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Analysis of the *pelE* Promoter in *Erwinia chrysanthemi* EC16

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The *pelE* gene of *Erwinia chrysanthemi* strain EC16 encodes an extracellular pectate lyase protein that is important in virulence on plants. Control of *pelE* expression is complex, because the gene is regulated by catabolite repression, substrate induction, and growth-phase inhibition. A Tn*7*-*lux* reporter gene system was employed to define DNA sequences comprising the *pelE* promoter. When EC16 cells were grown on medium containing sodium polypectate, *pelE* transcriptional start sites were observed only at 95 and 96 bases upstream of the translational start site. However, DNA sequences required for *pelE* expression were also shown by deletion analysis to reside between 196 and 215 base pairs upstream of the translational start site. In addition to these upstream elements, two putative operator sequences that interact with negative regulatory factors occurred downstream of the transcriptional start. Finally, deletion of three bases from a putative catabolite gene activator protein binding site in the *pelE* promoter eliminated activity. The data demonstrate that the *pelE* promoter is complex and suggest that it interacts with several regulatory proteins.

Additional keywords: gene regulation, pectic enzymes.

Pectate lyase (PL) enzymes are important in the bacterial soft-rot diseases caused by *Erwinia* spp. (Boccara *et al.* 1988; Collmer and Keen 1986; Kotoujansky 1987; Ried and Collmer 1988). Most strains of *E. chrysanthemi* Burkholder, McFadden and Dimock produce five extracellular pectate lyase enzymes, but strain EC16 produces only four (Barras *et al.* 1987; Tamaki *et al.* 1988). In this latter strain, PLb and PLc have moderately basic pI values, whereas PLa is acidic and PLe is strongly basic. DNA sequence data also disclosed a DNA remnant of the coding region of an additional gene encoding a strongly basic PL that is produced by other *E. chrysanthemi* strains (Tamaki *et al.* 1988). It was therefore concluded that the absence of a fifth PL in strain EC16 was caused by a prior deletion event, which removed most of the coding region for one of the *pel* structural genes. Collmer *et al.* (1991) have also recently shown that additional *pel* genes are harbored by strain EC16, which are only expressed when the bacteria are grown in the presence of plant extracts or cell walls.

Despite the fact that the characterized *E. chrysanthemi* *pel* genes occur in two tandem arrays on the chromosome, DNA sequence and reporter gene data showed that all of them are independently regulated (Keen and Tamaki 1986; Reverchon *et al.* 1986; Tamaki *et al.* 1988). For instance, Beaulieu and van Gijsegem (1990) recently showed that *pelA* in *E. chrysanthemi* strain 3937 was specifically induced by an extract from plant tissue. The genes encoding the basic PLs are generally inducible when the bacteria are grown on plant tissue or culture medium containing the substrate polygalacturonic acid; the neutral PLs are produced at a significant basal level but induced to higher activities in the presence of polygalacturonic acid (Pupillo *et al.* 1976; Tamaki *et al.* 1988). Generally, pectic enzyme production in *Erwinia* spp. is induced by metabolic degradation products of polygalacturonic acid, as first shown by Tsuyumu (1977).

Of the PLs produced by strain EC16, several lines of evidence show that PLe is the most important for bacterial virulence in plants (Barras *et al.* 1987; Ried and Collmer 1988). DNA sequence data also previously identified sequences 5′ to the *pelE* coding region that might account for the observed substrate induction by polygalacturonic acid and catabolite repression (Keen and Tamaki 1986). In this paper, we describe experiments designed to define elements comprising the *pelE* promoter by employing promoter fusions with a Tn*7*-*lux* cassette.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains, plasmids, and Tn*7*-*lux* integrants are shown in Table 1. Bacteria were routinely grown in or on M9 or LB media (Maniatis *et al.* 1982) or on King's medium B (KMB, King *et al.* 1954). Bacto agar (Difco Laboratories) was added to 1.5% for solid medium plates. Pectate lyase production was assayed on YC-pectate plates prepared as previously described (Keen *et al.* 1984). *Escherichia coli* (Migula) Castellani and Chalmers strains and *E. chrysanthemi* strain EC16 (EC16) were grown at 37° and 28° C, respectively. Media were amended with apppropriate antibiotics (Sigma, St. Louis, MO) at the following concentrations: ampicillin (Ap, 50 μg/ml), streptomycin (Sm, 25 μg/ml), spectinomycin (Sp, 50 μg/ml), and gentamicin (Gm, 15 μg/ml). For long-term storage, bacteria were maintained at −80° C in 15% glycerol.

General DNA manipulations. Large-scale plasmid and chromosomal DNA preparations were carried out by CsCl isopycnic gradient centrifugation in the presence of

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Table 1. Bacterial strains, bacteriophage, plasmids, and *Tn7-lux* integrants used

	Description	Source or Reference
Bacterial strains		
Escherichia coli		
DH5α		BRL
MV1193		Vieira and Messing 1987
SM10		Simon *et al.* 1983
Erwinia chrysanthemi		
EC16	Wild-type strain	A. Chatterjee, Univ. of Missouri
Ec16::Tn*7*E*lux* series	EC16 integrants of various Tn*7-lux pelE* promoter constructs	This work
Phage		
M13K07	Helper phage	Vieira and Messing 1987
Plasmids		
pUC18	Cloning plasmid	Yanisch-Perron *et al.* 1985
pUC118/119	Ap^r cloning/sequencing vectors	Vieira and Messing 1987
pUC129	Ap^r cloning plasmid	Keen *et al.* 1988
pMTL20p	Ap^r cloning plasmid	Chambers *et al.* 1989
pBluescript KS+	Ap^r cloning vector	Stratagene
pHSK728/729	$Ap^r/Sm^r/Sp^r$ Tn*7-lux* transcriptional fusion vectors	H. Shen, unpublished
pMON7181	Gm^r helper plasmid carrying genes for Tn*7* transposition	Barry 1988
pPEL743	Clone carrying *pelE* and 5′ sequences	Tamaki *et al.* 1988
pPEL7432	2.0-kb *Hin*dIII-*Sal*I fragment from pPEL743 cloned into the same sites of PUC119	This work
pPEL744	1.1-kb *Kpn*I-*Eco*RI fragment containing the *pelE* 5′ region and the 3′ end of *pelA* cloned in the same sites of pUC18	This work
pPEL763	496-bp *Ssp*I-*Eco*RI fragment from pPEL744 cloned into the *Sma*I-*Eco*RI sites of pUC118	This work
pPEL764	235-bp *Hpa*I-*Eco*RI fragment from pPEL744 cloned into the *Sma*I-*Eco*RI sites of pUC118	This work
pBSE1	Plasmid used for *pelE* 5′ deletions; a 596-bp *Bgl*II-*Eco*RI fragment from pPEL744 was cloned into the *Bam*HI-*Eco*RI sites of pBluescript KS+; deletions were subconed as *Sac*II-*Eco*RI fragments	This work
pElux81	Same as pBSE1, but cloned in pHSK728	This work
pPEL790	656-bp *Bgl*II-*Mlu*I fragment from pPEL744 cloned into the same sites of pMTL20p	This work
pElux790	Same insert as pPEL790 but cloned into the *Bam*HI-*Xba*I sites of pHSK729	This work
pElux91	Same as pElux81, but cloned in pHSK729	This work
pElux83	*Bam*HI-*Eco*RI fragment from pPEL764 cloned in the same sites of pHSK728	This work
pElux780	Oligonucleotide mutant in which bases +43 to +46 of the *pelE* promoter were deleted (TGAA) and the resulting *Bgl*II-*Mlu*I fragment was cloned into pHSK729 so that the promoter was in the same orientation as the *lux* cassette	This work
pElux781	Oligonucleotide mutant in which bases +32 to +35 of the *pelE* promoter were deleted (AAAC) and the resulting 595-bp *Bgl*II-*Eco*RI fragment was cloned into pHSK728 so that the promoter was in the same orientation as the *lux* cassette	This work
pElux782	Oligonucleotide mutant in which bases +17 to +20 of the *pelE* promoter were deleted (ATTT) and the resulting 595-bp *Bgl*II-*Eco*RI fragment was cloned into pHSK728	This work
pElux783	Oligonucleotide mutant in which bases -41 to -43 of the *pelE* promoter were deleted (TGA) and the resulting fragment recloned into pHSK728 as with pElux782	This work
pElux784	Oligonucleotide mutant in which the deletions in both pElux781 and pElux782 were introduced into the *pelE* promoter and the resulting fragment recloned in pHSK728 as with pElux782	This work
pPEL791	506-bp *Xho*I, *Bgl*II-*Bsa*BI fragment from pPEL790 cloned into the *Xho*I-*Eco*RV sites of pUC129; pPEL790 DNA was sensitive to overlapping *dam* methylation for restriction by *Bsa*BI and the plasmid was therefore increased in a *dam*$^-$ *E. coli* strain	This work
pPEL792	148-bp *Bsa*BI-*Mlu*I, *Xba*I fragment from pPEL790 (grown in a *dam*$^-$ strain as noted above) cloned into the *Eco*RV-*Xba*I sites of pUC129	This work
pPEL793	459-bp *Xho*I,*Bgl*II-*Bsa*I fragment from pPEL790 endfilled and cloned into the *Eco*RV site of pUC129	This work
pPEL794	201-bp *Bsa*I-*Mlu*I, *Xba*I fragment from pPEL790 endfilled and cloned into the *Eco*RV site of pUC129	This work
pPEL794a	Same as pPEL794, but opposite insert orientation	This work
pElux794a	*Kpn*I-*Xba*I insert fragment from pPEL794 cloned into the same sites of pHSK729, in the same orientation as the *lux* cassette	This work
pElux794	*Kpn*I-*Xba*I insert fragment from pPEL794 cloned into the same sites of pHSK729, in the opposite orientation as the *lux* cassette	This work
pElux832-pElux834	Selected exoIII deletions of the *pelE* promoter region of pElux81	This work
placlux	*lac* promoter region subcloned from pUC18 as a 232-bp *Pvu*II-*Eco*RI fragment and subcloned into the *Sma*I-*Eco*RI sites of pHSK728	This work

ethidium bromide (Maniatis *et al.* 1982). Mini-preparations of plasmid DNA were prepared by the method of Zhou *et al.* (1990). Subcloning of DNA fragments was generally achieved by recovery from low melting point agarose gels (Crouse *et al.* 1983). Southern blot hydridizations were carried out using the Genius kit according to the manufacturer's protocols (Boehringer Mannheim). DNA sequencing and primer extension experiments were analyzed on standard 6% polyacrylamide, 8 M urea sequencing gels, using methods previously employed in our laboratories (Tamaki *et al.* 1988).

DNA sequencing. Nucleotide sequence analyses were performed by the dideoxy chain termination method with the Sequenase kit (U. S. Biochemicals, Cleveland, OH). Deletion clones for sequencing were constructed in the vector pBluescript KS+ (Stratagene, La Jolla, CA). Single-strand templates were generated by superinfection of *E. coli* MV1193 cells with the helper phage M13K07 (Vieira and Messing 1987) as previously described (Tamaki *et al.* 1988). Data were analyzed with the software package of Pustell and Kafatos (1984).

Tn*7-lux* system. The Tn*7-lux* system of Barry (1988) was utilized for insertion into the EC16 chromosome of cassettes including cloned promoter fragments ahead of the *lux* operon (H. Shen, S. Gold, and N. Keen, unpublished). This system was employed to continuously monitor the promoter activity of various *pelE* deletion mutants during growth on medium supplemented with glucose and/or polypectate. Cloned DNA fragments were ligated into the carrier plasmids, pHSK728 or pHSK729, which contain the *lux* cassette from *Vibrio fischeri* (Beijerink) Lehmann and Neumann in a pUC19-based plasmid (Fig. 1). As described in detail later, the resulting constructs were electroporated into EC16 cells containing the helper plasmid, pMON7181, which includes the Tn*7* transposase functions (Barry 1988), and integrated into the single chromosomal Tn*7* site.

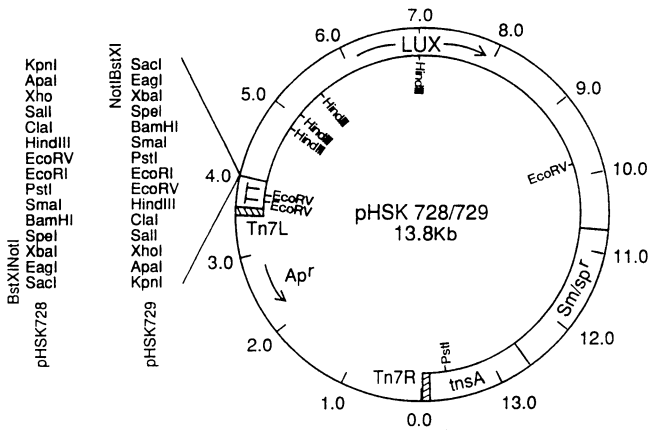

Fig. 1. Restriction map of pHSK728 and pHSK729, used to clone various promoter elements and introduce them into the chromosome of *Erwinia chrysanthemi* EC16. The plasmids were constructed by H. Shen and are fully described elsewhere. They include the ampicillin resistance gene and replication origin of pUC19 as well as the *tnsA* gene and left and right Tn*7* borders (Barry 1988), the *lux* cassette from *Vibrio fischeri* (Baldwin *et al.* 1989), a gene conferring resistance to streptomycin and spectinomycin (Ubben and Schmitt 1987), and the *pelE* gene transcriptional terminator (TT) (Keen and Tamaki 1986). The two polylinkers for cloning of promoter fragments were derived from pBluescript KS+ and are located upstream from the first translational start of the *lux* operon.

Deletions in the *pelE* promoter. Plasmid pBSE1 (Table 1) was used to construct unidirectional deletions in the 5′ region upstream from the *pelE* coding sequence. The plasmid was digested with *Not*I, endfill protected with alpha-thiotriphosphate deoxynucleotides in the presence of Klenow fragment, and then digested with *Xba*I. Deletions were performed by the method of Henikoff (1984) with the Erase-a-Base kit (Promega Biotec, Madison, WI). Selected clones were then sequenced to identify the exact breakpoints of each deletion.

After localization of the *pelE* promoter region with exoIII deletions, additional subclones were obtained by restricting pPEL790 with *Bsa*I or *Bsa*BI (see Fig. 2) as described in Table 1. The promoter regions upstream and downstream from these restriction sites were recloned into pUC129, and the deletion endpoints were confirmed by DNA sequencing.

To further define putative *cis*-acting elements in the *pelE* promoter, oligonucleotide site-directed deletion mutations were introduced by preparing appropriate oligonucleotides and selecting mutants by the method of Kunkel *et al.* (1987). Appropriate primers were synthesized and annealed to single-strand DNAs, prepared as for sequencing. The double mutant in Elux784 was constructed by annealing the mutated primer used for the construction of pElux782 to single-strand DNA from pElux781 (Table 1). These and the other mutations noted in Figure 2 and Table 2 were all confirmed by DNA sequencing of the relevant regions. All exoIII deletion clones were recloned into pHSK728 as *Eco*RI-*Sac*II fragments. The other deletion constructs

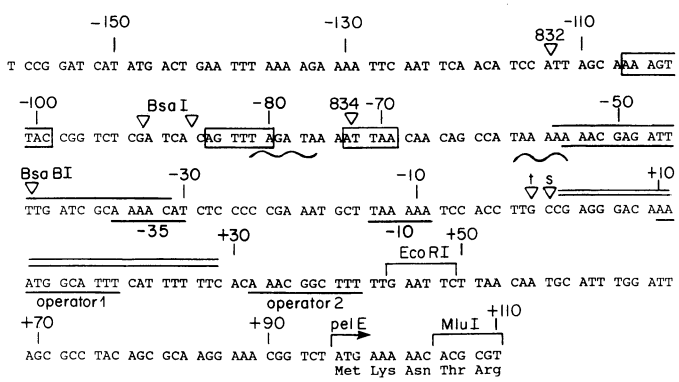

Fig. 2. Summary of promoter analysis of the *pelE* gene in *Erwinia chrysanthemi* EC16. Numbering is based on the transcriptional start sites (shown with arrows labelled 'ts') deduced from primer extension experiments. The translational initiation ATG codon is shown at base +96 with an arrow as well as the first five triplets of the coding region. *Eco*RI and *Mlu*I restriction sites utilized for the construction of *lux* fusions are shown as well as the cleavage sites for *Bsa*I and *Bsa*BI (arrows), used to construct deletion clones noted in the text. Two deletion sites are shown for *Bsa*I, a consequence of the endfill reactions used to construct Elux793 and Elux794a (see Table 1). The −35 and −10 boxes defined by primer extension experiments are underlined. Sequences with homology to −10 and −35 boxes occurring at −81 and −59 are underscored with wavy lines. Exonuclease III deletions that affected *pelE* promoter activity are denoted by numbered arrows. A sequence closely matching the *E. coli* consensus CAP site (de Crombrugghe *et al.* 1984) is shown by a single overline and a *kdgR* binding site (Reverchon *et al.* 1989) is shown with double overlines. Two putative operator sequences are underlined. Boxed sequences indicate conserved bases occurring in several *pelD/E* genes discussed in the text.

in pUC129 or pMTL20 were recloned into pHSK728 or pHSK729 by using appropriate polylinker restriction enzyme sites (Table 1). To ensure accuracy of all constructs in pHSK728 or pHSK729, CsCl-purified DNAs were prepared and the *pelE* promoter regions were confirmed by sequencing. These purified DNAs were then electroporated into EC16 (pMON7181) cells and, following transpositional insertion of the loaded *lux* cassettes into the EC16 chromosome, the resulting cells were assayed for bioluminescence.

Introduction of DNA into *Erwinia* cells. The Tn*7* helper plasmid, pMON7181 (Barry 1988), was introduced into EC16 cells by conjugation from *E. coli* strain SM10 (Simon *et al.* 1983). Transconjugants were selected on M9 medium amended with Gm and confirmed as derived from EC16 by the ability to produce pectate lyase on YC-pectate plates. The various promoter constructs cloned in the Tn*7-lux* vectors pHSK728 or pHSK729 (Fig. 1) were introduced into EC16 (pMON7181) by electroporation. For preparation of competent cells, cultures in 250 ml of LB supplemented with Gm and grown at 37° C were harvested at an optical density at 600 nm of 0.5–0.7. The cells were cooled to 0° C in an ice bath and pelleted by centrifugation for 5 min at 5,000 × *g*. The cells were then resuspended in 100 ml of ice-cold 10% glycerol in water (filter sterilized) and again pelleted. The cells were washed three times in this way, and the final pellet was resuspended in 10 ml of ice-cold 10% glycerol. The cells were used directly for electroporation or frozen over dry ice and stored at −80° C before use.

One-tenth to one microgram of mini-preparation or CsCl-purified DNA of the various pHSK promoter constructs was introduced into competent EC16 (pMON7181) cells with a Gene Pulser apparatus (Bio-Rad, Richmond, CA), set at 5 kV/cm, 25 μF, and 200 ohms. The competent cells (100 μl, kept on ice) were mixed with 1–10 μl of DNA in Tris-EDTA in ice-cold Bio-Rad cuvettes with a 0.2-cm electrode gap. Immediately after electroporation, 0.5 ml of LB at room temperature was added, and the cells were incubated for 2.0–2.5 hr at 28° C with shaking (greatly reduced efficiency was observed if the cells were incubated at 37° C). Transformants were selected by plating the mixture onto plates of LB Gm-Sm. The plates were incubated for 24 hr at 37° C and single colonies were selected for analysis. The above protocol resulted in 10^2 to 10^3 colonies per microgram of DNA electroporated, using either fresh or frozen competent cells.

To cure electroporated cells of plasmids and permit selection of Tn*7-lux* transposition events, electroporated Sm^r-Gm^r colonies were restreaked onto LB Sm plates and rapidly growing single colonies were transferred to 5 ml of nonsupplemented LB medium in tubes and grown overnight at 37° C. Ten microliters of the cell suspension was transferred after about 12 hr to a fresh LB tube. Similar transfers were repeated a total of three or four times, after which 20–100 μl of the cell suspension was plated onto LB Sm plates. Single Sm^r/Sp^r colonies were transferred onto LB plates amended with Ap or Gm or onto YC pectate plates to test for pectic enzyme production. Cured colonies with the Sm^r Sp^r Gm^s Ap^s pel^+ phenotype were retained for *lux* analyses and stored at −80 C in LB medium with 15% glycerol. After three rounds of growth in unsupplemented LB medium, 50–90% of the Sm^r/Sp^r colonies were cured of plasmids, as determined by antibiotic sensitivity.

Bioluminescence assays. Light production by EC16 *pel-lux* integrants was measured by using a Beckman LS3801 scintillation counter equipped with a single photon monitor. Bacteria (about 10^8/ml) were grown at 23° C in standard scintillation vials containing 5 ml of LB medium or in microfuge tubes with 0.5 ml of LB medium. Replicate tubes of the same bacteria were also grown on LB medium supplemented with sodium polypectate at 0.1% (w/v) or with 0.1% sodium polypectate and 0.2% glucose. KMB or M9 media were substituted for LB in some experiments. Light production was measured periodically for 1 min, with continuous cycling over a period of 6 hr or longer. Aliquots (0.2 ml) of the cell suspensions were periodically removed and the optical density at 600 nm was determined. Bacterial cell numbers were calculated assuming a conversion factor of 8×10^8/ml/optical density unit (Maniatis *et al.* 1982).

Expression of the *pel-lux* constructs in EC16 was also measured when the bacteria were inoculated onto potato tuber pieces. Cylinders (1.2 cm diameter × 1 cm thick) were cut from store-bought Russet-Burbank tubers with a #6 cork borer, and wells 0.4 cm wide and 0.5 cm deep

Table 2. Production of light on various media by *Erwinia chrysanthemi* EC16 cells carrying Tn*7-lux* fusions with various deletions or mutations of the *pelE* promoter region

Construct	Description	Light Production[a]		
		LB	LB/NaPP	LB/NaPP glucose
Elux790	656-bp *pelE* promoter	−	+	−
Elux81	596-bp *pelE* promoter	−	+	−
Elux91	596-bp inverted promoter	−	−	−
Elux83	235-bp *pelE* promoter	−	+	−
Elux832	161-bp 5′ deletion mutant	−	+	−
Elux834	121-bp 5′ deletion mutant	−	−	−
Elux780	Deletion of bp +43 to +46	−	+	−
Elux781	Deletion of bp +32 to +35	+	+	+
Elux782	Deletion of bp +17 to +20	+	+	+
Elux783	Deletion of bp −41 to −43	−	−	−
Elux784	Deletion of bp +32 to +35 and +17 to +20	+	+	+
Elux791	Deletion of sequences 3′ to bp −43	−	−	−
Elux792	Deletion of sequences 5′ to bp −43	−	−	−
Elux793	Deletion of sequences 3′ to bp −86	−	−	−
Elux794a	Deletion of sequences 5′ to bp −90	−	+ (reduced)	−
Elux794	Same as Elux794a except promoter inverted	−	−	−
laclux	*E. coli lac* promoter	+	+	+

[a] NaPP = sodium polypectate, added to 0.1% where noted; glucose was added at 0.2% where stated; + denotes significant light production by EC16 cells carrying the noted *pelE* promoter regions; − no light production relative to promoter negative controls. Experiments were performed as in Figures 4 and 5, with cells grown in microfuge tubes with 0.5 ml of LB medium. Constructs are described in Table 1 and Figure 3. Numbering is as shown in Figure 3. Mutants Elux781, Elux782, and Elux784 were active much earlier than the wild-type promoter (see Figure 5); Elux781 was somewhat slower (1–2 hr) than Elux782 or Elux784, which were active earlier and to higher activity than the *laclux* positive control. Elux794a gave reduced and delayed expression relative to the wild-type promoter (see Figure 5).

were cut into them with a #1 cork borer. The wells were inoculated with 2.5×10^7 bacterial cells. After various growth periods at 23° C, replicate potato tuber slices were homogenized in 10 ml of sterile distilled water with a Tissuemiser Mark II (Tekmar Co., Cincinnati, OH). Bacterial numbers were established as colony-forming units after dilution plating on LB Sm. Light assays were performed as described above with a single tuber cylinder added to each scintillation vial. All experiments were repeated at least twice with two or more replicates in each experiment.

Primer extension analysis. RNA was isolated from EC16 *pel-lux* integrants by the method for rapid isolation of RNA described by Ausubel *et al.* (1987). For purification of *pelE* message, EC16::Tn*7*-Elux81 cells were induced in LB medium amended with sodium polypectate. Light production was assayed and cells were harvested when 10 μl of cell suspension emitted about 10^8 cpm. Bacterial cells were then pelleted for RNA extraction. RNA integrity was determined by visualization of ribosomal RNA bands on denaturing agarose gels in the presence of formaldehyde (Ausubel *et al.* 1987). For primer extension experiments, 25 μg of total RNA was annealed in S1 hybridization solution with 5×10^4 cpm of a ^{32}P end-labeled 24-mer oligonucleotide (oligo-E, 5′GCTGTTACAACGGCAGCCAGTAAA3′), prepared by the UCR Bio-Instrumentation Laboratory).

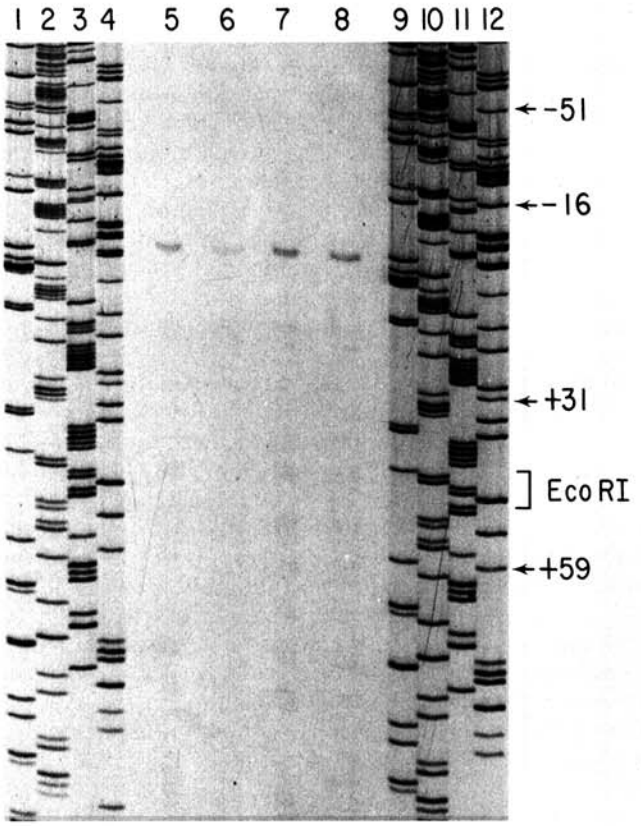

Fig. 3. Primer extension experiment using RNA from *Erwinia chrysanthemi* EC16 cells grown on LB-polypectate medium. Lanes 1 through 4 and 9 through 12 = GATC DNA sequence tracks; lanes 5 through 8 = replicate RNA lanes using a *pelE* primer for extensions. The termini occurred at −96 or −97 bp relative to the translational start of *pelE* (see Fig. 2).

This oligonucleotide hybridized to the coding strand beginning at base 36 after the translational start codon (Keen and Tamaki 1986). Extension reactions were performed according to section 4.8 (Ausubel *et al.* 1987) and products were fractionated on sequencing gels using dideoxy sequencing reactions of single-strand DNA from plasmid pPEL7432 primed with oligo E as size markers.

RESULTS

Mapping the transcriptional initiation site of the *pelE* gene. RNA was extracted from polypectate-induced EC16 cells and used in primer extension experiments. Extension terminated sharply at G and C bases located 96 and 95 bp upstream from the translational start of *pelE,* and these bands showed approximately equal intensity (Fig. 3). No other significant start sites were detected. The experiment therefore established the transcriptional start sites for *pelE* under inducing conditions as shown in Figure 2. Inspection of the DNA sequence of this *pelE* region disclosed the presence of a possible sigma 70 promoter with a −10 box (TAAAAA) upstream of the transcriptional start sites and a poor −35 box (AAAACA).

Bioluminescence from *pel-lux* integrants. Promoter activity of the *pelE* gene 5′ DNA was assayed by measuring light production from *E. chrysanthemi* EC16 cells carrying various *pel-lux* transcriptional fusions transposed into the single chromosomal Tn*7* site (H. Shen, S. Gold, and N. Keen, unpublished). All reported data are for cells grown on LB medium. However, similar results were obtained when bacteria were grown on M9-glycerol medium (data not shown). Somewhat more rapid induction of the *pelE* promoter was observed when cells were grown on KMB medium, a result at least partially due to more rapid growth rates. Nevertheless, the patterns of polypectate induction and glucose repression by the *pelE* wild type as well as the various mutant promoters were the same on all media. All anti-orientation promoter constructs (e.g., EC16::Tn*7*-Elux91, Table 1, hereafter called Elux91) were negative for light production (Table 2). Bacteria carrying the promoterless pHSK728 integrant, EC16::Tn*7*-*lux*, also did not produce light under any conditions and served as a negative control in all experiments (data not shown). The *lac* promoter construct, *laclux*, was used as a constitutive, positive control (Tables 1 and 2, Fig. 4).

Promoter activity was not detected from bacteria carrying a large fragment of *pelE* 5′ DNA (647 bp, Elux81, Table 1) when the cells were grown in unsupplemented LB medium (Fig. 4A), but light was produced by the addition of polypectate to the medium (Fig. 4B). Expression of the *pelE* promoter, even in the presence of polypectate in the medium, was only detected after 5–10 hr, considerably later than from cells expressing the *lac* promoter (Fig. 4B). The relatively late induction of the *pelE* promoter is a reflection of growth-phase inhibition, previously described for *Erwinia pel* genes (Hugouvieux-Cotte-Pattat *et al.* 1986). The addition of glucose at a concentration equal or greater than the polypectate repressed the induction of *pelE* (Fig. 4C). Glucose repression was only temporary and delayed promoter activity, presumably until the glucose was metabolized.

When EC16 cells carrying Elux81 were inoculated onto potato tuber slices (Fig. 4D), light production mirrored the pattern seen on polypectate medium, but the curves were temporally delayed. Cells carrying the *lac* promoter (*laclux*) also produced light in potato tubers, but Elux91 was devoid of activity, as expected (Fig. 4D). The experiments in culture media and potato tissues therefore established that the Tn*7-lux* system with EC16 cells carrying the *pelE* promoter region exhibited the regulation known to occur for production of PLe (Chatterjee *et al.* 1985; Hugouvieux-Cotte-Pattat *et al.* 1986).

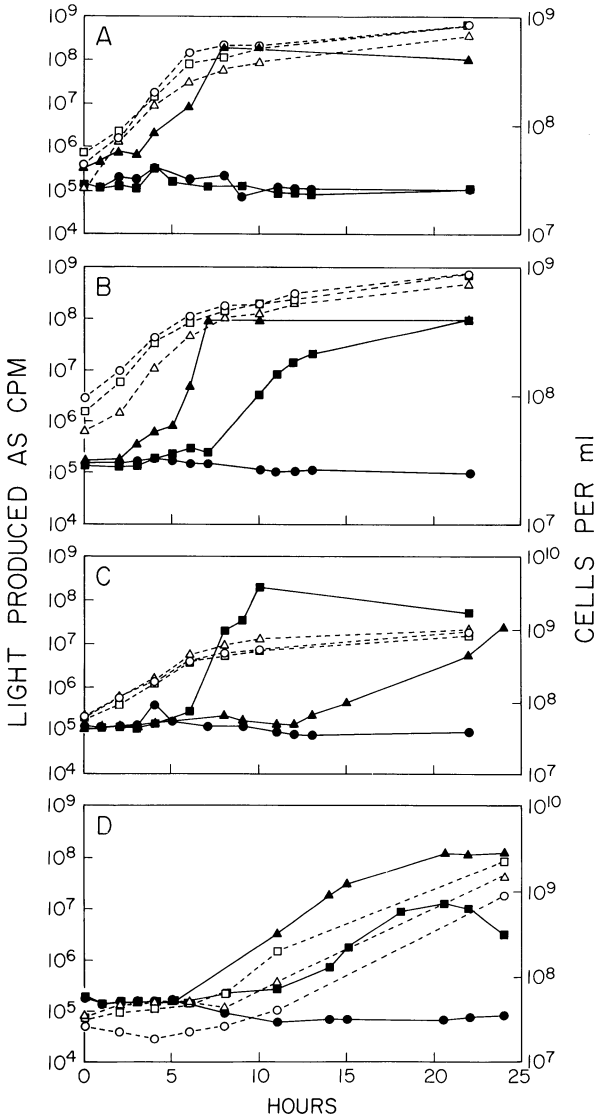

Fig. 4. Light production (as cpm) and cell numbers or colony-forming units (in the case of potato tubers) attained on various media by *Erwinia chrysanthemi* EC16 cells carrying the Tn*7-lux* cassette with various promoter sequences. Background values with the scintillation counter in single photon mode ranged from 0.5 to 1.1 × 10^5 cpm. Cultures were grown at 23° C in scintillation vials with 5 ml of LB medium supplemented as indicated or on potato tuber cylinders. Liquid cultures were initiated with about 2 × 10^8 cells and potato tuber slices were inoculated with 2.5 × 10^7 cells. Dashed lines and open symbols denote cells per milliliter and solid lines and points indicate light emitted. All data points are the average of two replicate treatments. **A,** Growth on LB medium only: (△ - △) = EC16::*laclux*; (□ - □) = EC16::Elux81; (○ - ○) = EC16::promoterless *lux* only; **B,** growth on LB medium supplemented with 0.1% sodium polypectate; cultures are as denoted in A; **C,** EC16::Elux81 cells were grown on LB medium only (○ - ○), LB medium supplemented with 0.1% sodium polypectate (□ - □), or LB medium supplemented with 0.1% sodium polypectate and 0.2% glucose (△ - △); **D,** potato tuber slices inoculated with the same bacteria as used in A and B. Labeling is as shown in A.

Deletions in the *pelE* promoter region. To further define DNA elements important for promoter activity, exonuclease III and restriction site deletions were made from both ends of the *pelE* promoter region. These DNA fragments were then cloned into pHSK728 or pHSK729, the resultant Tn*7-lux* cassettes were transposed into the EC16 chromosome, and the bacteria were assayed for light production. Elux790 and Elux780, which had 3′ termini at the *Mlu*I site within the *pelE* coding region (Fig. 2) yielded indistinguishable data from Elux81 and Elux83, which had 3′ termini at the *Eco*RI site located at +45 (Fig. 2, Table 2). These data indicated that no essential promoter elements resided 3′ to the *Eco*RI site and permitted use of this restriction site in most of the succeeding constructions.

Experiments were carried out to determine the 5′ extent of the *pelE* promoter. The promoter regions in pPEL7432, pPEL744, pPEL763, and pPEL764 (Table 1) all yielded full promoter activity in the *lux* assay when EC16 cells were grown in the presence of polypectate. Elux83, carrying 235 bp of DNA from pPEL764 (Table 1) also yielded expression data similar to Elux81 (carrying 596 bp of 5′ DNA) and therefore contained all regions of importance (Table 2). Of the several exoIII deletions assayed, Elux832, mapping 208 bp 5′ of the ATG initiation codon (−112 relative to the transcriptional start, Fig. 2) had fully regulated promoter activity (Table 2). However, the next exoIII deletion (Elux834), occurring at −72 (Fig. 2), was completely devoid of *pelE* promoter activity on any medium or when the bacteria were grown on potato tubers (Table 2). All exoIII deletions with breakpoints occurring 3′ to that in Elux834 were also devoid of promoter activity when the cells were grown on any of the media tested (data not shown).

Two additional sets of restriction site deletions were analyzed to more fully characterize the *pelE* promoter. In the first, the enzyme *Bsa*I, which has a unique cleavage site in the *pelE* promoter region (staggered cuts at −86 and −90, Fig. 2) was employed, and regions upstream and downstream of this site were recloned into pHSK729. The region upstream of the *Bsa*I site (Elux793) proved to be devoid of promoter activity (Table 2, Fig. 5). Cells carrying the region downstream of the *Bsa*I site in correct orientation with the *lux* cassette (Elux794a) produced light only under polypectate induction, but activity was delayed relative to the wild-type promoter and the magnitude attained was lower (Table 2, Fig. 5). As expected, the control anti-promoter construct (Elux794, Table 1) of the same DNA was entirely devoid of activity (Table 2). These data confirmed the occurrence of elements in the upstream region of the *pelE* promoter that are essential for full induction in the presence of polypectate.

The *pelE* promoter region was also restricted at a unique *Bsa*BI site occurring at −43 (Fig. 2). The construct Elux791 (including the region upstream of the *Bsa*BI site) and

Elux792 (comprising the region downstream of the *Bsa*BI site) were both completely devoid of promoter activity on any medium (Table 2). These experiments indicated that the −10/−35 boxes upstream of the transcriptional start sites (Fig. 2) were not, by themselves, sufficient for promoter activity. Because the *Bsa*BI site occurs within the putative catabolite gene activator protein (CAP)-binding site (Fig. 2), the results indicate the necessity of this and possibly other upstream elements for *pelE* promoter function.

Oligonucleotide site-directed mutations. DNA sequence data previously identified a perfect AT-rich palindromic sequence in the *pelE* promoter region (+10 to +20, Fig. 2) which might be important in regulation. A similar but imperfect palindromic sequence is also located at +32 to +41. To test whether these regions are involved in promoter regulation, oligonucleotide site-directed mutations were constructed. A deletion mutation in the perfect palindromic sequence (Elux782, Table 1) led to constitutive expression of the promoter on all tested culture media, including those containing glucose (Table 2, Fig. 5). Expression of Elux782 in *E. chrysanthemi* EC16 cells was even faster and to a higher level than the *laclux* control (data not shown). The addition of polypectate to the medium, however, resulted in an additional approximately twofold enhancement relative to cells grown in LB only, indicating that substrate induction still occurred. A mutation in the imperfect palindromic sequence (ELux781, Table 1) also led to constitutive expression of the promoter on all tested culture media (Table 2), but the timing and magnitude of light expression were somewhat delayed relative to Elux782 (Fig. 5). Also, the addition of polypectate to cells carrying Elux781 increased light production by three- to fourfold relative to growth on LB only (data not shown). A mutant in which the TGAA residues at position +43 of the imperfect palindrome were deleted (Elux780) did not affect light production relative to the native promoter (Table 2). The terminal G of the imperfect palindromic sequence accordingly did not appear important for regulation. The experiments therefore identified two putative negative regulatory sequences, called operator 1 and operator 2 in Figure 2. Consistent with these results, the double operator mutant (Elux784) behaved very similarly to Elux782. Somewhat surprisingly, promoter activity was not reduced in Elux781, Elux782, or Elux784 when glucose was added to LB medium or to LB containing polypectate, indicating that catabolite repression was not functional with these promoter mutants (Table 2).

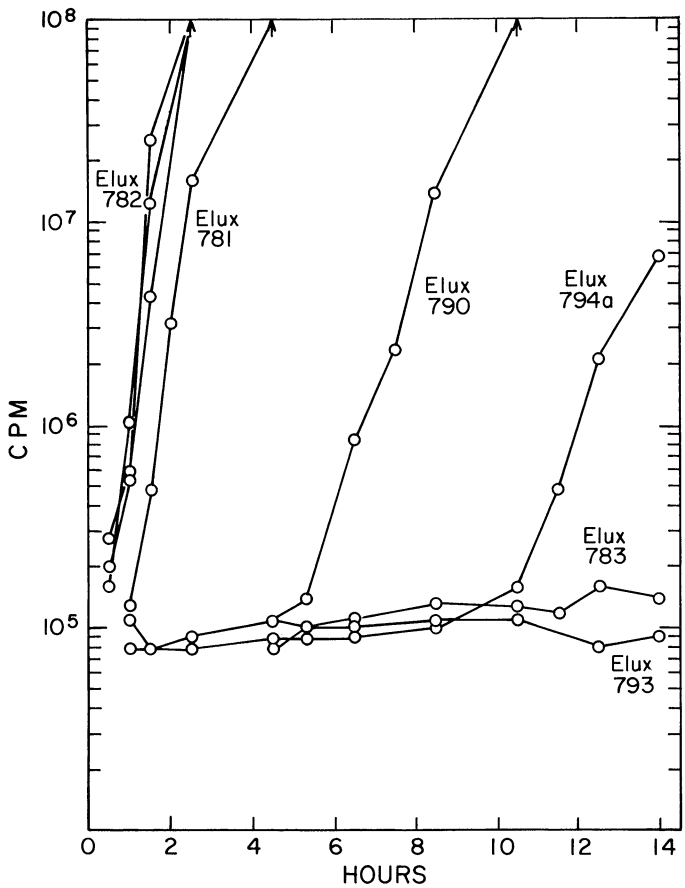

Fig. 5. Production of light (as cpm) by *Erwinia chrysanthemi* EC16 cells containing various *lux* integrants. The three curves for Elux782 denote cells grown in LB medium only, LB plus polypectate, or LB plus polypectate plus glucose. Addition of sodium polypectate to LB medium reproducibly resulted in a two- to threefold faster induction with cells carrying Elux782. Cells with Elux781 were grown only on LB medium. All other data are for cells grown in LB medium containing sodium polypectate, because all of these constructs yielded no expression above the background level of about 10^5 cpm on LB medium or LB polypectate medium supplemented with glucose. Points denoting cells carrying Elux783 or Elux793 are representative of background levels observed for all negative constructs. Baseline values are not shown for certain of the active constructs at the earlier time points that did not exceed 1.1×10^5 cpm. Cells carrying Elux780 gave curves very similar to Elux790 and the data are therefore not plotted; Elux784 gave curves very similar to Elux782 and the data are therefore not plotted.

To test the role of the putative CAP-binding site identified by DNA sequencing of the *pelE* promoter region (Keen and Tamaki 1986), a site-directed mutant was constructed in which the TGA residues at −43 (Fig. 2) were deleted (Elux783, Table 2). This mutation was expected to lower promoter function by destroying the putative CAP-binding site. When the promoter of Elux783 was fused to the *pelE* structural gene and this construct was assessed for production of PLe in *E. coli* cells, promoter function was very low, as expected (Tsuyumu *et al.* 1991). In *E. chrysanthemi* EC16 cells, Elux783 also resulted in negligible promoter activity (Table 2, Fig. 5). These results therefore confirm the role of the CAP site (Fig. 2) and indicate that *pelE* is a CAP-activated promoter.

DISCUSSION

Because of the relatedness of *E. chrysanthemi* to *E. coli*, its promoters would be expected to share several general features. Collado-Vides *et al.* (1991) recently reviewed the characterized *E. coli* promoters, which were classified as simple, if controlled by a single regulatory system, but complex if multiple regulators occurred. The results in this paper indicate that the *pelE* gene of *E. chrysanthemi* EC16 is driven by a complex, sigma 70 promoter. This promoter is regulated by catabolite repression and substrate induction (Fig. 4). Further, the *pelE* promoter is affected by growth-phase inhibition such that significant expression occurs only relatively late in culture growth (Chatterjee *et al.* 1985; Hugouvieux-Cotte-Pattat *et al.* 1986; our Fig. 4).

DNA sequence data (Keen and Tamaki 1986) identified AT-rich perfect and imperfect palindromic sequences 5′ to the *pelE* coding region (operators 1 and 2, Fig. 2), which may function as operators in substrate induction. Reverchon *et al.* (1989) subsequently identified a region including operator 1 as the *cis*-acting element for a general negative regulator, called *kdgR* (Reverchon *et al.* 1991), of many different pectic enzyme genes in both *E. carotovora* (Jones) Bergey *et al.* and *E. chrysanthemi*. However, Tsuyumu *et al.* (1991) recently showed in gel shift assays that an oligonucleotide containing the imperfect palindromic sequence shown in Figure 2 (operator 2) specifically bound to a factor in extracts from *E. chrysanthemi* but not from other bacteria, including *E. carotovora*. This factor may be a regulatory protein that interacts with both of the operators in Figure 2. It is therefore possible that operator 1 has another function in addition to contributing a portion of the *kdg* box. This conclusion is supported by the finding that oligonucleotide site-directed mutagenesis affecting only operator 2 also resulted in high level, constitutive expression of the *pelE* promoter in strain EC16 (Elux781, Table 2). Because the sequences surrounding operator 2 are not highly homologous to the *kdg* box, operators 1 and 2 may interact with a negative regulatory protein unrelated to KdgR. Furthermore, because mutation of either operator resulted in largely constitutive promoter activity, the two operators must function cooperatively to effectively repress transcription. In this regard, it is noteworthy that the spacing between the two operators (22 bp) would place them on the same side of the DNA double helix and thereby facilitate their contact with a repressor protein(s). Similar spacing of duplicated, cooperative operators has been observed for the ArgR and TyrR repressors in *E. coli* (see Collado-Vides *et al.* 1991).

DNA sequence analysis previously identified a sequence occurring 5′ to the *pelE* coding region and centered at −43.5 (Fig. 2) that had high homology to the *E. coli* CAP-binding site (Keen and Tamaki 1986). Because *E. carotovora* (Mount *et al.* 1979) and *E. chrysanthemi* (Hedegaard and Danchin 1985; Hugouvieux-Cotte-Pattat *et al.* 1986) produce adenyl cyclases similar to *E. coli*, the *pelE* CAP-binding site may account for the observed catabolite repression of *pelE*. The three-base deletion in Elux783 (Table 1) indeed eliminated promoter function in both *E. coli* (Tsuyumu *et al.* 1991) and *E. chrysanthemi* (Table 2, Fig. 5), presumably because it destroyed the spacing between the important diad symmetry elements CGAGA at −51 and TCGCA at −40 in the wild-type promoter (de Crombrugghe *et al.* 1984; Steitz 1990). These results therefore indicate that *pelE* has a CAP-activated promoter, as is the case with several other bacterial promoters (Collado-Vides *et al.* 1991). Moreover, a reasonable −10 box occurs in the correct position relative to the observed transcriptional start sites of the *pelE* gene (Fig. 2), but a very poor −35 box is located 16-bp upstream. These features are typical of the −10/−35 boxes of promoters regulated by catabolite repression (Collado-Vides *et al.* 1991; de Crombrugghe *et al.* 1984). The −35 box of the *pelE* promoter also overlaps the putative CAP-binding site at −43.5, as has been noted with other *E. coli* genes (Collado-Vides *et al.* 1991; de Crombrugghe *et al.* 1984; Ushida and Aiba 1990). Like certain other promoters, *pelE* also contains a second putative CAP site centered at −74.5 (Fig. 2), but its function has not been demonstrated.

The DNA sequence of *pelE* failed to disclose promoters with high homology to consensus *E. coli* −10 and −35 boxes (Keen and Tamaki 1986), although sequences with a moderate fit were identified in the *pelE* 5′ DNA, 177 bp 5′ from the translational start (59 and 81 bp upstream from the transcriptional start sites, Fig. 2). Deletion analyses indeed disclosed that an essential element(s) resides between 209 and 169 bp 5′ of the translational start (−112 and −72 bp relative to the transcriptional start sites, Fig. 2). Deletion of this region removed the −35 box at base −81. However, the primer extensions showed no evidence for a transcript resulting from this promoter motif (Fig. 3). Furthermore, deletion Elux794a, made at the *Bsa*I site at −90 (Fig. 2) yielded a promoter that contains the −35 box and gave low, polypectate inducible activity, but not constitutive expression (Fig. 5). This experiment thus confirms the importance of the upstream region but indicates that the elements at −59 and −81 are probably not functional in *E. chrysanthemi* as direct components of a sigma 70 promoter.

Three domains occur in the upstream region of the *pelE* promoter, shown boxed in Figure 2, which are also conserved in the promoter regions of three other *pelD/E* genes from different *E. chrysanthemi* strains (van Gijsegem 1989; Reverchon *et al.* 1989). Furthermore, the spacing between the boxed elements is identical in all three genes. Although physical data are required to test the possibility, one or more of these upstream DNA elements may interact with a DNA-binding protein(s) that positively activates transcription. It is interesting to note in this regard that Hugouvieux-Cotte-Pattat and Robert-Baudouy (1989) isolated a mutant in *E. chrysanthemi* strain B374, called *pecA*, and a similar mutant, called *pecL*, from strain 3937 that reduced PL production by bacteria grown in the presence of polypectate. In addition, *pecN* and *pecH* mutants have recently been isolated from strain 3937 that are involved in the positive control of *pel* gene expression (G. Condemine, N. Hugovieux-Cotte-Pattat, and J. Robert-Baudouy, personal communication). Although these mutations have not yet been characterized and some of them may occur in the same gene, their putative regulatory proteins could interact with the upstream *pelE* promoter region that we have identified.

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