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Environmental Conditions Affect Transcription of the Pectinase Genes of *Erwinia chrysanthemi* 3937

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To depolymerize plant pectin, the phytopathogenic enterobacterium *Erwinia chrysanthemi* produces a series of enzymes which include a pectin-methyl-esterase encoded by the *pem* gene and five isoenzymes of pectate lyases encoded by the five genes *pelA*, *pelB*, *pelC*, *pelD*, and *pelE*. We have constructed transcriptional fusions between the pectinase gene promoters and the *uidA* gene, encoding β-glucuronidase, to study the regulation of these *E. chrysanthemi* pectinase genes individually. The transcription of the pectinase genes is dependent on many environmental conditions. All the fusions were induced by pectic catabolic products and responded, to different degrees, to growth phase, catabolite repression, temperature, and nitrogen starvation. Transcription of *pelA*, *pelD*, and *pelE* was also increased in anaerobic growth conditions. High osmolarity of the culture medium increased expression of *pelE* but decreased that of *pelD*; the other pectinase genes were not affected. The level of expression of each gene was different. Transcription of *pelA* was very low under all growth conditions. The expression of the *pelB*, *pelC*, and *pem* genes was intermediate. The *pelE* gene had a high basal level of expression. Expression of *pelD* was generally the most affected by changes in culture conditions and showed a low basal level but very high induced levels. These differences in the expression of the pectinase genes of *E. chrysanthemi* 3937 presumably reflect their role during infection of plants, because the degradation of pectic polymers of the plant cell walls is the main determinant of tissue maceration caused by soft rot erwiniae.

The enterobacterium *Erwinia chrysanthemi* induces soft rot disease in a wide range of plants. This pathogen produces several extracellular enzymes that attack components of the plant cell wall, particularly pectic enzymes that play a major role in the maceration of plant tissue (9). To depolymerize and catabolize pectin, *E. chrysanthemi* produces a series of enzymes. Pectin-methyl-esterase (PME) removes methoxyl groups linked to carbon 6 on some galacturonate residues of the chain (Fig. 1). Pectate lyases (PL) then cut the internal glycosidic bonds of pectic polymers by a β-elimination reaction (Fig. 1). Other pectic enzymes, such as polygalacturonase or pectin lyase, are produced by some *Erwinia* strains (9, 28, 49). The *E. chrysanthemi* 3937 secretes one PME and five isoenzymes of PL (PLa, -b, -c, -d, and -e) (3). The nomenclature of PL isoenzymes refers to their pIs, from the most acidic, PLa, to the most basic, PLe. Analysis of the end products generated by each isoenzyme was carried out with the PLs of *E. chrysanthemi* EC16 (38, 48). The oligomers predominantly produced by each PL vary in lengths from dimers to dodecamers for PLa, trimers and tetramers for PLb or PLc, and mainly dimers for PLe, reflecting differences in their catalytic properties. Pectinase genes from a variety of *Erwinia* species have been cloned (for a review, see reference 28). In *E. chrysanthemi* 3937, isolation of the six genes *pem*, *pelA*, *pelB*, *pelC*, *pelD*, and *pelE* revealed that the PME and each PL isoenzyme are encoded by an independent transcriptional unit (13, 14, 37, 43). These genes are arranged into two clusters, widely separated on the bacterial chromosome (21); the genes *pelB* and *pelC* form one cluster, and *pelA*, *pelD*, *pelE*, and *pem* belong to the second cluster (Fig. 2).

In *E. chrysanthemi*, production of pectinases is inducible by pectin, its demethylated derivative polygalacturonic acid (PGA), or the monomer, galacturonate. Analysis of mutants blocked in different steps of pectinolysis has shown that the real intracellular inducers are catabolic products, mainly 2-keto-3-deoxygluconate (KDG) or its derivatives (10, 23, 24, 34). Inducibility by pectic derivatives is in part mediated by the KdgR repressor, and in vitro experiments showed that the specific binding of the KdgR repressor to its operator is inhibited in the presence of KDG (34, 35). However in a *kdgR*-deficient strain, PL synthesis is still inducible in the presence of PGA, demonstrating that other regulatory genes are involved in PL induction (25). Different loci involved in *pel* regulation, beside *kdgR*, were identified among the mutants affected for PL synthesis, including *pecI* (25), *pecS* (41), *pecY* (4), *pecZ* (26), and *pecX* (16).

Physiological studies demonstrated that PL synthesis is subject to other types of regulation, such as catabolite repression or induction in late exponential growth phase (20, 27). PL synthesis could also vary with temperature (36), oxygen tension (16), or iron concentration (46). The regulatory genes involved in these additional regulatory circuits are not identified. Therefore, it appears that the regulation of the PL genes is complex, both at the physiological and genetic level, and probably requires several regulatory systems. Moreover, since the total PL activity results from the expression of five different genes, it was not possible to determine the contribution of each gene in a particular inducing condition.

We report here the construction and analysis of operon fusions between each *E. chrysanthemi* 3937 pectinase gene and the reporter gene *uidA* (encoding β-glucuronidase [GUS]). These genetic fusions permitted us to follow individually the expression of each gene. We have analyzed *pel* gene regulation in response to pectin catabolites, plant

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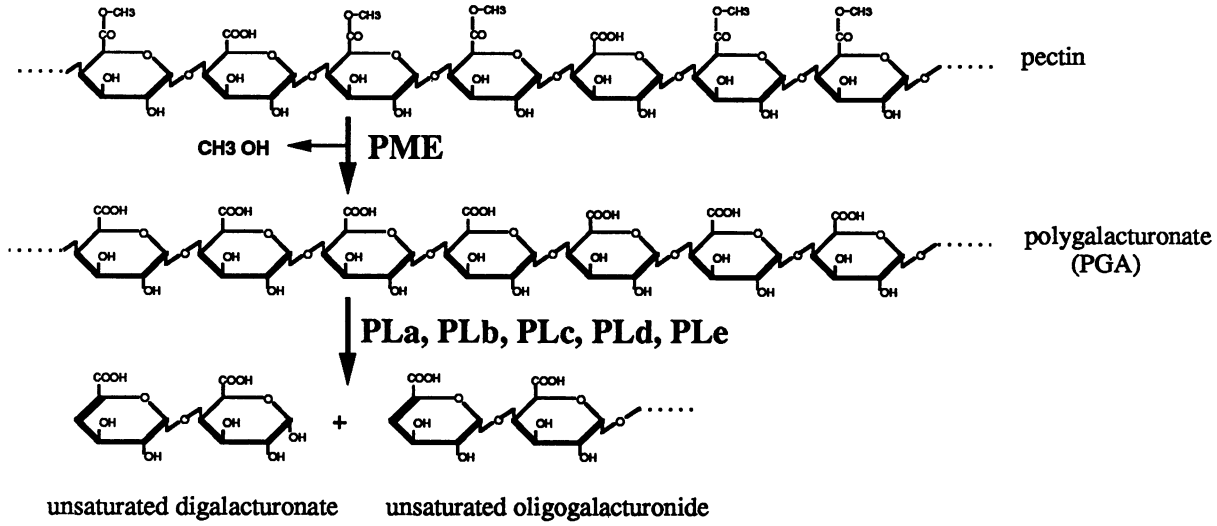

FIG. 1. Degradation of pectin by *E. chrysanthemi* 3937. PME, encoded by the *pem* gene, demethoxylates pectic polymers to give PGA and methanol. PGA is cleaved into oligomers and dimers by the action of PL, encoded by the *pel* genes. The *E. chrysanthemi* 3937 possesses five *pel* genes, *pelA* to *pelE*, coding for five PL isoenzymes, PLa to PLe. These enzymes, which act directly on the pectic polymer, are secreted by the bacteria. In contrast, further degradation of the oligogalacturonides appears to be the function of intracellular enzymes (not shown).

products, or environmental stresses such as oxygen limitation, temperature, or osmolarity fluctuation.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1.

Media and growth conditions. Cells were grown in complete L medium or in synthetic M63 medium (32). When required, the media were solidified with Difco agar (15 g/liter). *E. chrysanthemi* cells were usually incubated at 30°C and *Escherichia coli* cells were incubated at 37°C. Carbon sources (glycerol, glucose, and galacturonate) were added at 2 g/liter, except for polygalacturonate (PGA) (grade II, Sigma Chemical Co.), which was added at 4 g/liter. A crude carrot extract was prepared by homogenization of carrots, filtration through 0.45-μm-pore-size filters, and sterilization by autoclaving. This extract was used at the final concentration of 1% (vol/vol).

Liquid cultures were grown in a shaking incubator (220 rpm). Enzymatic assays were usually performed on cultures grown at 30°C in M63 glycerol minimal medium. To induce pectinases, this medium was supplemented with PGA and plant extract. Semianaerobic conditions were achieved without shaking in liquid cultures layered with paraffin oil by using M63 minimal medium supplemented with fumarate (2.5%) as an electron acceptor. Nitrogen starvation was performed in the following medium: M63 deprived of $(NH_4)_2SO_4$ but supplemented with arginine (200 μg/ml) as a nitrogen source. Phosphate starvation was carried out by using the low-phosphate medium MinA-P (44) supplemented with 1 mM phosphate. Mitomycin was used as inducer of the SOS system at a final concentration of 0.5 μg/ml. High osmolarity was obtained by the addition of NaCl (final concentration, 0.3 M) to M63 minimal medium. Such high-salt concentration provoked precipitation of PGA. To test the effect of osmolarity, the induction was performed with galacturonate instead of PGA.

When required, antibiotics were added at the following concentrations (micrograms per milliliter): kanamycin (Km), 20; ampicillin (Ap), 50; tetracycline (Tc), 10; and chloramphenicol (Cm), 20.

Plate and enzyme assays. Clones producing PL were detected on medium containing PGA. After growth, plates were flooded with a solution (10%) of copper acetate, which forms a blue complex with the polymer, leaving clear haloes around clones producing PL. PME activity was tested by using the semiquantitative colorimetric method previously described (25).

Assays of PL, GUS, and β-galactosidase were performed on toluenized cell extracts. PL activity was determined by the degradation of PGA to unsaturated products that absorb at 235 nm (33). Specific activity is expressed as micromoles of unsaturated products liberated per minute per milligram (dry weight) of bacteria. GUS activity was measured by

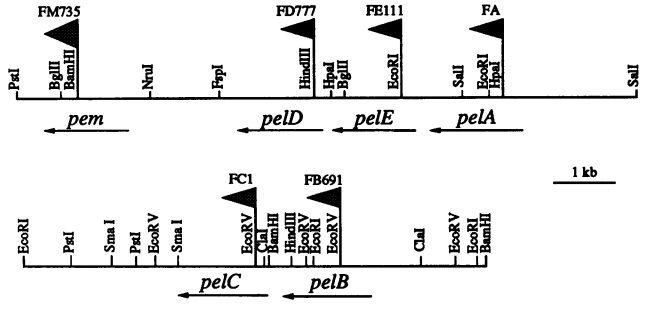

FIG. 2. Physical map of the two clusters encoding pectinases in *E. chrysanthemi* 3937. These maps show the location and transcription direction of the pectinase-encoding genes, *pem*, *pelA*, *pelB*, *pelC*, *pelD*, and *pelE*. Restriction sites used in this study are indicated. Insertions of the *uidA*-Km cassette leading to fusions are indicated by flags, with the number of the corresponding fusion plasmid.

TABLE 1. Bacterial strains, phages, and plasmids

Strain, phage, or plasmid	Genotype,[a] phenotype, or description	Reference or origin
E. chrysanthemi		
3937	Wild type	29
L2	*lmrT*(Con) *lacZ2*	22
A876	*lmrT*(Con) *lacZ2 kdgR*::*lacZ* Km	This work
A1696	*lmrT*(Con) *lacZ2 pelA*::*uidA* Km	C. Bourson
A1888	*pelA*::*uidA* Km	Transduction of 3937 by a phi-EC2 stock made on A1696
A1787	*pelB*::*uidA* Km	This work
A1880	*pelC*::*uidA* Km	This work
A1798	*pelD*::*uidA* Km	This work
A1828	*pelE*::*uidA* Km	This work
A1789	*pem*::*uidA* Km	This work
Phages		
phi-EC2	*E. chrysanthemi* transducing phage	39
MudI681	*lacZ* Km	7
Plasmids		
pBR322	Ap^r Tc^r	Laboratory collection
pUC8	Ap^r	Laboratory collection
pBS-Ap	Bluescript SK+, Ap^r	Stratagene
pBS-Cm	Bluescript KS+, Cm^r	Stratagene
pBSH	pBS-Ap derivative with a *Sma*I-*Eco*RV deletion	This work
pUIDK1	pBR322 derivative bearing the *uidA*-Km cassette, Ap^r	2
pUIDK31	pSU9 derivative bearing the *uidA*-Km cassette, Cm^r	2
pN496	pBS-Cm derivative bearing the *uidA*-Km cassette, Cm^r	Ligation of pUIDK1 *Hin*dIII with pBS-Cm *Hin*dIII
pROU2	RP4 derivative, Ap^r Km^r Tc^r *kdgR*$^+$ *kduD*$^+$ *ogl*$^+$	42
pN420	pBR322 derivative with a 2.6-kb *Bam*HI-*Cla*I fragment, Ap^r *pelB*$^+$	This work
pGP1	pBS-Cm derivative with a 1.8-kb *Hin*dIII-*Sal*I fragment, Cm^r, *pelC*$^+$	This work
pN715	pBR322 derivative with a 2.8-kb *Eco*RI-*Fsp*I fragment, Ap^r *pelD*$^+$	This work
pHD108	pBSH derivative with a 2.9-kb *Hin*dIII-*Sal*I fragment, Ap^r, *pelE*$^+$	This work
pNM7	pUC8 derivative with a 1.7-kb *Nru*I-*Pst*I fragment, Ap^r *pem*$^+$	This work
pFB691	pN420 derivative with a *uidA*-Km insertion in the *Eco*RV site, *pelB*::*uidA*$^+$	This work
pFB671	pN420 derivative with a *uidA*-Km insertion in the *Eco*RV site	This work
pFC1	pGP1 derivative with a *uidA*-Km insertion in the *Eco*RV site, *pelC*::*uidA*$^+$	This work
pFC2	pGP1 derivative with a *uidA*-Km insertion in the *Eco*RV site	This work
pFD777	pN715 derivative with a *uidA*-Km insertion in the *Hin*dIII site, *pelD*::*uidA*$^+$	This work
pFD775	pN715 derivative with a *uidA*-Km insertion in the *Hin*dIII site	This work
pFE111	pHD108 derivative with a *uidA*-Km insertion in the *Eco*RI site, *pelE*::*uidA*$^+$	This work
pFE112	pHD108 derivative with a *uidA*-Km insertion in the *Eco*RI site	This work
pFM735	pHD108 derivative with a *uidA*-Km insertion in the *Bam*HI site, *pem*::*uidA*$^+$	This work
pFM732	pHD108 derivative with a *uidA*-Km insertion in the *Bam*HI site	This work

[a] Genotype symbols are according to Bachmann (1). In addition, *lmrT*(Con) indicates that the transport system encoded by the *lmrT* gene and able to mediate entry of lactose, melibiose, and raffinose into the cells is constitutively expressed (22).

following the degradation of *p*-nitrophenyl-β-D-glucuronide into *p*-nitrophenol that absorbs at 405 nm (2). Specific activity is expressed as nanomoles of products liberated per minute per milligram (dry weight) of bacteria. β-Galactosidase activity was measured by following the degradation of *o*-nitrophenyl-β-D-galactoside into *o*-nitrophenol, at 420 nm (32). Specific activity is expressed as nanomoles of product liberated per minute per milligram (dry weight) of bacteria.

Recombinant DNA techniques. Preparations of plasmid DNA, restriction digestions, ligations, DNA electrophoresis, and transformations were carried out as described by Sambrook et al. (45).

In vitro construction of *uidA* fusions. The *uidA*-Km cassettes (2) included a promoterless *uidA* gene that conserved its Shine-Dalgarno sequence. Insertion of this cassette in a gene in the correct orientation generated a transcriptional fusion. The cassettes are flanked by various polylinkers containing multiple restriction sites. The cassette can be isolated after digestion with an appropriate restriction enzyme and inserted in the gene of interest cut with a compatible restriction enzyme. The kanamycin resistance marker allows the selection of plasmids which have incorporated the cassette. Construction of the different *uidA* transcriptional fusions was carried out as follows (Fig. 3).

(i) **Construction of the *pelB*::*uidA* fusion.** The 2.6-kb *Bam*HI-*Cla*I fragment containing the *pelB* gene was cloned in plasmid pBR322. The resulting plasmid pN420 was linearized with *Eco*RV and ligated with the 3.8-kb *uidA*-Km cassette obtained by *Sma*I digestion of pN496. Ap^r Km^r clones were selected and checked for their production of pectinase and GUS. Restriction analysis of the plasmids confirmed the localization and orientation of the *uidA*-Km cassette. Plasmids pFB671 and pFB691 contained the *uidA*-Km cassette inserted into the *pelB* gene. In pFB691, the *uidA* insertion is oriented with the same direction of transcription as *pelB*, giving rise to a *pelB*::*uidA* fusion (Fig.

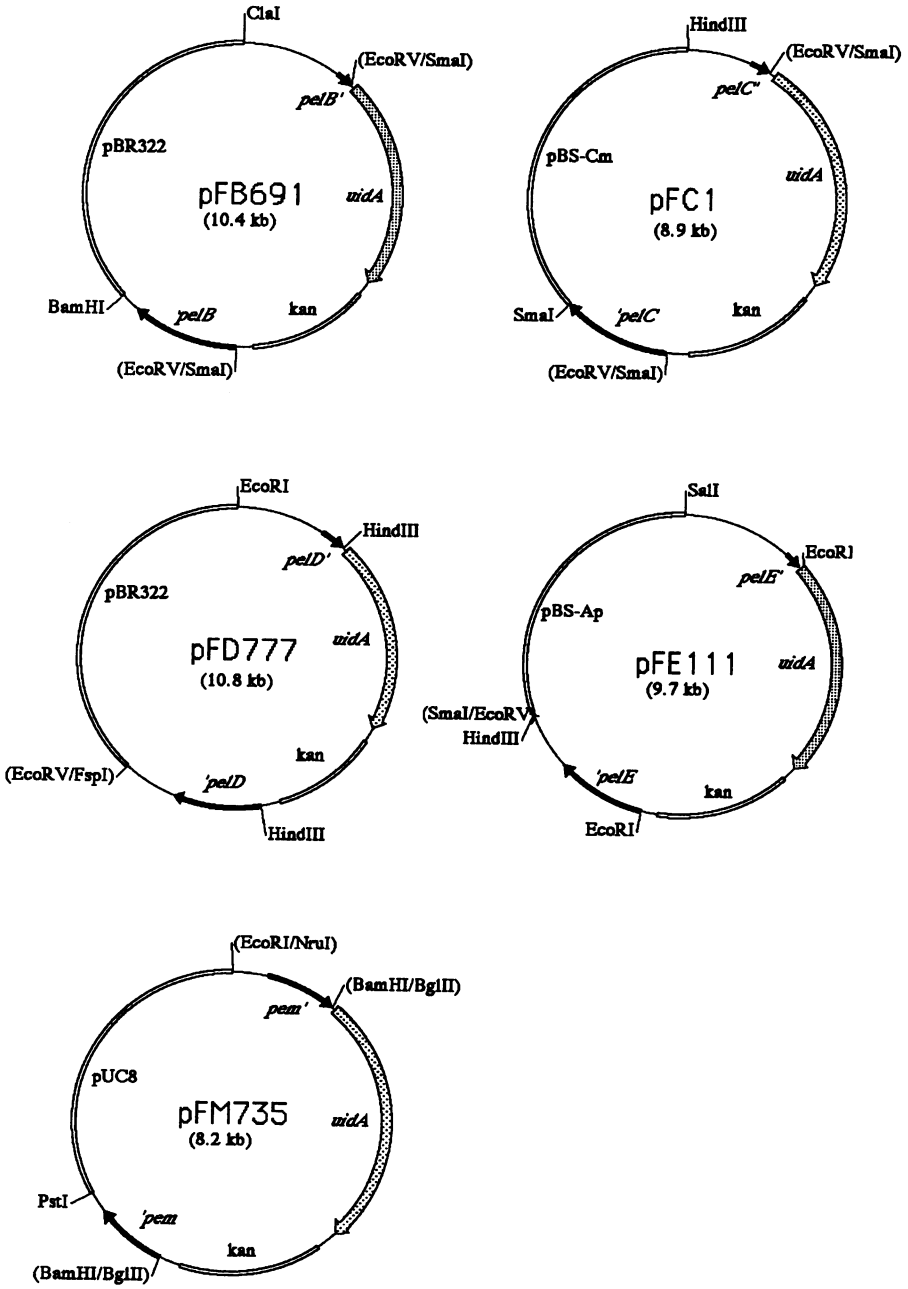

FIG. 3. Insertion of *uidA*-Km cassettes in the pectinase genes. The *uidA* gene (the structural gene for GUS) is indicated by large, dotted arrows, while the pectinase genes, *pem* and *pel*, are shown by small, black arrows. Restriction sites used for subcloning or insertion of the *uidA*-Km cassette are indicated.

3); in pFB671 the *uidA*-Km cassette is inserted in the opposite direction.

(ii) **Construction of the *pelC*::*uidA* fusion.** The 1.8-kb *Sma*I-*Hin*dIII fragment containing the *pelC* gene was first subcloned in plasmid pBS-Cm, and the resulting plasmid, pGP1, was linearized with *Eco*RV. The *uidA*-Km cassette obtained by *Sma*I digestion of pUIDK1 was then inserted into the *pelC* gene to give plasmids pFC1 and pFC2, in which the *uidA*-Km insertion is oriented in the same direction as *pelC* (pFC1) (Fig. 3) or in the opposite direction (pFC2).

(iii) **Construction of the *pelD*::*uidA* fusion.** The 2.8-kb *Fsp*I-*Eco*RI fragment containing the *pelD* gene was first subcloned in plasmid pBR322 digested by *Eco*RI and *Eco*RV. The resulting plasmid, pN715, was linearized with *Hin*dIII. The *uidA*-Km cassette, obtained after *Hin*dIII digestion of pUIDK1, was then inserted into the *pelD* gene to give plasmids pFD777 and pFD775, in which the *uidA* insertion is oriented in the same direction as *pelB* (pFD777) (Fig. 3) or in the opposite direction (pFD775).

(iv) **Construction of the *pelE*::*uidA* fusion.** The 2.9-kb *Hin*dIII-*Sal*I fragment containing the *pelE* gene was first subcloned in plasmid pBSH (pBS-Ap derivative deleted of

the *Eco*RI site by a *Sma*I-*Eco*RV deletion). The resulting plasmid, pHD108, was linearized with *Eco*RI and ligated to the *uidA*-Km cassette obtained by *Eco*RI digestion of pN496. Plasmids pFE111 and pFE112 contain the *uidA*-Km cassette inserted into the *pelE* gene, in the same direction as *pelE* (pFE111) (Fig. 3) or in the opposite direction (pFE112).

(v) Construction of the *pem*::*uidA* fusion. The *pem* gene was present on the 6-kb *Pst*I-*Eco*RI fragment inserted in the vector pUC8. Deletion of the *Nru*I-*Eco*RI fragment was realized after digestion and action of the Klenow enzyme to fill in the *Eco*RI protruding ends. The resulting plasmid, pNM7, containing the 1.7-kb *Pst*I-*Nru*I fragment was linearized with *Bam*HI and ligated with the *uidA*-Km cassette obtained by *Bgl*II digestion of pUIDK31. Plasmids pFM735 and pFM732 contained the *uidA*-Km cassette inserted into the *pem* gene in the same direction as *pem* (pFM735) (Fig. 3) or in the opposite direction (pFM732).

Recombination of the *uidA* fusions into the *E. chrysanthemi* chromosome. Mutations constructed in vitro by insertions of kanamycin resistance cassettes were introduced into the *E. chrysanthemi* chromosome by marker exchange recombination between the chromosomal allele and the plasmid-borne mutated allele. The recombinant was selected after two successive cultures in low-phosphate medium in the presence of kanamycin, conditions in which pBR322 derivatives are very unstable (44). The PL profile in the resulting strains of *E. chrysanthemi* was analyzed by electrofocusing as previously described (3) to verify that the PL isoenzyme encoded by the mutagenized gene was absent. In the case of PME, the test for the detection of this enzyme was used to check that the corresponding gene was inactivated. The strains A1787, A1789, A1798, A1828, and A1880 were retained, since they had a *pel* or *pem*::*uidA* fusion.

The *pelA*::*uidA* fusion was previously constructed by C. Bourson (6a) by introduction of the *uidA*-Km cassette into the *Hpa*I site located inside the *pelA* gene (Fig. 2). This *pel*::*uidA* fusion present in a *lacZ E. chrysanthemi* mutant (Table 1) was transduced in the wild-type strain 3937 by using a stock of the transducing phage Phi-EC2 (37) grown on strain A1696 and by selecting for the kanamycin resistance of the cassette.

In vivo construction of the *kdgR-lac* fusion. A *lacZ* mutant of *E. chrysanthemi*, L2, was mutagenized in vivo by infection with phage MdI1681 (7). Mutants with a derepressed expression of the *pel* genes were selected by using the PL in situ detection, as previously described (20). Mutants simultaneously showing a derepression of the *kdgT* transport system, i.e., able to grow with KDG as the sole carbon source, were most probably affected in the *kdgR* gene (11, 12). Those expressing β-galactosidase might contain a *kdgR*::*lacZ* transcriptional fusion. Transduction of the Mu insertion (Km^r) was performed to verify the linkage between the Mu insertion, the β-galactosidase synthesis, the growth with KDG as a carbon source, and the derepressed expression of the *pel* genes. The location of the Mu insertion in the *kdgR* gene was confirmed by chromosomal mapping of the Km^r marker, strongly linked to the *ogl* gene (40), and by complementation of the mutation with plasmid pROU2 (42) bearing the wild-type *kdgR* gene. Strain A876, containing a *kdgR*::*lacZ* fusion, was kept for analysis.

RESULTS

Individual expression of pectinase genes during growth phase. It was observed previously that the level of PL synthesis varies during bacterial growth (19). The expression of each *pel-uidA* fusion was followed during growth under conditions inducing PL synthesis (Fig. 4). The GUS specific activity represents the expression of the fused gene while the PL specific activity represents the expression of the four nonmutated *pel* genes. The expression of all the fusions strongly increased when the cells entered the late exponential growth phase (Fig. 4). Similar curves were obtained when PL activity was measured (Fig. 4). The GUS maximum expression was transient and was followed by a decline. In contrast, the PL specific activity reached a constant level, which probably corresponds to an equilibrium between the rate of synthesis and degradation of the enzymes. Since PL are rather stable enzymes in these conditions, the plateau probably corresponds to a low level of newly synthesized enzyme. The expression of *pem* was similarly dependent on growth phase, showing a peak followed by a steady state at the same phases as *pel* genes (Fig. 4). In conclusion, the maximum synthesis of pectinases occurs in a short period during which the cells slow down their growth. The increase due to the end of exponential growth phase approximately corresponds to a factor of 5, 10, 15, 60, 6, and 10 for *pelA*, *pelB*, *pelC*, *pelD*, *pelE*, and *pem*, respectively.

Involvement of *kdgR* in the growth-dependent regulation. The *pel* promoters are recognized by the negative regulatory protein KdgR, which represses their expression in the absence of inducer. The growth-phase-dependent expression of the *pel* genes could be the consequence of variations in KdgR activity. For instance, a reduced synthesis of KdgR during late exponential growth could result in derepression of the *pel* genes. To examine this possibility, expression of the *kdgR* gene was determined by using a *kdgR*::*lac* fusion (Fig. 5). We found that the expression of *kdgR*, estimated by β-galactosidase activity, was roughly constant during the exponential growth of the cells. In contrast, this expression was low during the latency and stationary phases of growth, i.e., when cells did not multiply (Fig. 5). The relatively high expression of *kdgR* could account for the higher repression of the *pel* gene expression during log phase. In the *kdgR* mutant A876, *pel* gene expression was higher than in the wild-type strain in the absence of inducer and did not appear dependent upon the growth phase. However, PL production in the *kdgR* mutant remained growth phase dependent in the presence of inducer, with a 30-fold increase factor (Fig. 5). Moreover, in the wild-type strain, the *pel* gene expression was growth phase dependent, both in noninduced and induced conditions (with 3- and 40-fold factors, respectively), i.e., when KdgR is or is not active (Fig. 5). Therefore, the presence of an active *kdgR* gene is not necessary for the growth-phase-dependent expression of the *pel* genes, but KdgR can be responsible of an increased repression of the *pel* genes in the log phase of growth.

Contribution of each *pel* gene in the total PL activity. Comparison between the levels of expression of each gene can be determined by taking the GUS values obtained in the plateau of the growth curve (Fig. 4; Table 2). The *pelD* and *pelE* fusions were highly expressed, about 5-fold higher than *pelB*, *pelC*, or *pem* and 2,000-fold higher than *pelA*, whose expression is very low compared with that of the other *pel* genes.

The PL activity in each mutant can also give information about the contribution of the corresponding enzyme in the total PL activity, corresponding to the sum of PLa, PLb, PLc, PLd, and PLe enzymatic activities. There was a decrease of PL activity in all the *pel* mutants. When *pelE* was mutated, the PL activity decreased to about 50% of that of the wild-type strain. When *pelA*, *pelB*, *pelC*, or *pelD* was

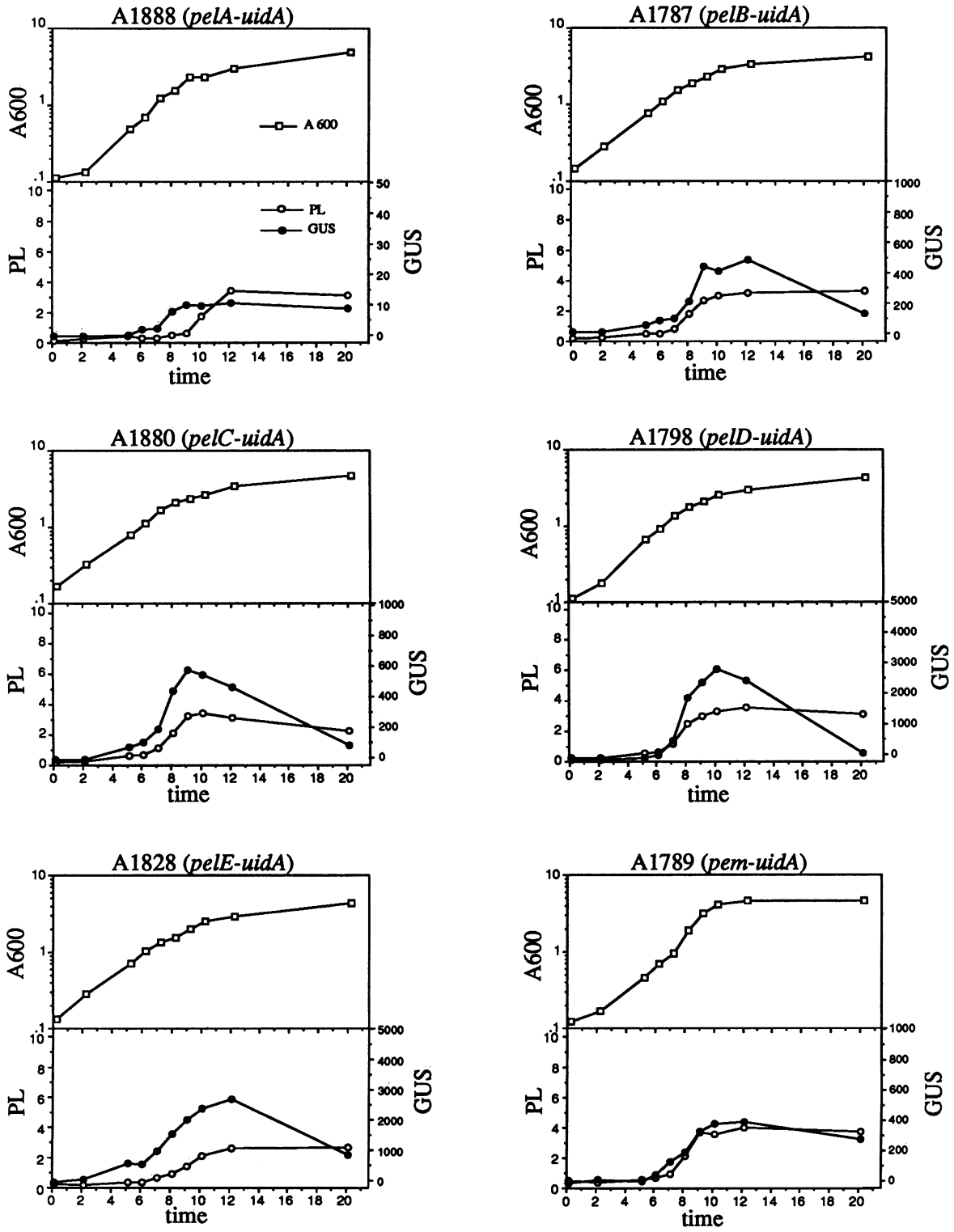

FIG. 4. Growth and expression of pectinase genes in *E. chrysanthemi* 3937. Growth of the different fusion strains in M63 minimal medium supplemented with PGA and plant extract was followed by measuring A_{600} (□). PL (○) and GUS (●) specific activities were determined on each sample and are expressed in micromoles per minute per milligram (dry weight) of bacteria and in nanomoles per minute per milligram (dry weight) of bacteria, respectively.

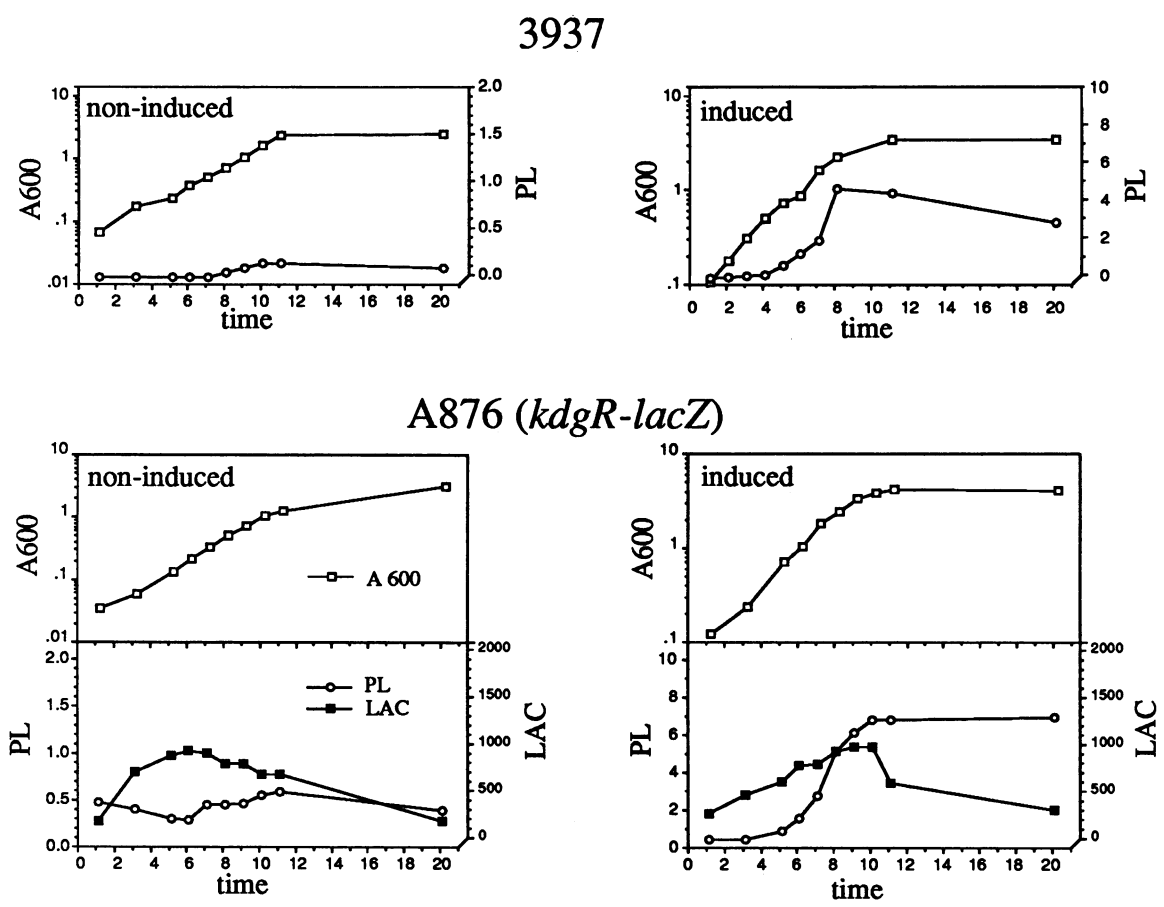

FIG. 5. PL synthesis and expression of the *kdgR* gene during bacterial growth. Growth and induction of PL synthesis were followed in the wild-type strain 3937 and in the *kdgR*::*lac* mutant A876 in glycerol M63 minimal medium supplemented with PGA and plant extract for the inducing conditions. β-Galactosidase specific activity reflected the expression of the *kdgR*::*lacZ* fusion. Growth was followed by measurement of A_{600} (□). PL (○) and β-galactosidase (LAC) (■) specific activities were determined on each sample and are expressed in micromoles per minute per milligram (dry weight) of bacteria and in nanomoles per minute per milligram (dry weight) of bacteria, respectively.

inactivated, the remaining PL activity was, in each case, about 70% of that in the wild type. However the reduction in PL activity due to each PL was not simply additive, since the sum of the decreases due to the absence of each PL isoenzyme would be higher than 100%. Therefore, the absence of one PL isoenzyme probably affected the synthesis of the others, for instance, by reducing the formation of metabolic intermediates that are inducers of *pel* expression.

Effect of various growth conditions on total PL production.

TABLE 2. Contribution of each *pel* gene to total PL activity

Strain (main genotype)	Sp act[a]	
	GUS	PL
3937 (Wild type)	ND	4.9
A1888 (*pelA*::*uidA*)	12	3.3
A1787 (*pelB*::*uidA*)	522	3.1
A1880 (*pelC*::*uidA*)	611	3.3
A1798 (*pelD*::*uidA*)	2,980	3.4
A1828 (*pelE*::*uidA*)	2,871	2.6

[a] The specific activities of GUS and PL correspond to the level obtained at the end of the bacterial growth (from Fig. 4 and 5); specific activities are expressed in nanomoles minute^{-1} milligram^{-1} and in micromoles minute^{-1} milligram^{-1}, respectively. ND, not detectable.

To determine the conditions that affect *pel* expression, we first tested the total PL production of the wild-type strain 3937, in various conditions (Table 3). The expression of the pectinase genes is growth phase dependent, and the following assays were performed at the beginning of the stationary phase. Total PL activity was low but detectable in noninducing conditions, i.e., glycerol-grown cells. Pectic derivatives, pectin, PGA, or the monomer galacturonate provoked a three- to sixfold increase of PL synthesis. This induction was augmented 6- to 12-fold when plant extract (1%) or $CaCl_2$ (1 mM) was added to medium with pectin or PGA. Plant extracts or $CaCl_2$ alone had no effect when added to glycerol or galacturonate cultures. Ca^{2+} ions are known to be necessary for PL activity. We found that plant extract had a similar effect: if the assay medium was deprived of $CaCl_2$, the addition of plant extract activated PL to the same extent as $CaCl_2$ (data not shown). Moreover, the addition of EGTA, which complexes Ca^{2+} ions, suppressed the effect of plant extract or $CaCl_2$ (Table 3). We conclude that both plant extract and $CaCl_2$ had the same effect: by increasing the activity of the low PL basal level, they favor the formation of intracellular inducers, which in turn provoke a better induction of PL synthesis. Plant cell walls are known to contain calcium at an approximate concentration of 1 mM (8); such concentration is highly sufficient for PL activation (33). Plant

TABLE 3. Production of PL in wild-type strain 3937[a]

Carbon source and inducer	Particular growth condition	Growth (A_{600})	PL sp act
Glycerol			
None		3.72	0.12
PGA		3.35	0.38
Pectin		1.30	0.54
Galacturonate		2.64	0.76
PGA + plant extract		3.37	4.65
Pectin + plant extract		1.83	3.25
PGA + $CaCl_2$		2.67	3.43
Plant extract		3.65	0.18
$CaCl_2$		3.06	0.19
Galacturonate + plant extract		3.26	0.86
Galacturonate + $CaCl_2$		2.80	0.98
PGA + plant extract + EGTA		2.94	0.29
PGA + $CaCl_2$ + EGTA		2.37	1.21
Glucose			
None	Catabolite repression	5.30	0.06
PGA + plant extract	Catabolite repression	4.29	1.47
Glycerol			
None	25°C	2.09	0.80
PGA + plant extract	25°C	2.44	3.63
None	37°C	3.08	0.04
PGA + plant extract	37°C	4.55	0.61
None	Anaerobiosis[b]	0.78	1.10
PGA + plant extract	Anaerobiosis	0.65	4.43
None	Phosphate starvation[c]	1.60	0.15
PGA + plant extract	Phosphate starvation	3.30	3.96
None	Nitrogen starvation[d]	0.24	0.07
PGA + plant extract	Nitrogen starvation	0.76	0.32
None	High osmolarity[e]	0.96	0.24
Galacturonate	High osmolarity	1.41	1.07
None	+ Mitomycin	2.39	0.26
PGA + plant extract	+ Mitomycin	2.34	4.21

[a] Cultures were performed in the absence or presence of inducing compounds, in different physiological conditions. Carbon sources (glycerol and glucose) were added at 2 g/liter. Other compounds were added at the following concentrations: PGA, 4 g/liter; pectin, 4 g/liter; galacturonate, 2 g/liter; plant extract, 1% (vol/vol); $CaCl_2$, 1 mM; EGTA, 10 mM; and mitomycin, 0.5 μg/ml. All the cultures were performed in M63 minimal medium except for phosphate starvation (see footnote c). Each experiment was repeated at least three times; the values obtained for a significant experiment are indicated.

[b] Anaerobiosis was achieved by using M63 supplemented with fumarate (2.5%) as an electron acceptor, in cultures grown without shaking that were layered with paraffin oil.

[c] Phosphate starvation was achieved in MinA-P medium supplemented with 1 mM phosphate. In MinA-P plus phosphate (50 mM), the PL values were similar to those in M63 medium (data not shown).

[d] Nitrogen starvation was performed in M63 deprivated of $(NH4)_2SO_4$ but supplemented with arginine (200 μg/ml) as a nitrogen source.

[e] High osmolarity was obtained by the addition of 0.3 M NaCl to M63.

extract or $CaCl_2$ had no effect on induction by galacturonate (Table 3), but inducer formation from galacturonate does not need the action of PL. Other cations, Na^+ or K^+ (100 mM), had no effect on PL expression (data not shown).

The presence of an easily metabolizable carbon source, such as glucose, caused a two- to threefold repression of PL production (Table 3). Growth temperature strongly affected PL production. *E. chrysanthemi* cells were usually cultured at 30°C. At 25°C, the noninduced level increased about sevenfold but the induced level was similar to that at 30°C. In contrast, at 37°C, both induced and noninduced levels decreased by a factor of 3 to 7. Like low temperature, anaerobiosis increased the noninduced PL level about sixfold. Phosphate limitation did not affect PL production. In contrast, nitrogen starvation provoked a strong decrease, about 15-fold, in PL production in inducing conditions. A high osmolarity of the medium, obtained by addition of 0.3 M NaCl, provoked a slight increase in PL production, mainly in the absence of inducer (twofold). The effect of an acidic pH could not be evaluated because of strong growth inhibition and low solubility of the inducing compounds at pH 6. Induction of the SOS system by addition of mitomycin had no effect on the PGA-induced level but increased PL basal activity twofold (Table 3). Assay of pectin lyase activity showed that this activity was induced about fourfold in the cultures containing mitomycin but was insensitive to the presence of pectin derivatives (data not shown). Induction of pectin lyase production depending on the SOS system was previously analyzed with other *E. chrysanthemi* strains (49).

Effect of growth conditions on the expression of each pectinase gene. The *uidA* fusions were assayed in various growth conditions to follow expression of the pectinase genes individually (Table 4). The PL activity was assayed in each case to verify that the corresponding growth conditions had an effect similar to that expected from the wild-type analysis (Table 5). In the absence of inducer, the different fusions were expressed at different levels. The basal level of *pelE* was high, the *pelB* and *pelC* basal levels were intermediate, and *pelA* and *pelD* were expressed at low basal levels.

TABLE 4. Effects of various environmental conditions on the independent transcription of each pectinase gene[a]

Growth condition and inducer	GUS sp act					
	A1888 (*pelA*::*uidA*)	A1787 (*pelB*::*uidA*)	A1880 (*pelC*::*uidA*)	A1798 (*pelD*::*uidA*)	A1828 (*pelE*::*uidA*)	A1789 (*pem*::*uidA*)
Standard						
None	5.1	89	92	23	303	58
PGA + plant extract	10.1	525	495	2,792	1,540	383
Galacturonate	7.2	125	150	216	1,128	111
+ Mitomycin, none	4.2	85	79	24	337	48
Anaerobiosis, none	12.6	91	61	63	1,687	52
25°C						
None	11.3	78	131	102	1,112	209
PGA + plant extract	20.3	373	421	3,812	2,210	331
37°C						
None	4.4	37	80	16	230	56
PGA + plant extract	3.8	81	99	165	367	119
Glucose addition, PGA + plant extract	4.6	509	321	1,823	862	132
Nitrogen starvation, PGA + plant extract	3.7	4	7	80	22	11
High osmolarity						
None	4.1	70	88	17	1,888	52
Galacturonate	6.2	142	146	71	3,702	156

[a] Each fusion was assayed, in the absence or presence of inducing compounds (PGA plus plant extract or galacturonate), at different temperatures or in different physiological conditions by cultivation in the specific media described for Table 3. Standard conditions were obtained by cultivation in M63 medium containing glycerol as a carbon source, at 30°C and with shaking (200 rpm). To ensure reproducible results, each culture was carried out at least three times. The value obtained for a significant experiment is indicated.

In the presence of PGA and plant extract, the transcription of the fusions was significantly stimulated. Induction ratios were ca. 2 for *pelA*; ca. 5 for *pelB*, *pelC*, *pelE*, and *pem*; and greater than 100 for *pelD*, demonstrating that all genes are induced by pectin-degradative products but with different efficiencies. Induction by galacturonate was very low for *pelA*, *pelB*, and *pelC*, about twofold for *pem*, fourfold for *pelE*, and ninefold for *pelD*. Since galacturonate is an easily metabolizable carbon source, it may provoke catabolite repression of the expression of pectinase genes, counterbalancing its inducing properties.

Induction of the SOS system by addition of mitomycin, which induces pectin lyase production, did not affect transcription of *pem* or *pel* genes. The low increase found for the total PL activity in the presence of mitomycin (Table 3) could be explained by a nonspecific activity of the pectin lyase, induced in these conditions, on the substrate of the PL assay, PGA. As found for total PL activity, anaerobiosis increased the expression of the genes *pelA*, *pelD*, and *pelE* in noninduced conditions, 2.5-, 3-, and 5-fold, respectively. Expression of *pelB*, *pelC*, and *pem* was not affected by anaerobiosis.

TABLE 5. Effects of various environmental conditions on the global synthesis of PL[a]

Growth condition and inducer	PL sp act					
	A1888 (*pelA*::*uidA*)	A1787 (*pelB*::*uidA*)	A1880 (*pelC*::*uidA*)	A1798 (*pelD*::*uidA*)	A1828 (*pelE*::*uidA*)	A1789 (*pem*::*uidA*)
Standard						
None	0.10	0.11	0.12	0.10	0.03	0.09
PGA + plant extract	3.69	2.07	2.20	3.20	2.80	2.93
Galacturonate	0.38	0.55	0.39	0.45	0.30	0.85
+ Mitomycin, none	0.15	0.23	0.14	0.34	0.11	0.16
Anaerobiosis, none	0.38	0.50	0.48	0.68	0.43	0.66
25°C						
None	0.23	0.35	0.46	0.60	0.26	0.76
PGA + plant extract	3.55	1.84	1.73	3.27	2.67	2.27
37°C						
None	0.04	0.05	0.08	0.07	0.03	0.06
PGA + plant extract	0.43	0.16	0.13	0.14	0.17	0.47
Glucose addition, PGA + plant extract	1.45	0.96	1.11	0.83	0.98	0.94
Nitrogen starvation, PGA + plant extract	0.28	0.23	0.21	0.19	0.13	0.16
High osmolarity						
None	0.17	0.15	0.15	0.16	0.04	0.14
Galacturonate	0.57	0.64	0.39	0.65	0.22	0.67

[a] The PL values indicated in this table were obtained for the same experiments as the GUS values given in Table 4.

Decreasing the temperature to 25°C elevated the expression of the genes *pelA*, *pelD*, *pelE*, and *pem* in the absence of inducer two- to fivefold. Increasing the temperature to 37°C repressed the transcription of all the genes tested, 3-fold for *pelA* and *pem*; ca. 5-fold for *pelB*, *pelC*, and *pelE*; and ca. 15-fold for *pelD* in inducing conditions. In the absence of inducer, increasing the temperature of growth has less repression effect. In the presence of a readily utilizable carbon source, such as glucose, a decrease in transcription of most of the fusions was observed. This repression was 1.5-fold for *pelC* and *pelD* and 2-fold for *pelA*, *pelE*, and *pem*. In conditions of nitrogen starvation, GUS synthesis was strongly inhibited in all the fusion strains, with a decrease of 3-fold for *pelA*, 30-fold for *pelD* and *pem*, 130-fold for *pelB*, and 270-fold for *pelC* and *pelE*. PL activity was simultaneously strongly repressed in each strain (Table 5).

Osmolarity of the medium had diverse effects on the *pel* genes. High osmolarity had no effect on *pelA*, *pelB*, *pelC*, or *pem* expression but decreased *pelD* transcription about threefold in the presence of pectic inducer and increased *pelE* transcription sixfold in the absence of inducer. Increase of *pelE* expression was seen even in the presence of inducer (threefold factor), while the repression effect of high osmolarity on *pelD* was evident only in inducing conditions.

DISCUSSION

We have explored several aspects of the transcriptional regulation of the *E. chrysanthemi* pectinase genes. We analyzed both the total PL production due to the five *pel* genes in the wild-type strain and the individual expression of each pectinase gene in the fusion strains. As shown for many bacterial virulence genes (31), the expression of the *E. chrysanthemi* pectinase genes is controlled by several environmental conditions.

Environmental conditions controlling PL production. The basal level of PL production, i.e., in noninducing conditions, was low but significant (Table 3). As shown in many previous studies, the transcription of the *pel* genes is significantly elevated by culturing bacteria with pectins or derivatives, the most commonly used being PGA or galacturonate (induction ratios of 30- and 6-fold, respectively). Various environmental stimuli influenced the production of pectinases. Enzyme synthesis was very sensitive to growth phase, and the production of PL was low during the first hours of bacterial growth but increased ca. 40-fold when cells reached the end of the log phase (Fig. 3). The other factors influencing PL production were (Tables 3 and 5) nitrogen starvation, anaerobiosis, growth temperature, osmolarity of the growth medium, and presence of a readily utilizable carbon source. The effect of conditions that increase PL expression (for instance, anaerobiosis and low temperature) was more visible in the absence of pectic inducer, since the responses to these different inducing conditions were not synergistic. In contrast, the effect of conditions that decreased PL synthesis was more apparent in the presence of inducer (nitrogen starvation and high temperature), probably because the basal level of PL activity was low and difficult to estimate precisely. For these reasons both induced and noninduced conditions were generally tested. Conditions that showed no effect on total PL production, such as phosphate starvation, were not further studied. Conditions which affected total PL production were further tested, by using transcriptional fusions with each pectinase gene.

Individual expression of each pectinase gene. Comparison of the basal level of each *pel*::*uidA* fusion revealed the major role played by *pelE* in the production of PL in the absence of inducing factors (Table 4). The contribution by *pelB* and *pelC* was also significant. *pelE* expression was 3-fold higher than that of *pelB* or *pelC*, 13-fold higher than that of *pelD*, and 60-fold higher than that of *pelA*. Comparison of the higher expression levels showed that the *pelD* and *pelE* genes are responsible for the major PL production in inducing conditions; *pelD* and *pelE* are expressed 3- to 5-fold higher than *pelB* or *pelC* and more than 100-fold higher than *pelA*. The transcription of each *pel* gene responded at different levels to the following conditions: induction in the presence of PGA, increase at the end of the exponential growth phase, repression in conditions of nitrogen starvation, and effect of temperature. Genes of the *pelA*, *pelD*, and *pelE* group are more sensitive to physiological conditions such as anaerobiosis or catabolite repression than the *pelB* and *pelC* genes. This study of the *pel* regulation revealed further differences between the *pelB* and *pelC* family and the *pelA*, *pelD*, and *pelE* group. These two classes were previously identified by comparison of the nucleotide and amino acid sequences of the genes and their products (19), confirmed by the PL immunological properties (51), their enzymatic activities (38), and their role in plant infection (5). At the level of regulation of their expression, the *pelB* and *pelC* genes appeared very similar. In contrast, in the *pelA*, *pelD*, and *pelE* group, each gene behaved differently (low expression for *pelA*, high induction for *pelD*, and high basal level for *pelE*). It could be noted that among the environmental conditions tested, osmolarity is the sole condition differently affecting the transcription of the five pectinase genes. While a high osmolarity had no effect on *pelA*, *pelB*, and *pelC* expression, it lowered *pelD* expression (threefold) and increased *pelE* expression (sixfold).

The expression of *pem* generally followed that of the *pel* genes but was less sensitive to most of the environmental conditions tested. The basal level of *pem* was quite high; *pem* expression was induced about sevenfold in the presence of PGA and was mainly affected by growth phase, catabolite repression, and nitrogen limitation.

The regulatory genes involved in *pel-pem* expression. The mechanisms of pectinase gene regulation are not well identified, except for induction by pectin catabolic products, which is, at least partially, mediated by the *kdgR* gene product (42). The KdgR repressor binds to the nucleotide sequence corresponding to the KdgR operator, situated in the vicinity of the *pel* promoter (35), and probably prevents DNA polymerase binding. The KdgR repressor is able to interact with KDG, the true inducing molecule, in vitro and probably in vivo, provoking the dissociation of the repressor from the operator (35), thus allowing the transcription of the gene. By analogy with *E. coli*, it is assumed that catabolite repression occurs via the catabolite gene activator protein-cyclic AMP complex. The presence of regions showing homologies with the *E. coli* catabolite gene activator protein binding site in the 5′ upstream region of some *pel* genes (18, 40) confirmed this supposition. The mechanisms controlling the growth-phase regulation are not known. In *E. coli*, a new sigma factor, product of the *rpoS* gene (30), is proposed to be involved in the regulation of genes expressed at the end of growth. However, the transcription of the *pel* genes is not dependent on this factor (26; unpublished data), suggesting that other mechanisms may be involved. In *E. coli*, the *fnr* gene is involved in a positive control of genes expressed in anaerobiosis (47). However, the *pel* genes do not contain

sequences homologous to the Fnr binding site. Furthermore, the *pecX* gene, located near *pelA*, is thought to be involved in the regulation of *pel* genes in anaerobic conditions (16). Since PecX protein shows homology with reductase and globin, its role may be a sensor of oxygen limitation, rather than a direct regulatory protein. Sauvage et al. (46) recently showed that conditions of iron restriction also affect the production of PL in *E. chrysanthemi* 3937. Each *pel* gene shows a different sensitivity to iron regulation (45a). The products of the *cbrAB* operon, which control iron assimilation, are probably involved in this regulation (15). Other genes are thought to be involved in more specific control of some pectinase genes, such as *pecY* on *pem* expression (4) or *pecZ* on *pelB* and *pelC* expression (26). However, the signals or the conditions to which PecY or PecZ responds remain unknown, and the mechanisms responsible for this regulation of pectinase production are not identified. It should also be noted that the factors influencing PL synthesis do not act in synergy. For instance, the effects of anaerobiosis and PGA induction are not additive. Since different classes of mutants affected for PL regulation were obtained in various *E. chrysanthemi* strains (*pecS*, *pecI*, *cri*, *gpiR*, etc.) (41), it is now necessary to characterize the corresponding wild-type genes and their products in order to specify their roles in the regulation of PL production.

The expression signals of each *pel* gene and their roles in bacterial ecology. Comparison of the promoter regions of the five *pel* genes of 3937 (17, 26, 40) shows a good homology for *pelB-pelC* (68% homology from nucleotides −100 to +1), a significant homology between *pelD* and *pelE* (57%), and little homology between these two groups or between one of these groups and the *pelA* gene. The similar expression of the two genes encoding neutral PL may be related to the homologies found in their promoter regions (26). The reason for the existence of these two genes, which possibly result from the duplication of a common ancestor, is not clear. Analysis of mutants devoid of PLb or PLc showed that the role of these neutral isoenzymes in the infection process is secondary (5). Both neutral isoenzymes are conserved in the various analyzed *E. chrysanthemi* strains (6), and homologous enzymes exist in other *Erwinia* soft rot species such as *E. carotovora* or *E. atroseptica* (19). These enzymes might have a role, not at the first steps of plant infection, but during the maintenance of infection, by increasing the total PL activity. Another possibility is that neutral PL are mainly involved in the saprophytic life of the bacteria.

The *pelA* gene was expressed at a very low level under all the conditions tested. This result is in agreement with the lack of identifiable transcription initiation signals in the 5′ upstream region of the *pelA* open reading frame (17) and the failure to identify a functional promoter by in vitro experiments (6a, 17). The role of the acidic isoenzyme in bacterial pathogenicity (5) is not clear in view of its catalytic properties (38) or its expression level.

The level of transcription of the two genes encoding basic isoenzymes appeared quite different. The *pelD* expression shows a very low basal level but is very sensitive to induction and is strongly affected by all the conditions modifying pectinase production. The *pelE* gene is expressed at a high basal level and is less sensitive to environmental conditions. The *pelE* and *pelD* promoters are more similar to the classical promoter consensus (18, 40, 50), and this could explain the high transcription of these two genes in induced conditions. The *pelD* gene presents two KdgR binding sites overlapping the promoter which could be very efficient for repression, explaining the low basal level and the high inducibility of this gene. The *pelE* gene presents one KdgR box (40) which is probably not very effective for KdgR binding or for inhibition of RNA polymerase binding, allowing the high basal level of transcription of this gene. The KdgR boxes are not the sole sequences near the promoter that may be involved in regulation of *pel* expression. For instance, in the *pelE* gene of the *E. chrysanthemi* EC16 (18), four sequences near the promoter might be involved in negative or positive regulations. Two of these sequences are probably operators of the regulatory protein KdgR and the catabolite gene activator protein. The other sequences might bind unidentified proteins.

Another important difference between *pelD* and *pelE* is that high osmolarity provokes an increase in *pelE* expression but a decrease in *pelD* expression. Osmolarity in the intercellular space of plants is relatively low. However, disruption of the cells after maceration probably provokes an increase in the osmolarity of the infected plant tissue. The expression of *pelE* might be adapted to this condition. Analysis of mutants affected for one basic PL showed that both PLd and PLe are important for full infection of plants (5). In the case of PLe, we proposed that its high basal level is an important factor during plant infection. The high basal level of PLe permits the rapid degradation of plant pectin and the formation of inducers, which then accelerate the production of all pectinases. Infection of potato tubers with the strains containing the *pel-uidA* fusions showed that the *pelD* and *pelE* genes are the highly expressed in planta (unpublished results), confirming the major role of PLd and PLe isoenzymes during plant infection.

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