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Structure of Two Pectate Lyase Genes from *Erwinia chrysanthemi* EC16 and Their High-Level Expression in *Escherichia coli*

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The *pelB* and *pelE* genes from *Erwinia chrysanthemi* EC16, which encode different pectate lyase enzymes, were sequenced and expressed at a high level in *Escherichia coli*. The genes possessed little similarity to each other in 5′ signal regions, signal peptide sequences, coding sequences, or 3′ noncoding regions. Both genes contained their own promoters as well as sequences 3′ to the coding regions with considerable secondary structure which may function as rho-independent transcriptional termination signals. High-level expression plasmids were constructed with both genes, which led to 20% or more of *E. coli* cellular protein. The pectate lyases were secreted efficiently to the periplasm and, to a lesser extent, the culture medium. The mature proteins in *E. coli* periplasmic fractions were obtained in milligram amounts and high purity with a single-column affinity purification method. *E. coli* cells which produced high amounts of the *pelE* protein macerated potato tuber tissue as efficiently as *E. chrysanthemi* EC16 cells but cells producing high amounts of the *pelB* protein were less effective. Thus, the *pelE* gene product is an important pathogenicity factor which solely enables *E. coli* to cause a soft-rot disease on potato tuber tissue under laboratory conditions.

We cloned genes coding for two different pectate lyase (EC 4.2.2.2) enzymes from the phytopathogenic bacterium *Erwinia chrysanthemi* EC16 (13) and observed their expression in *Escherichia coli*. Pectate lyases have previously been shown to account largely or entirely for the maceration or soft rotting of plant tissue caused by *Erwinia* spp. (4). Confirming this, *E. coli* cells containing the cloned pectate lyase genes macerated plant tissue, albeit less efficiently than *E. chrysanthemi* (13). Several groups subsequently cloned similar genes from other strains of *E. chrysanthemi* (5, 14, 28, 34) and the related bacterium *Erwinia carotovora* (18, 29, 40). The genes that we cloned did not cross-hybridize (13), but coded for enzymes with similar physical properties (molecular weights of ca. 40,000 and isoelectric points of 8.8 and 9.8) which both catalyzed the random eliminative cleavage of sodium polypectate. These enzymes were efficiently secreted to the periplasm and, to a lesser extent, the culture medium of *E. coli*. For reasons discussed below, the cloned DNA fragments appear to contain *pelB* and *pelE*, described by others, and the mature proteins which they encode are PLb and PLe, respectively. Plasmids containing our *pel* genes were named pPL in the previous paper (13), but this designation was found to be already entered in the Plasmid Reference Center (17). Accordingly, our *pel* gene plasmid constructs have been renamed pPEL, a designation that we have registered in the Plasmid Reference Center (17).

The cloned *pelB* and *pelE* genes were both regulated by catabolite repression in *E. coli* (13) but were not induced by sodium polypectate, as occurs in *E. chrysanthemi* (4). Due to their pathogenic importance in diseases caused by *Erwinia* spp. and to their regulation properties, we elected to further study the cloned *pel* genes coding for the pectate lyase enzymes. Since multigene families coding for functionally similar proteins are uncommon in procaryotes, it was also of interest to compare the structures of the two genes. This paper presents the further subcloning and sequencing of these genes, their high-level expression in *E. coli*, and the demonstration that their high-level expression enables *E. coli* cells to efficiently macerate plant tissue.

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MATERIALS AND METHODS

Purification and assay of pectate lyases. *E. coli* cells (usually HB101 or JA-221; Table 1) containing various expression plasmids were grown into the stationary phase for ca. 16 h at 28°C, usually in 15 ml of L broth with 50 μg of ampicillin per ml in 50-ml shaken DeLong flasks. These bacteria were also grown in the same way on M9 or M9CA medium (19) with glucose at 2 mg ml^{-1}, thiamine hydrochloride at 2 μg ml^{-1}, and proline, tryptophan, and leucine all at 20 μg ml^{-1}. Cultures received isopropyl-β-D-thiogalactopyranoside (IPTG) at 1 mM either when initiated or after attaining an absorbance at 600 nm of 0.5 to 0.7. The resultant cells (ca. 0.12 g per flask) were harvested by centrifugation, washed with 0.2 M Tris hydrochloride (pH 8.0), and induced to form spheroplasts by the method of Witholt et al. (37), resulting in a final periplasmic fraction of 16 ml from each culture. The periplasmic fractions, culture fluids, and cellular fractions were assayed as described previously (13).

For purification of the pectate lyases, periplasmic fractions were dialyzed extensively against 5 mM Tris hydrochloride (pH 8.0), containing 0.1 mM $CaCl_2$ at 4°C and centrifuged at 20,000 × *g* for 10 min to remove traces of insoluble material. Pectate lyase activity was easily purified to near homogeneity by a modification of techniques based on the affinity of pectate lyases for agarose gel matrices (7). The dialyzed periplasmic fraction resulting from eight culture flasks (≈ 120 ml) was pumped through a 1.5 by 7.0-cm column of Bio-Rad A-1.5m or Bio-Rad CM Bio-Gel at ca. 3 ml min^{-1} at room temperature. The columns were washed with 0.1 mM $CaCl_2$–4 mM Tris hydrochloride (pH 8.0) and then eluted with a linear gradient composed of 60 ml of 5 mM Tris hydrochloride (pH 8.0) and 60 ml of 0.2 M NaCl in the same buffer. In later experiments the columns were bulk eluted with 0.2 M NaCl in the Tris hydrochloride buffer to release the enzymes. Fractions (3 ml) were collected and

TABLE 1. Plasmids and bacterial strains employed

Plasmid or strain	Genotype or Description	Source or Reference
E. coli		
HB101	F$^-$ *hsdS20* (*hsdR hsdM*) *recA13 ara-14 proA2 lacY1 galK2 rpsL20* (Str^r) *xyl-5 mtl-1 supE44* λ$^-$	(19)
JA-221	*hsdR* Δ*trpE5 leuB6 lacY recA thi* F'(*lacI*^{q} *lacZ*$^+$ *lacY*$^+$ *lacA*$^+$ *proA*$^+$ *proB*$^+$)	(20)
RB791	W3110 *lacI*^{q}*L8*	(1)
C600	F$^-$ *thr-1 leuB6 thi-1 supE44 lacY1 fhuA21* λ$^-$	(19)
706	F$^-$ *proA thr leu argH his lac phoSt rpsL lky-207*	(16)
M5219	Str^r *lacZ*(Am) *trp*(Am) (λ *bio*252 *c*I857 ΔH1)	(27)
E. chrysanthemi EC16		E. Chatterjee
Plasmids		
pIN$_{III}$113 A-1 and A-2		(20)
pCQV2		(26)
pKC30		(31)
pPLc2820	Deletion of *Pst*I site from pPLc2819	(27; Remaut, unpublished data)
pUC8		(38)
pUC18 and pUC19		(38)
pJRD158		(8)
pINK-1	Expression vector for transcriptional fusions	This paper
pPEL3	Cosmid clone encoding PLb and PLc; formerly pPL3	(13)
pPEL34	6.6-kb *Pst*I fragment from pPEL3 coding for PLb only; formerly pPL34	(13)
pPEL342	2.0-kb *Kpn*I-*Sca*I fragment from pPEL34 cloned in pUC18	This paper
pPEL343	2.0-kb *Sst*I-*Hin*dIII fragment from pPEL342 cloned in pINK-1	This paper
pPEL344	1.3-kb *Dra*I-*Hin*dIII fragment from pPEL342 cloned into the *Sma*I-*Hin*dIII sites of pINK-1	This paper
pPEL7	Cosmid clone encoding PLa and PLe; formerly pPL7	(13)
pPEL74	8.2-kb *Pst*I fragment from pPEL7 encoding for PLa and PLe; formerly pPL74	(13)
pPEL742	1.75-kb *Eco*RI fragment from pPEL74 encoding PLe only	(13)
pPEL7421	1.2-kb *Eco*RI-*Sal*I fragment from pPEL742, cloned in pUC8	This paper
pPEL7422	1.2-kb *Eco*RI-*Sal*I fragment cloned in pUC19	This paper
pPEL743	2.0-kb *Hin*dIII-*Sal*I fragment from pPEL74 cloned in pUC19	This paper
pPEL743J	2.0-kb *Hin*dIII-*Sal*I fragment from pPEL74 cloned in PJRD158	This paper
pPEL746	1.2-kb *Eco*RI-*Hin*dIII fragment from pPEL7421 cloned in pINK-1	This paper
pPEL711	pPEL7421 with 92-base-pair internal *Bcl*I fragment deleted	This paper
pPEL712	pPEL7421 with 361-base-pair internal *Eco*RV fragment deleted	This paper
pPEL747	Pectate lyase-negative translational fusion of *pelE*	Fig. 4, this paper
pPEL748	Mutant pectate lyase-positive translational fusion of *pelE*	Fig. 4, this paper
pPEL749	Pectate lyase-positive translational fusion of *pelE*	Fig. 4, this paper

assayed for pectate lyase activity (13) and absorbance at 280 nm. Peak fractions were pooled, dialyzed against distilled water at 4°C, and lyophilized. The white, fluffy enzymes were stored dry at −20°C. Care was taken to use dialysis tubing with 6,000- to 8,000-molecular-weight cutoff in the above steps, since tubing with a 12,000- to 14,000-molecular-weight cutoff resulted in significant loss of the 9.8 pectate lyase.

Electrophoresis of whole cell proteins. Cells of *E. coli* HB101 containing desired plasmids were grown for various periods at 28°C on 15 ml of L broth containing 50 μg of ampicillin per ml; some cultures received 1 mM IPTG at various times. The cultures were centrifuged, and the resultant cells (ca. 0.10 to 0.15 g, fresh weight) were washed once with 10 ml of 0.01 M Tris hydrochloride (pH 7.5). The pellets were then suspended in 0.25 ml of water, an equal volume of 2.5× Laemmli sample solution (15) was added, and the samples were boiled for 5 min. Samples were then electrophoresed directly on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels (1 mm thick) with a Laemmli buffer system (15). Protein standards (Sigma Chemical Co.) and purified pectate lyases were prepared and electrophoresed similarly. The gels were run at 160 V for 4 h at room temperature and directly stained with Coomassie blue R250 and destained, both as described previously (13).

Plant tissue maceration tests. Cells of *E. chrysanthemi* EC16 or log-phase *E. coli* cells carrying desired plasmids were grown on LB medium, and various cell concentrations were placed into wells on potato tuber cylinders. The cylinders (1.4-cm diameter) were cut from store-bought Russet Burbank potato tubers with a no. 7 cork borer and sliced to 7-mm lengths. A well 3 mm deep and 6 mm wide was made at one end of each cylinder with a no. 2 cork borer such that 0.1 ml of cell suspension could be added. The cylinders were then incubated on moistened filter paper in petri plates at 28°C and observed for maceration at various time intervals. Maceration severity was rated on a 0 to 4 scale by probing cylinders with a spatula to assess the degree of softening and tissue disintegration.

DNA manipulations. Subcloning and plasmid constructions were generally done by ligating desired DNA fragments recovered from low-melting-point agarose gels by the method of Crouse et al. (6), except that gelatin was added to 100 μg ml^{-1}. End-filling reactions used T4 DNA polymerase

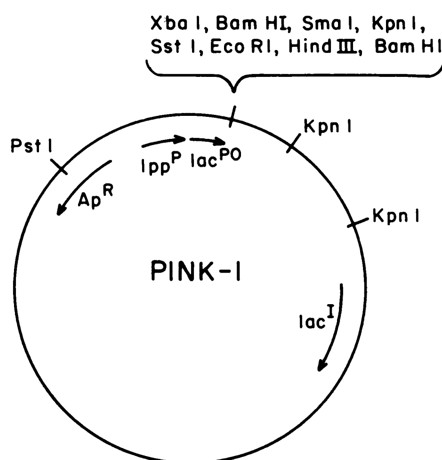

FIG. 1. Restriction map of pINK-1, constructed from PINIII[113] A-1 (20), showing polylinker used for insertion of *E. chrysanthemi pel* genes bearing their own translation signals.

(6). Constructs were checked by mini-boil plasmid extractions (6, 13) followed by restriction with the appropriate enzymes. The junction sequences of plasmids producing fusion proteins were confirmed by DNA sequencing as described below. Plasmid constructs were transformed into frozen, competent cells of *E. coli* by the method of Morrison (22). Other cloning details were as described previously (13, 19).

N-terminal amino acid sequencing. The purified mature PLb or PLe pectate lyases (50 pmol) were dissolved in 0.5% SDS, and microsequencing was performed with an Applied Biosystems 470A vapor-phase microsequencer. Fifteen amino acid residues were determined for each pectate lyase.

DNA sequencing. A series of BAL 31 deletions were generated from either end of the fragments and sequenced by the dideoxy chain termination method of Dean et al. (9). All data were confirmed by sequencing both strands. Two noncoding regions, both ca. 20 base pairs in length, associated with the insert DNA of pPEL342 could not be determined accurately by this method and were therefore sequenced by the Maxam and Gilbert method (21). Sequence data were analyzed by the computer programs of Pustell and Kafatos (25) and the BIONET system supplied through Intelligenetics Corp., Palo Alto, Calif.

RESULTS

Construction of pINK-1. To test the expression of the subcloned pectate lyase genes in *E. coli* with *E. chrysanthemi* translation signals, a plasmid was constructed from the *lac*-regulated expression vector pINIII[113] A-1 (20). Plasmid DNA was restricted at the *Xba*I and *Eco*RI sites, and the large fragment was ligated with pUC19 DNA cut with the same enzymes. The resulting construct contained most of the pUC19 polylinker region downstream from the triple *lac* UV5 promoters of pINIII and was called pINK-1 (Fig. 1). It permitted use of the strong regulated promoters with *E. chrysanthemi* genes carrying their own translational signals and allowed the insertion of DNA fragments with various restriction termini. The other advantages of the pINIII vectors, such as transcriptional termination sequences 3' to the inserted coding region and presence in the vector of the *lacI*^{q} gene, were retained.

Expression of the *pelB* gene in *E. coli*. The 6.6-kilobase (kb) *Pst*I fragment from pPEL34 (13) coding for PLb was further subcloned. Preliminary experiments indicated that the coding region resided on the left-hand side of the fragment, since a *Kpn*I-*Eco*RI fragment cloned in pUC18 retained the activity, but a *Bst*EII-*Pst*I fragment cloned in pJRD158 did not. A 2.0-kb *Kpn*I-*Sca*I fragment cloned in pUC18 conferred pectate lyase activity in *E. coli*, but a 1.3-kb *Kpn*I-*Eco*RV fragment was inactive. Accordingly, the *Kpn*I-*Sca*I fragment cloned in pUC18 was retained and designated pPEL342 (Fig. 2). This fragment was further mapped, and the additional restriction sites are shown in Fig. 2.

Pectate lyase production was high in *E. coli* cells carrying pPEL342 with or without IPTG (Table 2). However, a small increase with IPTG was consistently observed with pPEL342 but not the reverse insert orientation in pUC19 (data not shown). The same was true when the 2.0-kb *Kpn*I-*Sca*I fragment from pPEL342 was inserted into pINK-1 to give pPEL343. These suggestions for the direction of transcription were later confirmed by DNA sequence data. pPEL344, a pINK-1 construct lacking the *E. chrysanthemi* promoter regions (see Table 1 and Fig. 2 and 9) led to somewhat lower constitutive pectate lyase production, which was increased ca. threefold when cells were induced with IPTG (Table 2). Unlike the prior constructs, IPTG supplied at culture initiation severely inhibited the growth of cells carrying pPEL344.

High-level expression of the *pelE* gene. The 1.75-kb *Eco*RI insert in pPEL742 (13) was further restricted, giving a 1.2-kb *Eco*RI-*Sal*I fragment that retained PLe activity when cloned in pUC8 to yield pPEL7421 (Table 2). Expression of the 1.2-kb insert in pPEL7421 was found to be orientation dependent, since little pectate lyase activity was detected when the 1.2-kb *Eco*RI-*Sal*I fragment was reversed in pUC19 to yield pPEL7422 (Table 2). To retain the *E. chrysanthemi* transcription signals of the pI 9.8 pectate lyase, a 2.0-kb *Hin*dIII-*Sal*I fragment from pPEL74 was cloned in pUC19 to yield pPEL743. The same fragment was also cloned in pJRD158, and the resultant plasmid was called pPEL743J. Significantly, pPEL743 gave considerably less pectate lyase activity in HB101 than pPEL7421, and only a small increase in activity was observed when cells harboring pPEL743 were supplied with IPTG (Table 2). Somewhat more pectate lyase was produced by HB101 cells carrying pPEL743J, but this was still below the induced level for pPEL7421 and may be attributable to the reported high copy number of the vector, pJRD158 (8). These results raise the possibility that a regulatory DNA element in the 5' noncoding region of the *pelE* gene may be repressing expression in *E. coli*. In contrast, pPEL7421 lacks most of the 5' DNA and led to an unexpectedly higher uninduced pectate lyase level (Table 2).

High-level expression of the *pelE* gene was initially at-

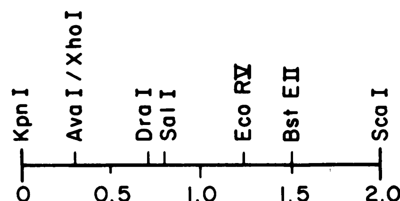

FIG. 2. Restriction map of a ca. 2.0-kb *Kpn*I-*Sca*I fragment isolated from pPEL34 which conferred production of PLb in *E. coli* HB101. The fragment was cloned into the *Kpn*I and *Hin*cII sites of pUC18, and the resultant construct was called pPEL342. Map units are in kilobase pairs. Sequence data subsequently showed the presence of two closely spaced *Ava*I-*Xho*I sites rather than one site.

TABLE 2. Production of pectate lyase by *E. coli* cells containing various expression plasmids

Plasmid[a]	*E. coli* strain	IPTG[b]	Pectate lyase U per g of cells (% of total activity)[c]			
			Periplasmic fraction	Intracellular fraction	Extracellular fraction	Total activity
pPEL342	HB101	No	4,500			
pPEL342	HB101	Yes	6,000			
pPEL343	HB101	No	7,500 (93)	200 (3)	350 (4)	8,050
pPEL343	HB101	Yes	8,060 (93)	160 (2)	490 (5)	8,710
pPEL344	HB101	No	3,100		30	
pPEL344	HB101	Yes, 7 h	8,900		55	
pPEL7421	HB101	No	310 (84)	20 (5)	40 (11)	370
pPEL7421	HB101	Yes	1,750 (93)	80 (4)	50 (3)	1,880
pPEL7422	HB101	Yes	55 (92)	<2	5 (8)	60
pPEL711	HB101	Yes	<0.2	<1	<0.2	<1
pPEL712	HB101	Yes	<0.2	<1	<0.2	<1
pPEL743	HB101	No	90 (88)	5 (5)	7 (7)	102
pPEL743	HB101	Yes	130 (86)	10 (7)	11 (7)	151
pPEL743J	HB101	No	520			
pPEL746	RB791	No	1,650 (88)	90 (5)	30 (7)	1,870
pPEL746	RB791	Yes	4,360 (95)	170 (4)	60 (1)	4,590
pPEL746	JA-221	No	4,830			
pPEL746	JA-221	Yes	8,750			
pPEL746	HB101	No	1,700 (90)	135 (7)	55 (3)	1,890
pPEL746	HB101	Yes	6,400 (94)	220 (3)	200 (3)	6,820
pPEL746	706	No	146 (32)	50 (11)	258 (57)	454
pPEL746	706	Yes	245 (7)	85 (3)	2,890 (90)	3,220
pPEL747	HB101	Yes	<0.2	<1	<0.2	<1
pPEL748	HB101	No	10,500 (91)	1,030 (9)	55 (<1)	11,585
pPEL748	HB101	Yes, 7 h	12,000 (89)	450 (3)	1,100 (8)	13,550
pPEL748	JA-221	No	3,280 (44)	600 (8)	3,630 (48)	7,510
pPEL748	JA-221	Yes, 5 h	6,780 (51)	720 (6)	5,590 (43)	13,090
pPEL749	HB101	No	63			
pPEL749	HB101	Yes	325 (86)	20 (5)	35 (9)	380

[a] pPEL342, 343, and 344 all contain the *pelB* gene; all other plasmids contain *pelE*.

[b] Unless otherwise noted, 1 mM IPTG was added at culture initiation; since IPTG severely inhibited the growth of cells containing pPEL344 or pPEL748, it was added after the noted culture times when cell density was between 0.5 and 0.7 at 600 nm; cultures were then harvested 5 or 7 h later. The fully induced level of total pectate lyase activity produced by *E. chrysanthemi* EC16 was about 500 U per g of cells (13).

[c] All cultures harvested after growth at 28°C on 15 ml of medium; growth was for 14 to 18 h in all cases except pPEL344 or pPEL748, where growth was for ca. 12 h; cell fractions were prepared and assayed as described in Materials and Methods.

tempted with several vectors carrying temperature-regulated phage lambda promoters (Table 1), but these constructs resulted in lower pectate lyase activity in *E. coli* at 37 and 42°C (100 to 200 U per g of cells) than at 32°C (200 to 600 U g^{-1}). To test whether the temperature dependence of enzyme production resulted from the use of lambda promoter vectors or was peculiar to the particular gene, the *lac*-regulated pPEL746 was examined. At lower temperatures, cells with pPEL746 produced substantially more pectate lyase in the periplasmic fraction and responded to induction by IPTG (Table 3). At 32 and 37°C, however, a large reduction in PLe activity was observed, and production was not increased by IPTG induction. Production of PLb directed by pPEL343 was also less at 37°C than at 28°C, but the decrease was much less than for PLe, and IPTG increased production of PLb at all temperatures (Table 3). The decreased expression of *pelE* at 37°C therefore appeared to explain the poor performance of constructs utilizing lambda promoters. Further expression experiments were accordingly conducted with *lac* promoter vectors in cells grown at 28°C.

Strain HB101 containing pPEL746 produced considerably higher PLe levels at 28°C than the comparable construct in pUC8, pPEL7421 (Table 2). Most of the pectate lyase activity encoded by pPEL746 occurred in the periplasmic fraction, even when IPTG was supplied. Strain JA-221 containing pPEL746 produced higher levels of pectate lyase than HB101, RB791, or 706. However, the *lac* repressor-overproducing JA-221 and RB791 strains did not yield lower levels of pectate lyase in the absence of IPTG than HB101. The periplasmic leaky strain, 706, resulted in extensive secretion of pectate lyase to the culture medium (Table 2) at all stages of growth. However, total activity was lower than with the other tested strains. The increased enzyme production conferred by pPEL746 in the presence of IPTG (Table 2, Fig. 3) was expected since the previous results (13) suggested that the *Eco*RI subcloning site was within or 3′ to the *E. chrysanthemi* promoter or the catabolite activator protein-binding site associated with the coding region of *pelE*. The constitutive level of pectate lyase production directed by pPEL746 in the absence of IPTG was unexpectedly high, however, even in strains RB791 and JA-221, which overproduce *lac* repressor (Table 2). Electrophoresis of whole *E. coli* cells bearing pPEL746 (Fig. 3) confirmed the high constitutive and induced expression of PLe but did not reveal a detectable preprotein band at ≈2 kilodaltons (kDa) above the secreted protein.

Various plasmid constructs were made to test expression of the *pelE* gene under the control of vector transcription and translation signals. An in-frame fusion with pINIII A-1, pPEL749 (Fig. 4 and 5), produced pectate lyase activity in *E. coli* as predicted, but levels were relatively low with or without IPTG in all cell fractions (Table 2). The reason for this relatively low expression is not known, although several amino acid changes at the N terminus of the pectate lyase preprotein occurred relative to the native preprotein (Fig. 5).

A control pectate lyase-negative construct of the *pelE* coding sequence was made by fusion with pINIII A-2 (Fig. 4). Of the resulting HB101 transformants, 47 were pectate lyase negative on the pectate plate assay (13) as expected; one of these was retained and called pPEL747. One transformant, however, was strongly pectate lyase positive and was also found to lack a reconstituted *Eco*RI site as seen in pPEL747 (Fig. 5). The pectate lyase-positive construct was called pPEL748. Sequencing confirmed that plasmids pPEL747 and pPEL749 had the predicted junction sequences (Fig. 5), but pPEL748 was found to be a mutant plasmid in which a 5'-terminal cytosine residue had been lost from the *Mlu*I 5' overhang during the T4 polymerase end fill. This accounted for the loss of the *Eco*RI site and for the regained pectate lyase activity, since the one-base deletion threw the pectate lyase coding region into frame with the translational start codon of the vector (Fig. 5). Unexpectedly, pPEL748 led to higher pectate lyase production in *E. coli* than any other tested construct (Table 2, Fig. 6); most of the activity was secreted into the periplasm, even in the presence of IPTG. However, pectate lyase production was also extensive in the absence of IPTG, and cells exhibited severe growth inhibition and premature lysis when IPTG was supplied at the initiation of culture growth. Although 95% or more of the pectate lyase activity was secreted into the periplasm and culture medium from IPTG-induced cells with pPEL748 (Table 2), electrophoresis of crude lysates from induced cells disclosed a putative preprotein band ca. 2 kDa above the secreted PLe protein (Fig. 6). Scanning of the lanes in Fig. 6 indicated that PLe and its preprotein constituted ca. 25% of the total cellular protein in induced cells (data not shown).

Maceration of potato tissue. As observed previously (13), *E. coli* cells containing only the plasmid vector pINIII^{113} A-2 did not macerate potato tuber cylinders at any tested concentration. Of the *pel* constructs, those expressing PLe (pPEL746 and pPEL748) were all more active than pPEL343 or pPEL344, which encode PLb (data not shown). Construct pPL748 in strains JA-221 and HB101 gave the highest macerating activity, which was comparable to or greater than that caused by *E. chrysanthemi* EC16. The periplasmic leaky strain 706 caused less maceration with plasmids pPEL746 and pPEL748 than strains JA-221 and HB101, despite its more efficient secretion of pectate lyase to the culture medium (Table 2). This may have been due to poorer viability of this strain carrying the high-expression pectate lyase constructs, since it transformed at 1% or less the frequency of the other *E. coli* strains. A detailed study of the

TABLE 3. Production of pectate lyase by *E. coli* HB101 cells containing pPEL746 or pPEL343 and grown at various temperatures[a]

Plasmid	IPTG	Pectate lyase activity (U per g of fresh cells) at:			
		25°C	28°C	32°C	37°C
pPEL746	No	1,680	1,600	300	100
pPEL746	Yes	5,550	6,100	1,300	50
pPEL343	No		5,210		1,760
pPEL343	Yes		6,150		2,150

[a] Cultures were grown for 24 h, ca. 14 h of which was stationary phase; IPTG at 1 mM was supplied at the initiation of culture growth on 15 ml of LB plus 50 μg of amplicillin per ml; flasks were shaken at ca. 100 reciprocal cycles per min; growth was similar in all cases, between 0.11 and 0.14 g (fresh weight) cells. Data are for periplasmic fractions only, but other fractions showed relative percentage values similar to those in Table 2.

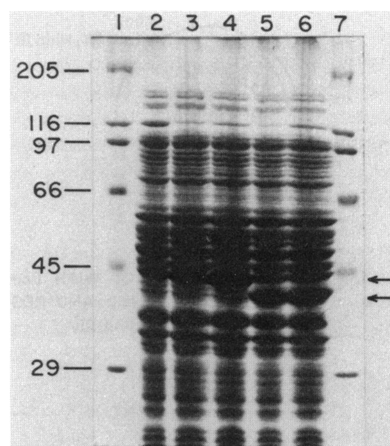

FIG. 3. SDS-gel electrophoresis of HB101 cells containing pINK-1, pPEL343, or pPEL746 grown with or without IPTG added at the start of culturing. Cultures were grown for 16 h at 28°C and, after washing, were treated with sample buffer as described in Materials and Methods, and 5 μl was applied to each lane. Lanes: 1, standards; 2, pINK-1 plus IPTG; 3, pPEL746 without IPTG; 4, pPEL746 with IPTG; 5, pPEL343 without IPTG; 6, pPEL343 with IPTG; 7, size standards. Arrows denote mature PLe (upper) and PLb (lower). Sizes of standard proteins in kilodaltons are noted on the left.

pathogenic properties of *E. coli* cells carrying the *pel* expression plasmids will be published elsewhere (Payne, Collmer, and Keen, manuscript in preparation).

Purification of the pectate lyases. HB101 cells harboring pPEL748 or pPEL344 grown at 28°C in the absence of IPTG for ca. 12 h into the stationary phase were a good source of PLe or PLb, respectively, for purification. Cells were harvested, the periplasmic fraction was prepared, and either the affinity or ion-exchange chromatographic technique described in Materials and Methods was used to purify the enzymes. Single symmetrical and coincident peaks of pectate lyase activity and material absorbing at 280 nm eluted from the CM Bio-Gel or Bio-Gel A-1.5m columns with an NaCl gradient (Fig. 7). Application of more than 3 to 5 mg of the enzymes overloaded the A-1.5m column, but as much as 10 mg adsorbed to the CM Bio-Gel column. Otherwise, the two columns performed similarly. Because no significant contaminating proteins were detected during the gradient, the columns were, after washing, eluted batchwise with 0.2 M NaCl in the Tris hydrochloride buffer to preparatively purify the pectate lyases. The suggestion of high purity noted in the elution profile from the columns (Fig. 7) was confirmed when the purified pectate lyase preparations were electrophoresed on SDS-polyacrylamide gels and when N-terminal amino acid analyses were performed. For instance, a single polypeptide which comprised 98% or more of the total was observed on SDS gels at the same position (estimated molecular mass, 43 kDa) as the PLe protein present in *E. coli* cells containing high-level expression plasmids (lane 6, Fig. 6).

DNA sequencing of the *pelE* gene. The data summarized in Fig. 8 revealed a single long open reading frame occurring between the *Eco*RI and *Sal*I sites of plasmid pPEL7421, as predicted from expression data. This is believed to encode the PLe preprotein. Confirming this suspicion, pPEL711 and pPEL712, lacking internal *Bcl*I and *Eco*RV fragments, respectively, were negative for pectate lyase (Table 2) as predicted. A purine-rich Shine-Delgarno sequence with an

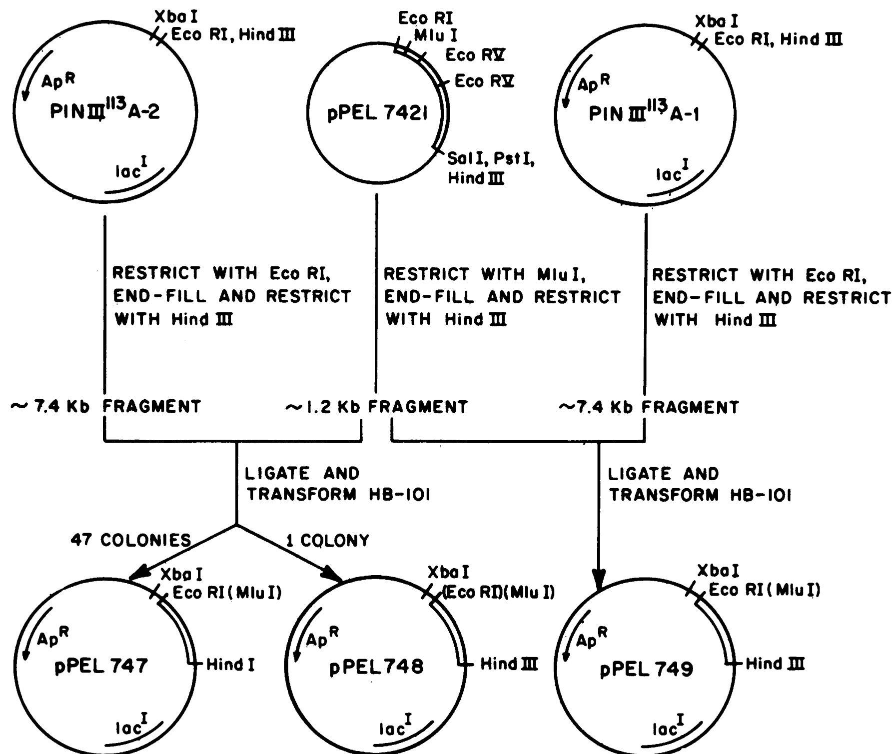

FIG. 4. Construction of fusion plasmids pPEL747, pPEL748, and pPEL749. End filling was done with T4 DNA polymerase, and fragments were isolated from soft agarose gels and ligated by the method of Crouse et al. (6).

internal AGGA was located at base 241 and was appropriately positioned 5′ to the presumed translational start codon at base 253. The peptide sequence at the amino-terminal end of the protein strongly resembles the signal peptide sequences of *E. coli* proteins that are efficiently secreted into the periplasm (36). This presumably accounts for the fact that the *E. chrysanthemi* PLe protein is efficiently secreted to the periplasm of *E. chrysanthemi* and *E. coli* cells. It is noteworthy that the latter bacteria secreted the pectate lyase very efficiently even when they contained high-expression plasmids (Table 2). Only the plasmid with the highest level of expression, pPEL748, resulted in concentrations of the putative preprotein high enough to be seen on SDS gels (Fig. 6).

The calculated molecular mass of the PLe preprotein from the amino acid sequence was 41,115 Da. Subtracting the amino acids comprising the signal sequence, the mature peptide was calculated to be 38,037 Da, about 5 kDa less than that estimated on SDS-gel electrophoresis, (13) (Fig. 3 and 6). This discrepancy is presumed to be due at least in part to the basic nature of this pectate lyase protein. Codon usage (data not shown) was very similar to that of genes which are highly expressed in *E. coli* (11). The *pelE* gene is preceded at the 5′ end by a sequence which shows a strong relationship to the consensus binding site for the *E. coli* catabolite activator protein. The *E. chrysanthemi* sequence beginning at base 103 shows homology in 9 of 13 positions with the consensus *E. coli* catabolite activator protein-binding sequence (10) and contains a properly positioned obligatory G(T/A)G sequence at base 107 (Fig. 8). It was not possible to assign putative promoter sequences with assurance, since computer searches disclosed no strong *E. coli* promoters 5′ to the coding region, but several weak promoter sequences were present. The sequence from bases 76 through 104, for instance, showed 67% homology with the *tac* promoter −35 and −10 sequences with 16-base spacing

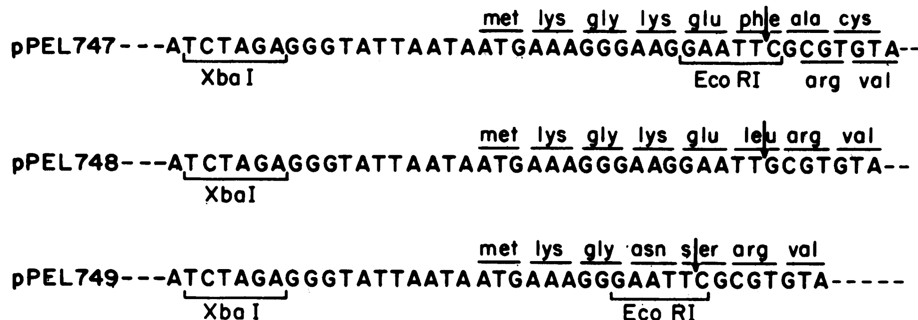

FIG. 5. Junction sequences of pINIII^{113} A-1 or A-2 translational fusions with the coding region of *pelE*, constructed as shown in Fig. 4. Arrows denote fusion sites of insert and vector DNA.

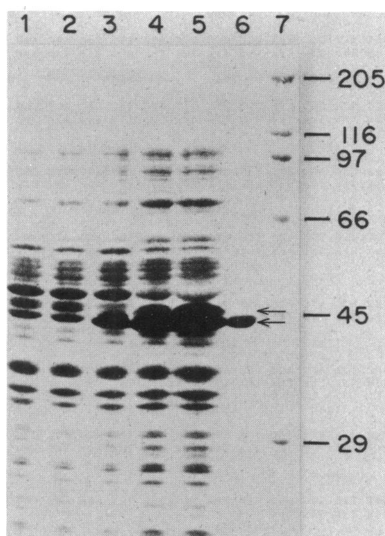

FIG. 6. SDS-gel electrophoresis of purified PLe or HB101 cells containing pINIII[113] A-2 or pPEL748. Cells were grown at 28°C on L broth plus 50 μg of ampicillin per ml, and IPTG was added to 1 mM when the cells attained an absorbance at 600 nm of 0.7. Cultures were harvested at intervals as noted in Materials and Methods, and 3-μl samples were applied to each lane. Size standards are denoted in kilodaltons at the right. The intense staining bands at ca. 44 and 46 kDa (arrows) are the mature pectate lyase and the presumed preprotein, respectively. Lanes: 1, cells containing pINIII A-2 grown without IPTG; 2, pINIII A-2 grown with IPTG for 7 h; 3, pPEL748 grown without IPTG; 4, pPEL748 with IPTG for 2 h; 5, pPEL748 with IPTG for 7 h; 6, 4 μg of mature PLe purified by affinity chromatography; 7, size standards. The photograph was underexposed to resolve the mature and presumed preprotein bands.

between the 6-base promoter elements (Fig. 8). However, physical experiments will be required to locate the *E. chrysanthemi* promoter elements with assurance. Of interest, an 11-base palindromic sequence occurred at positions 168 through 178, downstream from the putative promoter and catabolite activator protein-binding sites. It is possible that this could represent an operator sequence important in regulation of production of PLe by a *trans*-acting element produced by *E. chrysanthemi* EC16.

The region immediately 3' to the translational stop of the *pelE* gene (ca. positions 1430 through 1450, Fig. 8) was difficult to sequence with the dideoxy method until a deletion was obtained after base 1434. The resulting data revealed that this region would be predicted to function as a rho-independent transcriptional termination sequence (see Discussion). The sequence immediately following this stem-loop structure is A+T-rich and may function as a rho-dependent termination sequence (30).

Sequencing of the *pelB* gene. A single long open reading frame occurred in the predicted orientation in the 2.0-kb *KpnI*/*ScaI* insert of pPEL342 (Fig. 9). This is believed to represent the PLb preprotein. The presumed ATG translational start codon at base 714 is favorably positioned behind a Shine-Delgarno box with the same internal AGGA sequence as the *pelE* gene. Only one basic amino acid, lysine, was present in the putative signal peptide sequence. The calculated mass of the PLb preprotein was 40,213 Da, and that of the mature protein was 37,922 Da, which agrees well with the value of 39 kDa estimated by SDS-gel electrophoresis (13) (Fig. 3). Codon usage (not shown) was similar to that of highly expressed *E. coli* genes (11). A sequence beginning at base 516 (Fig. 9) was identified which would be expected to function as a strong promoter in *E. coli*, and a possible catabolite activator protein-binding site was present

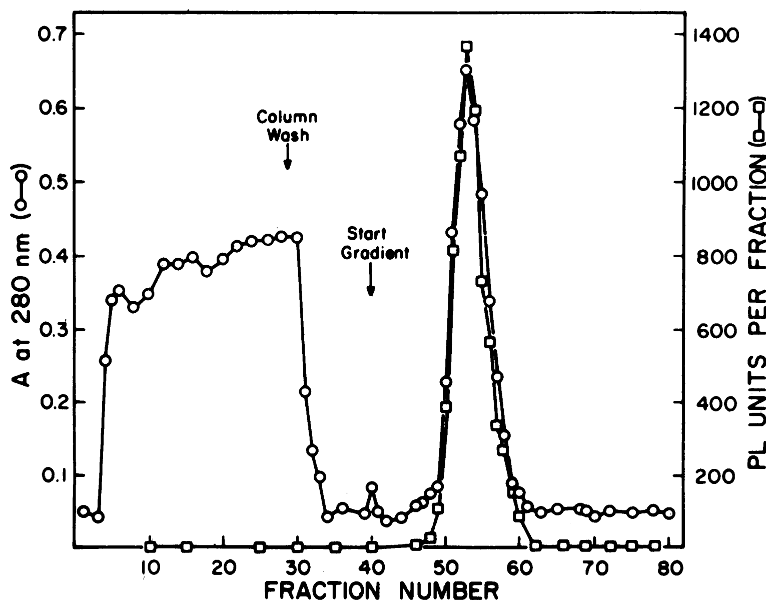

FIG. 7. Purification of PLe from the periplasmic fraction of *E. coli* HB101 cells containing pPEL746 and supplied with 1 mM IPTG. A 1.5- by 5.5-cm Bio-Gel A 1.5-m column was used as described in Materials and Methods and eluted with a linear NaCl gradient, ending at 0.2 M at fraction 80. Fractions of 3 ml were collected; 9,000 U of pectate lyase was applied to the column in 105 ml, and fractions 51 through 55 yielded 6,700 U of pectate lyase and a lyophilized dry weight of 5.2 mg after dialysis against water. Activities of several batches of similarly purified enzyme gave from 1,100 to 1,350 U of pectate lyase per mg of lyophilized enzyme.

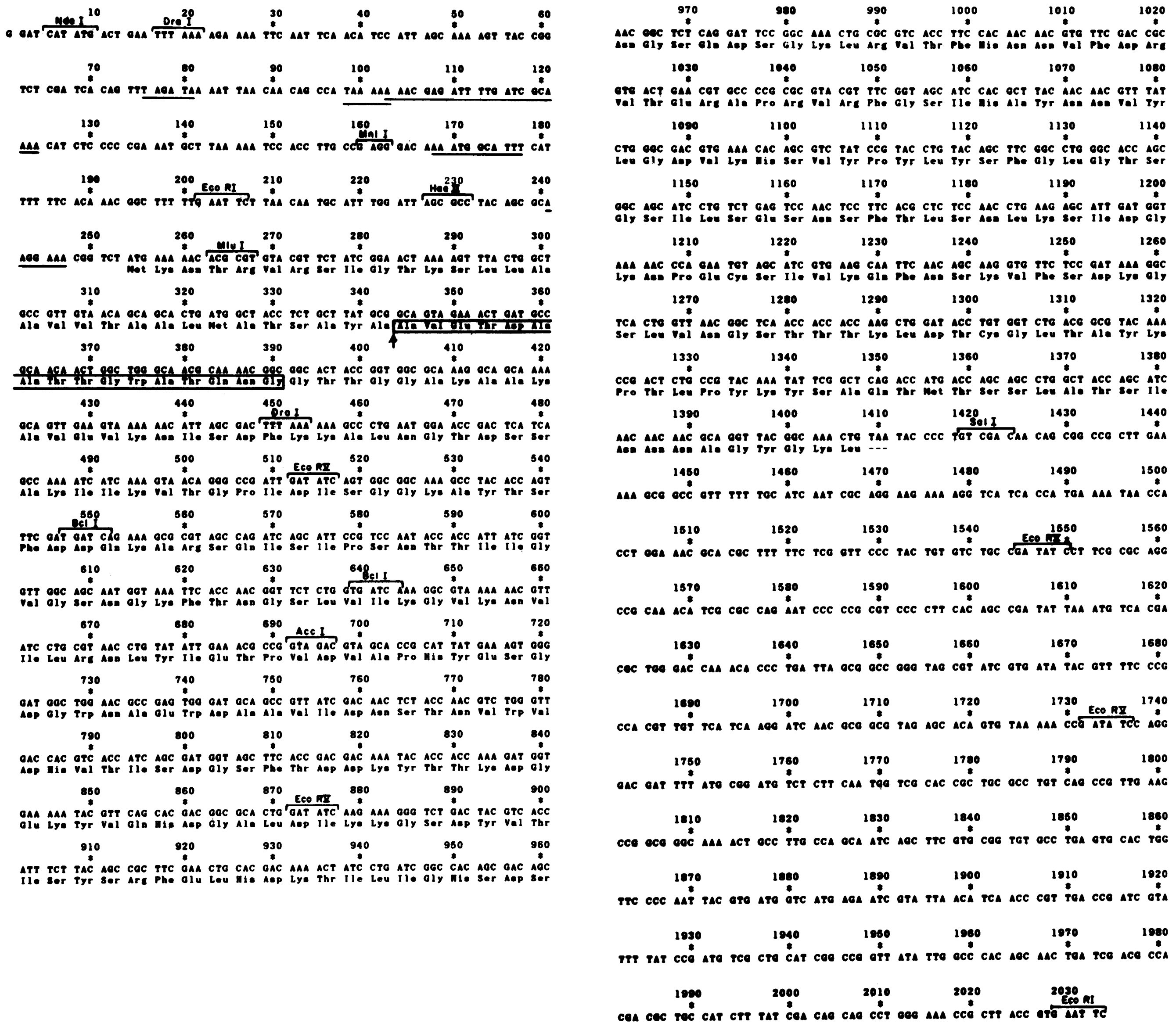

FIG. 8. DNA sequence determined for the coding region of the *pelE* gene and associated 5' and 3' sequences. The predicted amino acid sequence of the preprotein is shown along with selected endonuclease restriction sites. Possible signal sequences discussed in the text are underlined. The determined N-terminal amino acid sequence of the mature protein is boxed, and the deduced signal peptidase cleavage site is shown with an arrow.

beginning at base 558. The 11-base palindromic sequence observed in the *pelE* gene was not present in the 5' region of the *pelB* gene.

Two regions of pPEL342 proved difficult to sequence with the dideoxy method. One of these occurred between bases 295 and 320, and accurate sequencing required use of the Maxam-Gilbert method. As discussed below, this region would be expected to function as a rho-independent transcriptional terminator in *E. coli*. The second region occurred approximately between bases 1870 and 1910 (Fig. 9). Considerable secondary structure was suggested by the observed compression of gel bands in this region which were not satisfactorily resolved even by Maxam-Gilbert sequencing. We are currently attempting to isolate deletions in this region to clarify the sequence.

N-terminal amino acid sequencing. Purified preparations of the mature PLb and PLe proteins isolated from *E. coli* periplasmic fractions both had N-terminal alanine residues (Fig. 8 and 9). The first 15 N-terminal amino acids of both proteins were found to agree exactly with the respective sequences predicted from the DNA sequence data (Fig. 8 and 9). These observations permitted deduction of the amino acids constituting the signal peptide sequences of both preproteins.

DISCUSSION

Although sequence data have not been reported for *pel* genes from other *E. chrysanthemi* strains, the EC16 gene coding for the pI 9.8 pectate lyase appears analogous to the

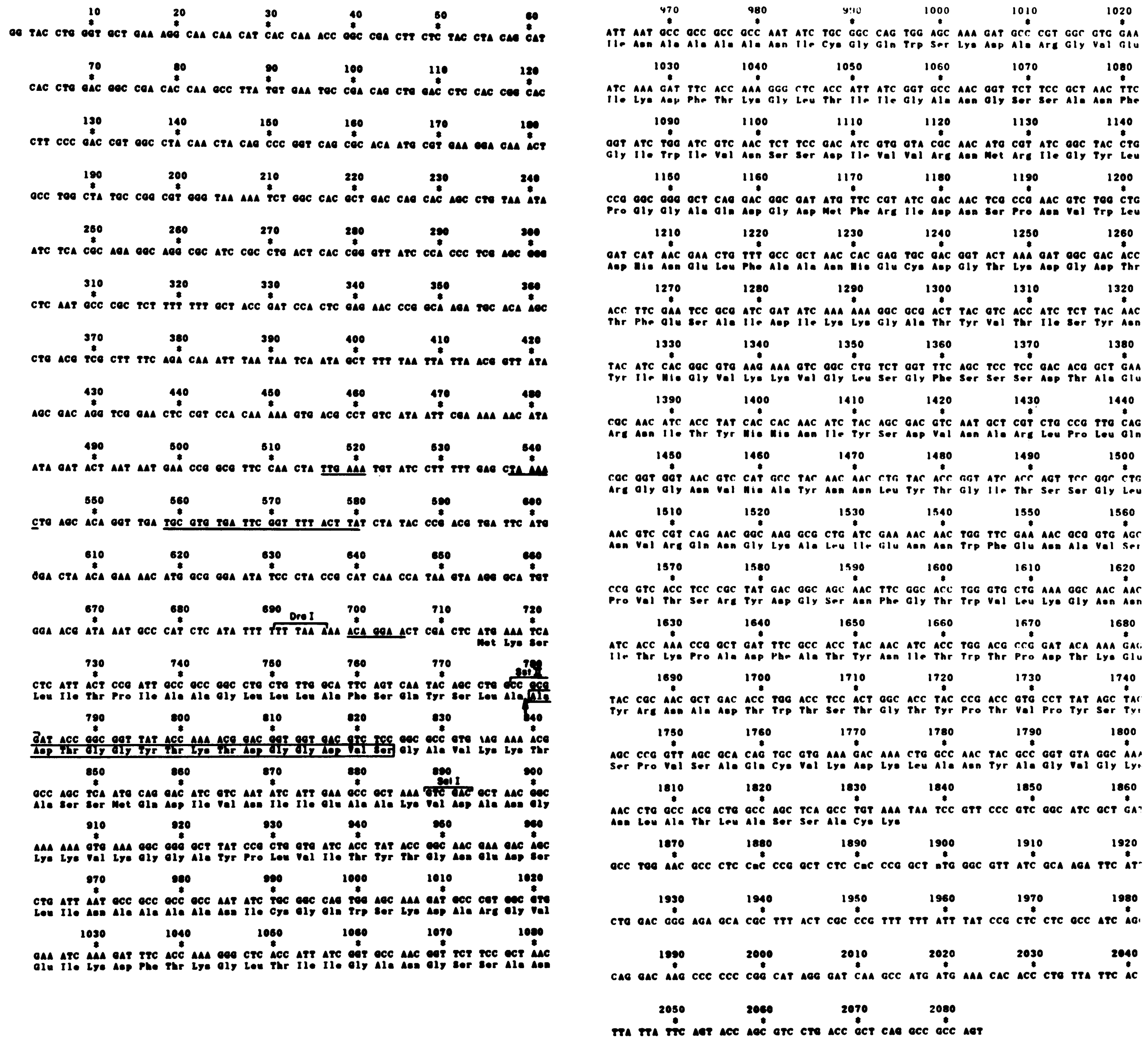

FIG. 9. DNA sequence determined for the coding region of the *pelB* gene and flanking DNA in pPEL342. The predicted amino acid sequence of the preprotein is shown, and selected restriction sites are noted. Possible signal sequences discussed in the text are underlined. The determined N-terminal amino acid residues of the mature protein are boxed, and the deduced signal peptidase cleavage site is denoted with an arrow. The G+C-rich region from bases 1,880 through 1,900 could not be accurately determined with either dideoxy or Maxam-Gilbert sequencing. Ambiguous bases are denoted by n, but the region may contain other errors.

pelE gene defined in other strains of *E. chrysanthemi* (14, 28, 34). Our pI 9.8 enzyme also conforms to the same pectate lyase isolated from strain EC16 by Thurn et al. (Abstract, VI International Conference on Plant Pathogenic Bacteria, Beltsville, Md., 1985) and reported by them to have an isoelectric point of 9.9. This agrees closely with our value, but neither matches well with the pI values reported for the *pelE* gene products from certain other strains of *E. chrysanthemi* (14, 28). The basis of these differences has not been established.

Subclone pPEL342 has been shown by sequencing to code for only one pectate lyase with a pI of 8.8 as previously determined by column electrofocusing (13). The gene coding for this enzyme appears to be *pelB*, described from other strains of *E. chrysanthemi* (5, 14, 28, 34). It was subcloned from a pHC79 cosmid clone, pPEL3 (13), and column electrofocusing originally resolved only one pectate lyase enzyme from *E. coli* cells bearing this clone. However, Gary Wilcox subsequently resolved two different pectate lyase enzymes with slightly different molecular weights encoded by pPEL3 (unpublished data), a conclusion subsequently confirmed by K. K. Thurn and A. K. Chatterjee (Abstract, VI Int. Conf. Plant Pathogenic Bacteria). Subclones pPEL34 and pPEL342, however, produced only one detectable pect-

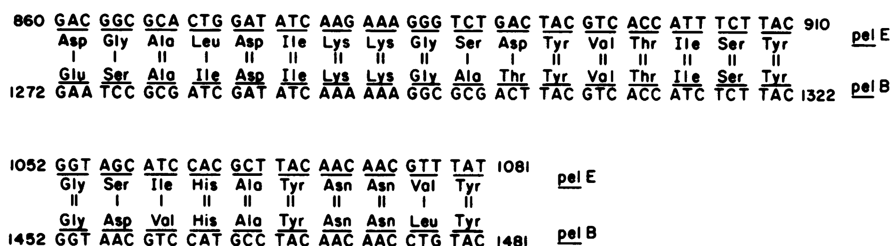

FIG. 10. Amino acid sequences of two different regions of the *pelB* and *pelE* genes which exhibit significant homology. The DNA base numbering at the 5′ and 3′ termini of the sequences is from that in Fig. 8 and 9. Double lines connect identical amino acid residues, and single lines connect functionally homologous amino acids.

ate lyase. Collmer (personal communication) and Chatterjee (personal communication) have observed that the isoelectric point of this gene product conforms to PLb. It therefore appears that cosmid clone pPEL3 contains two different genes, *pelB* and *pelC*, and that only the *pelB* gene was subcloned during the construction of pPEL34. Kotoujansky et al. (Abstract, VI Int. Conf. Plant Pathogenic Bacteria) suggested that the *pelB* and *pelC* genes of strain B374 are separated by only a few hundred base pairs. A computer homology search of our sequence data in Fig. 9 revealed considerable DNA homology between bases 1 through 243 and 1599 through 1841. Furthermore, 67 of 80 amino acid residues are identical in the two translated sequences, and both are terminated by TAA. These data lead to the suspicion that bases 1 through 243 in Fig. 9 constitute the 3′ coding end of the *pelC* gene. Sequencing and expression of the gene will be required to test this prediction.

We did not detect genes analogous to *pelA* or *pelD* in our cosmid library from strain EC16, despite the fact that these genes have been reported to be linked to *pelE* in certain other *E. chrysanthemi* strains (14, 34). It is not clear whether these genes were not detected in the original cloning (13) or whether they are not well expressed in strain EC16 or *E. coli*. Kotoujansky et al. recently showed that *pelD* mapped close to one end of *pelE* in two different strains of *E. chrysanthemi* (Abstract, VI Int. Conf. Plant Pathogenic Bacteria). Inspection of our sequence data in Fig. 8 indeed disclosed the occurrence of a long open reading frame initiating at base 1489 and extending to the *Eco*RI terminus of the sequenced DNA. Neither of the other reading frames nor any frame of the reverse complement constituted an open reading frame of significant length. Further, the translated sequence beginning at base 1489 possesses a recognizable signal peptide sequence. This DNA may comprise the 5′

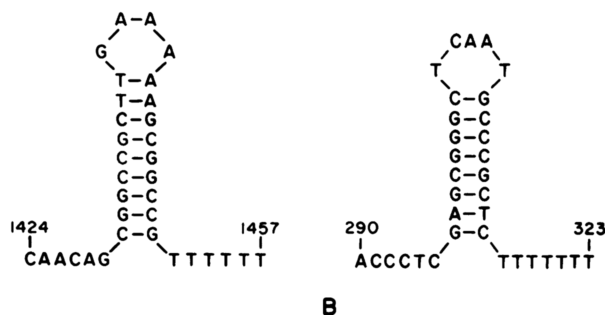

FIG. 11. Sequences at the 3′ noncoding ends of the *pel* genes which form RNA structures expected to function as rho-independent transcriptional terminators. (A) 3′ sequence of the *pelE* gene; (B) 3′ sequence of the putative *pelC* gene.

end of the *pelD* gene of strain EC16, but the possibility has not yet been tested. Significantly, this reading frame occurs immediately 3′ to the *pelE* transcriptional terminator (Fig. 8; see Fig. 11A), which might block expression.

Unlike *E. chrysanthemi* EC16, which efficiently secretes pectate lyase to the culture medium, the majority of both pectate lyases was found in the periplasmic space of *E. coli* cells, except for the periplasmic leaky strain, 706. It is noteworthy that Andro et al. (2) and Thurn and Chatterjee (33) have obtained data indicating that *E. chrysanthemi* produces specific gene products which are necessary for the efficient transport of pectate lyase and certain other proteins from the periplasmic space to the culture medium. The absence of these genes in *E. coli* would explain the predominantly periplasmic nature of the pectate lyase proteins.

Cells of *E. coli* containing pPEL343 or pPEL344 produced high levels of PLb, but did not macerate potato tubers as efficiently as cells producing PLe. It is therefore possible that PLe more efficiently macerates potato tuber tissue, a conclusion also reached by Thurn and Chatterjee with the purified enzymes (Abstracts, VI Int. Conf. Plant Pathogenic Bacteria). Although a relatively small proportion of PLe was secreted to the medium of *E. coli* (Table 2), cells with high-expression plasmids efficiently macerated potato tuber slices at minimal cell concentrations similar to *E. chrysanthemi* E16. Payne et al. (in preparation) have recently confirmed these findings and have also observed that *E. coli* cells producing PLe multiply in inoculated potato tubers. It is significant that the plasmid-directed synthesis of a single pectate lyase enzyme permitted the normally saprophytic *E. coli* to cause typical soft-rot disease symptoms in plant tissue. Thus, the cloned *pelE* gene is the sole requirement to convert *E. coli* into an efficient plant pathogen under laboratory conditions.

The mutant translational fusion plasmid pPEL748 led to considerably greater production of PLe in *E. coli* cells than the related construct, pPEL749 (Table 2). The latter construct resulted in one additional amino acid in the N-terminal region relative to the wild-type preprotein, whereas pPEL748 codes for two extra amino acids (Fig. 6 and 8). Although it is possible that other changes occurred in the sequence of pPL748, one reason for the lower expression of pPEL749 may be the introduction of AAT and TCG codons at the fusion junction (Fig. 5), both of which occur infrequently in genes that are highly expressed in *E. coli* (11). On the other hand, pPEL748 contains the codons GAA and TTG at the fusion junction; these codons are more frequently used.

Despite the fact that *pelB* and *pelE* both express well in *E. coli* and encode basic, secreted proteins of similar molecular weight which catalyze the same chemical reaction, the two genes are dissimilar both in the coding and flanking DNA regions (Fig. 8 and 9). Computer searching revealed only two

regions of 10 and 16 functionally homologous amino acid stretches in the two genes (Fig. 10). Both pectate lyase genes have signal peptide sequences which are cleaved between Ala Ala residues but otherwise are very different from each other (Fig. 8 and 9). Both signal sequences have clearly recognizable N, H, and C regions (35), but the *pelE* gene has a longer N region which contains three additional basic amino acid residues. Despite the differences in the sequences, secretion directed by both of the leader sequences appeared to be very efficient in *E. coli*, since even at the >20% total protein level directed by the high-expression constructs, most of the enzymes were secreted to the periplasm (Table 2).

The two *pel* genes also differ greatly in the 5′ noncoding regions. Although both genes have identifiable catabolite activator protein-binding sequences (10), they are very different (Fig. 8 and 9). Their function in *E. coli* is supported by the observation that deletion of the sites from the cloned genes abolished catabolite repression (13) and the demonstration that the *cya* gene from *E. chrysanthemi* complemented adenyl cyclase-deficient *E. coli* mutants (12). Coupled with the finding of Mount et al. (23) that *cya* mutants of the related bacterium *E. carotovora* are repressed for pectate lyase production in the absence of exogenous cyclic AMP, our results suggest that catabolite repression of the *E. chrysanthemi* pectate lyase genes functions by the same mechanism as in *E. coli*. Finally, unique sequences resembling rho-independent transcriptional terminators (32, 39) were observed 3′ to the *pelE* gene and the putative *pelC* gene (Fig. 11). The *pelB* gene also has a G+C-rich sequence 3′ to the coding region which may function as a transcriptional terminator, but we have not yet been able to accurately determine this sequence (Fig. 9). Significantly, *E. chrysanthemi* and *E. carotovora* (3, 24) have been shown to contain rho termination factors similar to *E. coli*. It is therefore of interest that the three different *pel* genes from *E. chrysanthemi* EC16 appear to be independently regulated and probably encode unique monocistronic transcripts.

Lei, Lin, Heffernan, and Wilcox (manuscript in preparation) sequenced one of several closely linked genes coding for pectate lyases in *E. carotovora* EC. The signal peptide sequence and coding sequence for the mature protein exhibited considerable DNA and amino acid sequence homology to our *pelB* gene from *E. chrysanthemi* (Fig. 9). In the common regions of the two proteins, 84% of the amino acids were identical. The major difference was that the *E. carotovora* protein was truncated by 46 amino acids at the carboxyl end. It is therefore probable that the *E. carotovora* gene evolved from the *E. chrysanthemi pelB* gene or its ancestral gene.

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