. 500 bp of intergenic DNA (Fig. 6). This is similar to the organization of the *pelB* and *pelC* genes in two other *E. chrysanthemi* strains (21, 25), although these genes have not been sequenced. Furthermore, we have identified sequences with considerable homology in the 5′ noncoding regions of the *pelB* and *pelC* genes which may function as promoter elements (Fig. 3). Significantly, however, overall homology between the 5′ DNAs of the *pelB* and *pelC* genes is low. The high homology of the coding regions (84% amino acid identity, including the signal peptide sequences) (Fig. 2) and the similarity of the protein products (pI values of 8.8 and 9.0) indicate that *pelB* arose via an evolutionary duplication of *pelC*. It is unclear why such a duplication was tolerated in *E. chrysanthemi*, but it should be noted that duplications of genes having high homology with *pelB* and *pelC* have been shown to occur in the related bacterium *Erwinia carotovora* (15; Lei, Hin, Wang, and Wilcox, submitted for publication).

An evolutionary duplication event presumably also gave rise to the clustered *pelA* and *pelE* genes of strain EC16, as well as to the subsequently deleted *pelD* gene (Fig. 5 and 7). The *pelA/E* genes have diverged more than the *pelB/C* genes, so that the protein products have very different pI values (4.6 for PLa and 9.8 for PLe). However, the mature PLa and PLe proteins have 62% amino acid identity, and the protein products exhibit similar catalytic properties in vitro (2), although not in plant tissue (Table 3).

```
1   MMNKASGRSFTRSSKYLLATLIAGMMASGVSAAELVSDKALESAPTVGWASQNGFT
                                          : : ::: ::: :
1           MKNTRVRSIGTKSLLAAVVTAALMATSAYAAVETDAATTGWATQNGGT

57  TGGA-AATSDNIYIVTNISEFTSAL-SAGAEAKIIQIKGTIDISGGTPYTDFADQK
    :::: ::       : ::: :  ::      ::::   : ::::::  :: : :::
49  TGGAKAA---KAVEVKNISDFKKALNGTDSSAKIIKVTGPIDISGGKAYTSFDDQK

111 ARSQINIPANTTVIGLGTDAKFINGSLIIDGTDGTNNVIIRNVYIQTPIDVEPHYE
    ::::: :: ::: :: :   :: :::: : :     ::: :: :: :: :: ::::
102 ARSQISIPSNTTIIGVGSNGKFTNGSLVIKGVK---NVILRNLYIETPVDVAPHYE

167 KGDGWNAEWDAMNITNGAHHVWIDHVTISDGNFTDDMYTTKDGETYVQHDGALDIK
     :::::::::  :      :: :::::::: :::: ::::::: ::::::::::
155 SGDGWNAEWDAAVID-NSTNVWVDHVTISDGSFTDDKYTTKDGEKYVQHDGALDIK

223 RGSDYVTISNSLIDQHDKTMLIGHNDTNSAQDKGKLHVTLFNNVFNRVTERAPRVR
     :::::::: :    :::: :::: : :  :: ::: ::  :::: ::::::::::
210 KGSDYVTISYSRFELHDKTILIGHSDSNGSQDSGKLRVTFHNNVFDRVTERAPRVR
                                                :  : :: :
                                              1 ALNRFDRRTPRLR

279 YGSIHSFNNVFKGDAKDPVYRYQYSFGIGTSGSVLSEGNSFTIANLSASKACK---
     ::::  :::  :: :  :: : :::: ::::: ::: ::::  ::
266 FGSIHAYNNVYLGDVKHSVYPYLYSFGLGTSGSILSESNSFTLSNLKSIDGKNPEC
    ::  ::::::: ::: :  : : :::: :::: ::: : ::  ::: : :   ::
 14 FGNVHAYNNVYTGDVNHKAYRYQYSFG--TSGSLLSENNAFTIDNLKKINGRDKEC

332 -VVKKFNGSIFSDNGSVLNG-SAVDLSGCGFSAYTS--KIPYIYDVQPMTTELAQS
     :: ::   ::: ::  :     :  ::  ::      :: :  : :: :: :
322 SIVKQFNSKVFSDKGSLVNGSTTTKLDTCGLTAY-KP-TLPYKYSAQTMTSSLATS
    : :: :: : ::::::  ::    :  ::          :::::::: :  :: :
 68 SVVKAFNGKIFSDKGSIINGASY-NLNGCGFGFNTYSAKIPYKYSAQTITTNLANS

384 ITDNAGSGKL
    :  ::: :::
376 INNNAGYGKL
    :  :::::::
123 ISSNAGYGKL
```

FIG. 5. Homology of the protein products of the *pelA* (top line) and *pelE* (second line) genes of *E. chrysanthemi* EC16 and the 3′ end of an ORF assumed to be the remains of the deleted *pelD* gene (third line).

The organization of the *pelA* and *pelE* genes in strain EC16 differs from that in two other strains of *E. chrysanthemi* (21). Whereas *pelA* and *pelE* are transcribed in the same direction of EC16 (Fig. 7), they appear to be transcribed divergently in the other two strains, as determined by the polarity of expression of *lacZ* insertions (21). These strains also produce a fifth PL protein, encoded by an additional gene, *pelD*, a functional copy of which does not occur in strain EC16 (2, 22). This gene encodes a PL with a pI of about 9.3 and has considerable homology with *pelE* (Kotoujansky, personal communication). The sequence data presented here establish why this gene is not functional in strain EC16. The short ORF located immediately 3′ to *pelA* (Fig. 4 and 5) has high

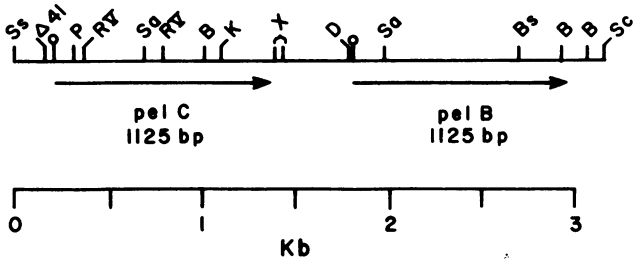


FIG. 6. Gene organization of the *E. chrysanthemi* EC16 *pelB* and *pelC* genes based on DNA sequence data presented here and elsewhere (12). Δ, Site of a deletion used in subcloning; for other abbreviations, see the legend to Fig. 7.

homology to the corresponding regions of PLa and PLe and almost certainly represents a remnant of the missing *pelD* gene. The deletion event presumed to have given rise to the truncated *pel* gene must have occurred relatively recently, because no detectable genetic drift was observed in the ORF (Fig. 5). Thus, while the ORF does not appear to retain a functional rho-independent transcriptional stop like the other *pel* genes, no mutations have yet occurred that destroyed ORF integrity (Fig. 4). The deletions and insertions observed in the truncated *pelD* gene relative to *pelA* and *pelE* were most likely introduced during its evolution as a functional gene because the *pelB/C* and *pelA/E* pairs exhibit similar features (Fig. 2, 4, and 5).

We previously observed that *pelB* and *pelE* have little homology, despite the fact that their gene products both catalyze random eliminative cleavages of sodium polypectate (2). Two short regions of conserved amino acids were noted in the two proteins, however. These conserved regions also occur in *pelA* and *pelC*, and one of them also occurs in the truncated *pelD* gene. The additional data have shown that certain conserved amino acids also precede region II (Fig. 8), and other amino acids between regions I and II are evolutionarily conserved substitutions. Structural or mutational analyses have not yet been undertaken to determine whether these conserved regions are essential for enzymatic activity.

The *pelB*, *pelC*, and *pelE* genes of strain EC16 were previously shown to possess sequences which would be expected to function as rho-independent transcriptional terminators after the translational stops (12). This suggests that these genes are independently expressed, consistent with expression and mutation studies (13, 21). We have now observed that a similar sequence follows the *pelA* gene (Fig.

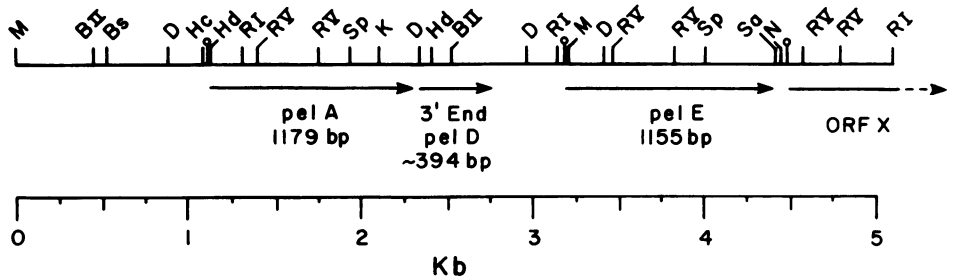


FIG. 7. Gene organization of the EC16 *pelA* and *pelE* genes and the deleted gene assumed to be *pelD* as determined from DNA sequence data given here and elsewhere (12). ORF X has no known function. A *pem* gene encoding pectin esterase occurs ca. 2.0 kb to the right of the translational stop of *pelE* (see map in reference 11). Arrows denote open reading frames; circles show translational initiation sites. Restriction site abbreviations: B, *Bgl*I; Bs, *Bst*EII; BII, *Bgl*II; D, *Dra*I; Hc, *Hinc*II; Hd, *Hin*dIII; K, *Kpn*I; M, *Mlu*I; N, *Not*I; P, *Pst*I; RI, *Eco*RI; RV, *Eco*RV; Sa, *Sal*I; Sp, *Spl*I; Ss, *Sst*I; Sc, *Sca*I; X, *Xho*I.

Erwinia chrysanthemi	pel B	AIDIKKGATY-VTISYNYI	30	VNARLPLQRGGNVHAYNNLYTG
	pel C	AVDIK-GASNTVTVSYNYI	29	VNARLPLQRGGLVHAYNNLYTN
	pel A	ALDIKRGSDY-VTISNSLI	34	VTERAPRVRYGSIHSFNNVFKG
	pel E	ALDIKKGSDY-VTISYSRF	34	VTERAPRVRFGSIHAYNNVYLG
	3'pel D		8	RTPRLRFGNVHAYNNVYTG
Erwinia carotovora	pel A	AVDIKKGSTN-VTVSYNYI	29	VNSRLPLQRGGQVHAYTNLYDG
	pel B	AVDIKKGSTN-VTVSYNYI	29	VNSRLPLQRGGLVHAYTNLYDG

FIG. 8. Amino acid homologies occurring in the four EC16 PL proteins as well as the carboxy terminus of PLd and the *E. carotovora* PLa and PLb proteins, which have high overall homology with the EC16 PLb and PLc proteins (15; Lei et al., submitted). Boldface letters denote conserved amino acids; numbers between regions show number of intervening amino acids.

4), which would be predicted to function as a rho-independent transcriptional terminator (10). This is of interest because *pelA* is poorly expressed relative to the other *pel* genes when *E. chrysanthemi* is grown under laboratory culture conditions (21, 22). A palindromic sequence also occurred after the translational stop of the deleted *pelD* gene (Fig. 4), but this was not followed by a poly(T) sequence. Thus, unlike the other EC16 *pel* genes, the deleted *pelD* gene would not appear to contain a rho-independent transcriptional terminator (10). This may be relevant to preliminary suggestions that the residual *pelD* ORF influences expression of the downstream *pelE* gene (1; Manulis and Keen, unpublished).

We previously identified an ORF (ORF X, Fig. 7) for which the Shine-Dalgarno box was located only 20 bp downstream from the transcriptional terminator of the EC16 *pelE* gene (12). This would be assumed to seriously limit transcription of the second ORF unless it is coupled to translation of *pelE* in the manner reported by Wright and Hayward (28) for the rho-indpendent transcriptional terminator of the *E. coli gal* operon. The ORF following *pelE* did not have significant homology with *pelE* but possessed a signal peptide sequence (12). We subcloned the region from the *Not*I site (Fig. 7) which occurs in one stem element of the *pelE* transcriptional terminator and 58 bp ahead of the ORF X start codon (12) to sites up to 3 kb downstream (data not shown). These constructs were assumed to destroy termination function and allow expression of the ORF from the pUC19 *lac* promoter. All constructs, however, failed to yield PL or detectable xylanase, protease, cellulase, or pectin lyase activities in *E. coli* cells when IPTG was added (data not shown). Thus, although the identity of the ORF is not known, it does not appear to encode a random chain-splitting PL.

We also observed that a strain EC16 gene encoding a pectin esterase occurs on a 2.2-kb *Sma*I-*Sst*I fragment located ca. 2.0 kb from the 3′ end of the *pelE* gene on clone pPEL74 (11; unpublished observations). Significantly, other *E. chrysanthemi* strains also contain a pectin esterase gene that maps at the same position (Kotoujansky, personal communication).

Similar to the previous results with *pelB* and *pelE*, the *pelA* and *pelC* genes from strain EC16 could be satisfactorily overexpressed in *E. coli* cells with pINK1, an expression plasmid derived from pINIII, constructed by Inouye and associates (18). Also similar to the previous results, the uninduced level of PL with these constructs was high despite the presence of the *lacI* gene on the vector. Recently, Hasan and Szybalski (7) constructed an invertible promoter vector, pNH8a. We found that this vector resulted in low uninduced levels of PLc (Table 2) but led to induced levels that were comparable to those of the *pelC* gene cloned in pINK1.

Barras et al. (2) reported that PLa is a much less efficient macerating factor for plant tissue than PLe, but both proteins have similar catalytic properties in vitro. We confirmed the poor maceration ability of the *pelA* protein (ca. 1,000 times less effective than the *pelE* protein against cucumber mesocarp tissue [Table 3]). However, Kotoujansky (13) showed that the *pelA* gene of *E. chrysanthemi* 3937 is essential for full pathogenicity on African violet plants. Furthermore, the *pelA* gene appears to occur in all strains of *E. chrysanthemi* tested to date (22), raising the possibility that it provides a selective advantage. These considerations raise the question of the function of PLa in plant pathogenesis. PLs with little plant-macerating ability and low pI values, similar to *pelA*, have also been described for the non-plant-pathogenic bacteria *Yersinia pseudotuberculosis* and *Klebsiella pneumoniae* (3, 17). The fact that these bacteria infrequently encounter plant tissue raises the possibility that the acidic PLs may have alternative but as yet unknown physiologic functions.

Since the *pelA* and *pelE* proteins from strain EC16 give similar specific activity in in vitro PL assays, the most obvious explanation for the low macerating activity of the *pelA* protein is its low pI value, as suggested by Tanabe and Kobayashi (26). This might impede the physical penetration of the protein into the negatively charged plant cell wall matrix. In order to test this possibility, we constructed several recombinant *pelA*/*pelE* genes. Three of these constructs resulted in little or no PL activity in *E. coli* cells, but pPEL780 and pPEL785 led to PL activity at levels similar to those of the parental *pelA* and *pelE* genes. The maceration activity of the recombinant PLea protein was greater than that of PLa but considerably less than that of PLe (Table 3). The recombinant protein was also less efficient at tissue maceration than PLb or PLc, which have pIs of 8.8 and 9.0, respectively. Furthermore, many PLs from erwinias and other organisms which efficiently macerate plant tissue possess isoelectric points in the range of 7.0 to 8.5 (13). Since the pI of the recombinant PLea protein (pH 7.05) was considerably higher than the pH of most plant intercellular fluids (pH 5 to 6), our data suggest that other factors in addition to the low pI value of PLa may contribute to its poor macerating efficiency. Additional work with recombinant gene constructions should further illuminate domains of the

proteins required for PL activity, plant tissue maceration, and pathogenicity.

ACKNOWLEDGMENTS

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