Expression of *Erwinia chrysanthemi* Pectinase Genes *pelL*, *pelI*, and *pelZ* During Infection of Potato Tubers

Sylvia Jafra,¹ Izabela Figura,¹ Nicole Hugouvieux-Cotte-Pattat,² and Ewa Lojkowska¹

¹Laboratory of Phytopathology, Department of Biotechnology, Intercollegiate Faculty of Biotechnology, University of Gdansk and Medical University of Gdansk, Kladki 24, 80-822 Gdansk, Poland. ²Laboratoire de Genetique Moleculaire des Microorganismes, UMR-CNRS 5577, INSA, Bat. 406, 20, Av. A. Einstein, 69621 Villeurbanne cedex, France

Accepted 14 June 1999.

Erwinia chrysanthemi mutants, containing transcriptional fusions of one of the minor pectate lyase genes (*pelI*, *pelL*, *pelZ*) with the reporter gene encoding β -glucuronidase activity, were studied for their ability to cause disease symptoms and to synthesize pectinases after inoculation of potato tubers. The strains affected in *pelI* and *pelL* genes displayed reduced virulence on potato tubers, demonstrating the important role of these isoenzymes in soft rot disease. Inactivation of the *pelZ* gene slightly influences the ability to macerate. Analysis of the bacterial population showed rapid multiplication of bacteria during infection. Similar kinetics of growth were observed for all mutants and for the wild-type strain. Comparison of the mutants and the wild-type strain showed that the *pelI*, *pelL*, and *pelZ* mutants synthesized reduced levels of Pels. The expression of *pelZ* is fivefold higher in planta than in bacterial cultures. In contrast, both *pelI* and *pelL* are highly (10-fold factor) induced in planta, which is characteristic of the plant-inducible pectate lyases.

The pathogenic bacterium *Erwinia chrysanthemi* causes various diseases in a wide range of plants. Pathogenicity results from the activity of extracellular enzymes responsible for the maceration process, which involves the depolymerization of pectins in the plant cell wall. In vitro, *E. chrysanthemi* produces a set of pectin-degrading enzymes such as pectin methylesterase, pectin acetyl esterase, pectate lyase, pectin lyase, and polygalacturonase (Collmer and Keen 1986; Barras et al. 1994; Shevchik et al. 1997). These pectinases are secreted into the external medium via a common secretion system, which is encoded by the *out* operon, essential for pathogenicity.

Pectate lyases seem to play a crucial role in the maceration of plant tissue (Barras et al. 1994). They cleave internal glycosidic bonds in polygalacturonic acid (PGA) or low-methylesterified pectins by β -elimination and exhibit reduced activity on a highly methylesterified or acetylated substrate. The pectin methylesterases and pectin acetyl esterases remove the esterified groups from pectin, liberating PGA, which is the substrate of pectate lyases (Laurent et al. 1993; Shevchik and Hugouvieux-Cotte-Pattat 1997). In *E. chrysanthemi*

3937, most of the Pel activity results from the cumulative action of the five major isoenzymes, PelA, PelB, PelC, PelD, and PelE (Bertheau et al. 1984; Ried and Collmer 1988), encoded by independent genes organized in two clusters on the bacterial chromosome (*pelA*, *pelD*, and *pelE*; *pelB* and *pelC*) (Hugouvieux-Cotte-Pattat et al. 1989). Deletion of the five major *pel* genes of *E. chrysanthemi* does not totally eliminate the ability to macerate plant tissues (Ried and Collmer 1988). This reveals the presence of a new set of Pels, which are probably synthesized by the bacteria after interaction with plant tissues (Beaulieu et al. 1993; Kelemu and Collmer 1993). With the use of various approaches, three new *pel* genes were cloned: *pelL*, *pelI*, and *pelZ* (Lojkowska et al. 1995; Alfano et al. 1995; Pissavin et al. 1996; Shevchik et al. 1997). Because of their low activity in synthetic medium they were first described as secondary or minor Pels.

Pectins and their derivatives induce synthesis of Pel isoenzymes. Transcription of the *pel* genes is regulated by environmental conditions such as growth phase, nitrogen starvation, anaerobiosis, osmolarity, temperature, and catabolite repression (Hugouvieux-Cotte-Pattat et al. 1992). Low concentrations of iron also favor the expression of *pel* genes (Sauvage and Expert 1994). Analysis of transcription of the major *pel* genes during plant infection shows differences in importance of each isoenzyme in pathogenicity (Lojkowska et al. 1993; Dorel et al. 1996; Masclaux et al. 1996). *E. chrysanthemi* attacks a wide range of plants and the role of each pectinase depends on the host plant (Beaulieu et al. 1993). The synthesis of each Pel in macerated tissues differs in various plant species (Beaulieu et al. 1993) and also in various types of tissues (Lojkowska et al. 1993). The variety of Pel isoenzymes may result from specific adaptations of the bacteria to macerate different plant tissues. Each Pel has particular individual properties (Lojkowska et al. 1995; Shevchik et al. 1997; Tardy et al. 1996; Pissavin et al. 1998) and can therefore be more or less adapted to the substrate and conditions present in the plant during the first step of infection. The minor pectate lyases PelL, PelI, and PelZ, despite their low activity on purified substrates, can play an important role during infection (Lojkowska et al. 1995; Shevchik et al. 1997; Pissavin et al. 1996).

Plant factors may influence the expression of genes involved in pathogenicity (Boccardo et al. 1988; Beaulieu et al. 1993; Dorel et al. 1996). In this study, we have analyzed the involvement of the *pelL*, *pelI*, and *pelZ* genes in pathogenicity

Corresponding author: Ewa Lojkowska; Telephone: +48 58 320 2248; Fax: +48 58 301 2708; E-mail: lojkowska@biotech.univ.gda.pl

by inoculating potato tubers with the appropriate *pelL::uidA*, *pelI::uidA*, or *pelZ::uidA* mutant. Using these genetic fusions, we have also compared the expression of *pelL*, *pelI*, and *pelZ* genes in rotting tuber tissues with that of the major *Pel* genes *pelA* and *pelE*.

RESULTS

Pathogenicity of the mutants on potato tubers.

Potato tubers were inoculated with the *E. chrysanthemi* wild-type strain 3937, the mutant A1744, which is deleted for six *pel* genes (Δpel) (Table 1), and mutants containing a *uidA* transcriptional fusion in *pelI*, *pelL*, and *pelZ* genes. This type of analysis was previously performed for the major *pel* genes (Lojkowska et al. 1993), and in this study we used *pelA* and *pelE* fusions as references.

Table 1. *Erwinia chrysanthemi* strains and bacteriophage

Strain	Characteristic	Reference or source
3937	Wild type	Laboratory collection
A1888	<i>pelA::uidA</i> , Km ^r	Hugouvieux-Cotte-Pattat et al. 1992
A1828	<i>pelE::uidA</i> , Km ^r	Hugouvieux-Cotte-Pattat et al. 1992
A3102	<i>pelI::uidA</i> , Km ^r	This work
A3103	A1744, <i>pelI::uidA</i> , Km ^r	This work
A1990	<i>pelL::uidA</i> , Km ^r	Lojkowska et al. 1995
A1994	A1744, <i>pelL::uidA</i> , Km ^r	Lojkowska et al. 1995
A1954	<i>pelZ::uidA</i> , Km ^r	Pissavin et al. 1996
A1744	PMV4116, $\Delta pelADEpaeY$, $\Delta pelBCZ$	Beaulieu et al. 1993
A2701	<i>lacZ2 pelI::uidA</i> , Km ^r	Shevchik et al. 1997
Phage Φ EC2	General transducing phage of <i>E. chrysanthemi</i>	Resibois et al. 1984

Rotting, estimated by the diameter of decay, progressed linearly (Fig. 1). After 24 h, the mutants affected in the gene of a minor *Pel* (*pelI*, *pelL*, or *pelZ*) exhibited a low maceration capacity. The disease severity of the *pelA* and *pelE* mutants was intermediate between that observed for the other mutants and the wild-type strain. After 48 h, the *pelZ* mutant exhibited a maceration similar to that of the *pelA* and *pelE* mutants, while the *pelI* and *pelL* mutants remained weak in macerating ability. After 72 h, about 40% reduction of maceration was observed after inoculation of potato tubers with the *pelI* and *pelL* mutants, compared with the wild-type strain (Fig. 1). No significant difference of ability to macerate was observed with the *pelE*, *pelA*, and *pelZ* mutants (Fig. 1). The Δpel mutant A1744 was able to macerate potato tuber tissue with a lower efficiency than single mutants, as maceration was reduced by 75% after 72 h, compared with the wild-type strain. In strain A1994 (Δpel , *pelI*) the reduction of maceration caused by *pelI* inactivation remained visible although this mutant was still able to macerate tuber tissue (Fig. 1). The maceration ability of A1744, which is deleted for the major isoenzymes, together with the clear reduction of maceration caused by either *pelI* or *pelL* inactivation, indicated the role played by these minor isoenzymes in the soft rot caused by *E. chrysanthemi* on potato tubers.

To analyze the bacterial multiplication after inoculation, we determined the total number of bacteria per inoculation site. This value is calculated by multiplying the number of bacteria per gram of rotted tissue (obtained by dilution plating) by the weight of rotted tissue.

Analysis of the bacterial population showed rapid multiplication of bacteria during the first 72 h after infection. The numbers of bacteria for the wild type and the *pelL* mutant per gram of rotted tissue are shown in Table 2. Similar kinetics of multiplication per gram of rotted tissue were observed for each mutant (*pelA*, *pelE*, *pelI*, *pelL*, *pelZ*) and for the wild-

Fig. 1. Comparison of the rotting capacity of the wild-type strain and *pel* mutants on potato tubers inoculated with the wild-type strain and mutants containing a fusion between one pectinase gene and the reporter gene *uidA*, using sterile pipette tips containing 50 μ l of bacterial suspension (2×10^7 CFU·ml⁻¹) and incubated in a dew chamber at 30°C. Disease severity was estimated by the rot progression. Every 24 h, the width of decayed tissue was measured. Standard deviation was calculated from three independent experiments for each mutant and was similar for the wild-type strain and mutants. For clarity, error bars are included only for the wild-type strain.

type strain. In contrast, the total number of bacteria for the wild-type strain per inoculation site is significantly higher than that for the *pelL* mutant. Analysis of the other mutants confirmed that the number of bacteria per inoculation site correlated with the ability to macerate the plant tissue.

Pectate lyase activity during infection.

In order to compare the ability of the mutants to produce Pels, estimation of pectate lyase activity in tuber tissue was performed 8, 12, 16, 20, 24, 48, and 72 h after inoculation (Fig. 2). Comparison of the mutants and the wild-type strain showed that the *pelI*, *pelL*, and *pelZ* mutants synthesized reduced levels of all Pels. Statistical analysis indicated that, each time during the first 3 days after inoculation, these mu-

tants produced significantly lower levels of Pels per gram of tissue than the wild-type strain. The low Pel activity in plant tissues inoculated with the *pelI* and *pelL* mutants is linked to a decrease of maceration (Figs. 1 and 2). However, despite reduced Pel activity, only a transient reduction of maceration was observed with the *pelZ* mutant (Figs. 1 and 2).

Expression of the *pel* genes during infections.

Estimation of *pel* gene expression in potato tubers was performed by measuring the β -glucuronidase (GUS) activity due to the reporter gene *uidA*. It was possible to differentiate various levels of expression of the fusions: *pelL* and *pelZ* are weakly expressed (up to 270 U per gram of tissue), but somewhat higher than *pelA* (Fig. 3A), whereas *pelI* is highly expressed (up to 2,600 U per gram of tissue) at a level similar to that of *pelE* (Fig. 3B).

During the first 16 h, expression of all the fusions is very low and there is no significant difference in their levels. When maceration becomes really apparent (which is at about 20 h), *pelA*, *pelL*, and *pelZ* remain weakly expressed (about 40 U per gram of tissue) while *pelE* and *pelI* are induced (250 U per gram of tissue). Two days after inoculation *pelA* is still weakly expressed (60 U per gram of tissue), while *pelL* and *pelZ* expression has increased further. In most cases, expression of the fusions reaches a plateau at 48 h. In the case of the *pelI* fusion, the expression still increases at 72 h (Fig. 3). The final level of expression of the *pelE* and *pelI* genes is about 10 times higher than that of *pelL* or *pelZ*.

Comparison of *pel* gene expression in potato tubers and in bacterial cultures.

The *pel* gene expression observed in potato tubers was compared with that obtained in bacterial cultures (Fig. 4). Synthetic media without inducer or with PGA or PGA and

Table 2. Bacterial multiplication between 8 and 72 h after inoculation of potato tubers^a

Time after inoculation	No. of bacteria per gram of rotted tissue ($\times 10^8$)		No. of bacteria per inoculation site ($\times 10^8$)	
	Wild-type strain	<i>pelL::uidA</i> mutant	Wild-type strain	<i>pelL::uidA</i> mutant
8 h	0.10	0.08	0.012	0.01
12 h	0.18	0.16	0.07	0.07
16 h	0.63	0.69	0.27	0.29
20 h	1.40	1.21	0.56	0.39
24 h	2.02	1.60	1.05	0.45
48 h	4.40	3.70	7.92	3.59
72 h	6.80	6.10	28.60	4.51

^a Potato tubers were inoculated with sterile pipette tips containing 50 μ l of bacterial suspension (2×10^7 CFU $\cdot$ ml⁻¹) and incubated in a dew chamber at 30°C. Number of bacteria was estimated 8, 12, 16, 20, 24, 48, and 72 h after inoculation of potato tubers with all tested strains. Number of bacteria in 1 g of rotted tissue was determined by dilution plating. Number of bacteria per inoculation site was calculated by multiplying number of bacteria per 1 g by weight of all rotted tissue.

Fig. 2. Activity of pectate lyase after inoculation of potato tubers. The wild-type strain and the three mutants containing a fusion between the minor *pel* genes and the reporter gene *uidA* were inoculated into potato tubers as described in Figure 1 caption. Estimation of pectate lyase activity in tuber tissue was performed 8, 12, 16, 20, 24, 48, and 72 h after inoculation in toluenized extracts of infected tissue. Number of bacteria in 1 g of rotted tissue was determined by dilution plating (three replicates) and the corresponding bacterial dry weight was calculated. Values of Pel activity were expressed per gram of rotting tissue. One unit of Pel activity was defined as the amount of enzyme required for liberation of 1 μ mol of unsaturated product per minute. Error bars indicate the standard deviation calculated from four independent experiments.

potato tuber extract were inoculated with each strain. With the wild-type strain the global Pel activity was induced in the presence of PGA and presented a higher induction in the presence of both PGA and potato tuber extract (Fig. 4A).

With the *pel* fusions, the three different levels of expression observed in planta were reproduced in synthetic media: *pelI* and *pelE* are expressed more than *pelL* and *pelZ*, which in turn are expressed more than *pelA*. The addition of PGA and PGA with potato tuber extract gave very different effects on the individual *pel* genes. The *pelA* and *pelI* fusions were better induced in the presence of PGA and potato tuber extract than with PGA only (Fig. 4B and D). The *pelE* and *pelL* fusions showed a better induction in the presence of PGA only (Fig. 4C and E). The *pelZ* expression was similar in the presence of PGA and of both PGA and potato tuber extract (Fig. 4F). Thus, the *pel* genes are not all subjected to hyperinduction in the presence of plant extracts.

Fig. 3. Expression of the *pel::uidA* fusions in inoculated tubers tissue. β -Glucuronidase (GUS) activity was determined 8, 12, 16, 20, 24, 48, and 72 h after inoculation of potato tubers with tested mutants as described in Figure 1 caption. Number of bacteria in 1 g of rotted tissue was determined by dilution plating (three replicates) and the corresponding bacterial dry weight was calculated. Values of GUS activity were expressed per gram of rotting tissue. GUS-specific activity was expressed as nanomoles of *p*-nitrophenol liberated from *p*-nitrophenyl- β -D-glucuronide per minute per gram of tissue. **A**, GUS activity of the weakly expressed fusions *pelA::uidA*, *pelL::uidA*, and *pelZ::uidA*. **B**, GUS activity of the highly expressed fusions *pelE::uidA* and *pelI::uidA*.

Both pectate lyase activity and expression of the fusions were induced more in rotting potato tubers than in synthetic medium with inducer. The in planta global Pel activity is increased by a fivefold factor, compared with PGA medium (Fig. 4A). The in planta induction of the *pelA*, *pelE*, and *pelZ* fusion is also about fivefold higher (Fig. 4B, C, and F), while the difference with the *pelI* and *pelL* reached a 10-fold factor (Fig. 4D and E), compared with PGA medium.

DISCUSSION

Pathogenicity of *E. chrysanthemi* is linked to the production of plant cell-wall-degrading enzymes. Development of the disease process depends on many environmental conditions (Perombelon and Kelman 1980; Maher and Kelman 1983; Beaulieu et al. 1993). Potato tissue appeared very suitable for investigation of the capacity for maceration of a wide range of *E. chrysanthemi* mutants (Beaulieu and Van Gijsegem 1992; Lojkowska et al. 1993). In this research, we have compared the importance of three different pectate lyase isoenzymes in maceration of potato tuber tissue.

The ability of *E. chrysanthemi* 3937 to macerate plant tissue results from the action of at least eight endo-pectate lyases. The disruption of any of the single major pectate lyase genes (*pelA*, *pelB*, *pelC*, *pelD*, *pelE*) does not significantly affect the maceration capacity of *E. chrysanthemi* on potato tubers (Fig. 1) (Lojkowska et al. 1993). In addition, an *E. chrysanthemi* strain in which the five major *pel* genes have been deleted remained capable of limited maceration on plant tissue (Fig. 1) (Beaulieu et al. 1993; Kelemu and Collmer 1993). This suggested the importance of the minor pectate lyases in the soft rot disease. Preliminary work indicated a role of PelL in pathogenicity since disruption of the corresponding gene decreases the virulence on *Saintpaulia* (Lojkowska et al. 1995). In contrast, *pelI* or *pelZ* mutations did not affect pathogenicity to these plants, but reduced the maceration observed on chicory leaves (Pissavin et al. 1996; Shevchik et al. 1997).

Mutants of *E. chrysanthemi* with transcriptional fusion of *pel* genes with the reporter gene *uidA* were used to investigate the role of the individual minor Pels (PelI, PelL, PelZ) in first steps of bacterial infection on potato tubers. For comparison, pectate lyase activity in the wild-type strain and expression of two major *pel* genes (*pelA* and *pelE*) were also analyzed. The progression of disease severity, bacterial growth, pectate lyase activity (which corresponds with the non-mutated *pel* genes), and GUS activity (indicating the level of expression of the fused gene) were determined 8, 12, 16, 20, 24, 48, and 72 h after inoculation of potato tubers.

After the first 24 h, all mutants affected in a minor pectate lyase gene (*pelL::uidA*, *pelI::uidA*, or *pelZ::uidA*) showed a 50% decrease of maceration capacity, compared with the wild-type strain (Fig. 1). However, the effect of the *pelZ* mutation is transient, since, after 72 h, the maceration caused by the *pelZ* mutant is similar to that caused by the *pelE* and *pelA* mutants, whose pathogenicity is not significantly different from that of the wild-type strain (Fig. 1) (Lojkowska et al. 1993). Preliminary results indicated that inactivation of the *pelL* gene leads to attenuation of the *E. chrysanthemi* virulence on potato tubers (Lojkowska et al. 1995; Jafra et al. 1997). In our studies, the *pelL* and *pelI* mutants showed a significant reduction of the capacity to cause soft rot on potato tubers even 72 h after infection

(Fig. 1). These results indicate an important role of both PelL and PelI isoenzymes in *E. chrysanthemi* pathogenicity.

All the mutants showed active multiplication in planta. Each time after infection, the number of bacteria per gram of

rotted tissue was similar for each strain (Table 2), as observed previously (Lojkowska et al. 1993). In contrast, the number of bacteria per inoculation site of the wild-type strain and the *pelL* mutant differed and were correlated with disease severity

Fig. 4. Expression of *pel* genes in potato tuber and bacterial cultures. Cultures were grown at 30°C in M63 + glycerol (Y) minimal medium supplemented with 2.5 g · liter⁻¹ polygalacturonic acid (PGA) or 1.5% potato tubers extract (PE) to late-log phase of growth. The wild-type strain and mutants containing a *pel::uidA* fusion were inoculated into potato tubers as described in Figure 1 caption. **A**, Pel activity produced by the wild-type strain in synthetic media and tuber tissue. Pel activity was determined in toluenized extracts of rotted tissue. Specific activity of Pel was expressed as micromoles of unsaturated products liberated per minute per milligram of bacterial dry weight. **B–F**, β-glucuronidase (GUS)-specific activity reflected expression of *pel* genes *pelA*, *pelE*, *pelI*, *pelL*, *pelZ*, respectively. GUS activity was determined in toluenized, rotted tissue extracts. Specific GUS activity is expressed as nanomoles of *p*-nitrophenol liberated from *p*-nitrophenyl-β-D-glucuronide per minute per milligram of bacterial dry weight.

(Table 2). Such correlation between bacterial multiplication and the ability to macerate tuber tissue was expected.

Disruption of one of the minor *pel* genes provokes a reduction of 70 to 80% of the pectate lyase activity in plant tissue (Fig. 2). Damage in one of the major genes influences the total pectate lyase activity to a lower extent, since only a 10 to 60% reduction was observed (Lojkowska et al. 1993). This indicates that the absence of expression of minor Pels could affect the synthesis of others. Our hypothesis is that minor Pels can attack particular constituents of the pectin matrix present in the plant cell walls, enabling better action of the other pectinases. Indeed, good cleavage of pectin is required for the formation of inducers of pectinolysis (Hugouvieux-Cotte-Pattat et al. 1996) which, in turn, enable use of pectin as a carbon source for bacterial growth. For instance, PelI, which acts at high calcium concentration (Shevchik et al. 1997), could perhaps disrupt calcium-bridged pectin chains. Thus, particular properties of minor Pels could explain why inactivation of one minor *pel* gene results in lower maceration of plant tissues associated with lower induction of Pel synthesis and bacterial multiplication.

Analysis of fusion expression during the infection process enables differentiation of two groups of minor *pel* genes. Determination of the expression of *uidA* fusions with *pelA*, *pelE*, *pell*, *pelL*, and *pelZ* indicated that, during the first 16 h of infection, their induction is very low, but similar. After the first 20 h, the differences become apparent: *pelE* and *pell* are highly expressed; expression of *pelL* and *pelZ* is moderate but higher than that of *pelA* (Fig. 3A and B). The level of *pelL* and *pelZ* expression is similar to that of the major *pel* genes *pelB* or *pelC* (Lojkowska et al. 1993).

We have compared the expression of each *pel::uidA* fusion and total pectate lyase activity of the wild-type strain after inoculation of potato tubers and synthetic medium with or without inducer. Kelemu and Collmer (1993) and Beaulieu et al. (1993) reported that bacteria synthesize the minor Pels only in plant tissue or in the presence of plant extract. In this work, we have observed that for each *pel* gene the highest induction takes place in plant tissue with increase factors of 10 for *pell* or *pelL* and 5 for *pelZ*, *pelA*, or *pelE* (Fig. 4). Comparing with PGA, the presence of plant extract did not increase expression of *pelE*, *pell*, or *pelZ*, and weakly induced that of *pelA* or *pell*. Thus, the effect of plant extract is very limited, compared with real infection conditions in plant tissue. A compound might be missing from the plant extract or an environmental factor may be different.

Different roles of each Pel in the cell wall degradation process could explain the diversity of the minor *E. chrysanthemi* Pels. Indeed, PelI, PelL, and PelZ belong to three different families of pectate lyases (families III, IV, and V, respectively) (Lojkowska et al. 1995; Pissavin et al. 1996; Shevchik et al. 1997). In contrast, the five *E. chrysanthemi* major Pels belong to the large family I of pectate lyases (Henrissat et al. 1995). The low homology observed between PelZ and PelC proteins and the fact that the *pelZ* gene is adjacent to the *pelC* gene suggest that PelZ could be a highly divergent member of family I (Pissavin et al. 1998). Indeed, in our study of the *pel::uidA* fusion on potato tubers, *pelZ* appeared more related by its behavior to *pelA* or *pelE* than to *pell* or *pelL*. The *pelZ* mutant is weakly affected for maceration, as observed for *pelA* and *pelE* mutants (Fig. 1). The expression of *pelZ* in planta is induced by a fivefold factor, similar to that observed for *pelA* or *pelE* induction (Fig. 4). In

contrast, both *pell* and *pelL* are induced in planta twice as much as *pelA*, *pelE*, and *pelZ* genes (Fig. 4). These genes indicated the characteristics of plant-inducible pectate lyases described by Kelemu and Collmer (1993). The important finding (from the point of view of disease development) is that the minor *pell* and *pelL* are expressed at high levels in tuber tissue; in the case of *pell*, as high as major *pelE* and much higher than *pelA*. This suggests a putative role of *pell* and *pelL* in disease development.

MATERIALS AND METHODS

Bacterial strains, potato tubers, and growth condition.

The derivatives of *E. chrysanthemi* 3937 used in this work are mutants containing a transcriptional fusion of *pelA*, *pelE*, *pell*, *pelL*, and *pelZ* genes with the reporter gene *uidA*. The strains are listed in Table 1.

The bacteria were grown in LB (Luria-Bertani) medium or M63 synthetic medium (Miller 1972) with glycerol (Y) (2 g · liter⁻¹), PGA (2.5 g · liter⁻¹) (Sigma Chemical, St. Louis, MO) or potato tuber extract as a carbon source or Pel inducer. Bacteria were grown in liquid medium at a temperature of 30°C for 24 h with shaking. The media were solidified with agar (15 g · liter⁻¹) when required. When appropriate, kanamycin was added to the media at a concentration of 20 µg · ml⁻¹.

Potato tuber extracts were prepared by homogenization of tubers followed by centrifugation and filtration through membranes. The extract was diluted (1/75) in M63 medium and sterilized by final filtration through a 0.22-µm porosity membrane.

Potato tubers (*Solanum tuberosum* L. 'Mila') were obtained from the experimental fields of the Institute for Potato Research, Bonin, Poland.

Construction of bacterial strains.

The *pell::uidA* fusion from the strain A2701 (Shevchik et al. 1997) was transduced into the wild-type strain 3937 and the Δpel mutant A1744 with phage $\Phi EC2$ (Resibois et al. 1984). Mutants obtained after transduction were selected with kanamycin resistance as a selective marker.

Determination of pathogenicity.

Potato tubers were washed and immersed twice in 10% sodium hypochlorite (Clorox) for 15 min, rinsed in sterile water, and air dried (Lojkowska et al. 1993). Sterile pipette tips containing 50 µl of bacterial suspensions (2×10^7 CFU · ml⁻¹) were inserted into the tuber parenchyma to a depth of 10 mm (Maher and Kelman 1983). Depending on the strains and size of the tuber, three or four tips were used for its inoculation. Ten tubers were inoculated with each mutant and incubated in high humidity (approximately 98%) at 30°C. Disease severity was estimated by the progression of rot. Tubers were cut vertically through each inoculation point and the diameter of rotting tissue was measured every 24 h up to 72 h following the inoculation.

Enzyme assays.

Enzyme assays were performed on toluenized extracts of cultures grown up to early stationary phase (24 h) or in bacterial cells collected from infected potato tuber tissue (this was taken from 10 potato tubers for each strain).

Pectate lyase activity was determined by spectrophotometric monitoring of the ability to form unsaturated products,

which absorb at 230 nm (Moran et al. 1968). The assay medium contained PGA 0.05%, 0.1 mM CaCl₂, 100 mM Tris-HCl pH 8.5. One unit of Pel activity was defined as the amount of enzyme required to produce 1 µmol of unsaturated product per minute. Specific Pel activity was expressed as micromoles of unsaturated products liberated per minute per milligram of bacterial dry weight.

GUS activity was measured by the degradation of *p*-nitrophenyl-β-D-glucuronide (PNPU) into *p*-nitrophenol that absorbs at 405 nm (Bardonnnet and Blanco 1992). Specific GUS activity was expressed as millimoles of *p*-nitrophenol liberated per minute per milligram of bacterial dry weight.

The number of bacteria in 1 g of rotted tissue was determined by dilution plating and the corresponding bacterial dry weight was calculated. The values of Pel and GUS activity were expressed either per gram of rotting tissue or per inoculation site. In the latter case, the number of bacteria was calculated by multiplying the number of bacteria per 1 g of rotted tissue and weight of all rotted tissue.

Statistical analysis.

Mean values and standard errors were calculated from four independent experiments.

ACKNOWLEDGMENTS

We thank Guy Condemine and Sylvie Reverchon for critical reading of the manuscript. This work was supported by a grant from the Polish State Committee for Scientific Research (KBN) 6P04A 03210, Tempus-Phare Programme JEP07191-94, and from the Centre National de la Recherche Scientifique and the Ministère de l'Éducation Nationale, de l'Enseignement Supérieur, de la Recherche et de l'Insertion Professionnelle. The ATP Franco-Polonaise 76066 supports the collaboration between N. H.-C.-P. and E. L. The receipt of a fellowship from the French government is gratefully acknowledged by S. J.

LITERATURE CITED

- Alfano, J. R., Ham, H. H., and Collmer, A. 1995. Use of Tn5tac1 to clone a *pel* gene encoding a highly alkaline, asparagine-rich pectate lyase isoenzyme from an *Erwinia chrysanthemi* EC16 mutant with deletion affecting the major pectate lyase isoenzymes. *J. Bacteriol.* 177:4553-4556.
- Bardonnnet, N., and Blanco, C. 1992. *uidA* antibiotic resistance cassettes for insertion mutagenesis gene fusion and genetic constructions. *FEMS Microbiol. Lett.* 93:243-248.
- Barras, F., Van Gijsegem, F., and Chatterjee, A. K. 1994. Extracellular enzymes and pathogenesis of soft-rot *Erwinia*. *Annu. Rev. Phytopathol.* 32:201-234.
- Beaulieu, C., Boccara, M., and Van Gijsegem, F. 1993. Pathogenic behavior of pectinase-defective *Erwinia chrysanthemi* mutants on different plants. *Mol. Plant-Microbe Interact.* 6:197-202.
- Beaulieu, C., and Van Gijsegem, F. 1992. Pathogenic behavior of several *mini*-Mu-induced mutants of *Erwinia chrysanthemi* on different plants. *Mol. Plant-Microbe Interact.* 5:340-346.
- Bertheau, Y., Madgidi-Hervan, E., Kotoujansky, A., Nguyen-The, C., Andro, T., and Coleno, A. 1984. Detection of depolymerase isoenzymes after electrophoresis or electrofocusing, or in titration curves. *Anal. Biochem.* 139:383-389.
- Boccara, M., Diollez, A., Rouve, M., and Kotoujansky, A. 1988. The role of individual pectate-lyase of *Erwinia chrysanthemi* strain 3937 in pathogenicity on *Saintpaulia* plants. *Physiol. Mol. Plant. Pathol.* 33: 95-104.
- Collmer, A., and Keen, N. T. 1986. The role of pectic enzymes in plant pathogenesis. *Annu. Rev. Phytopathol.* 24:383-409.
- Dorel, C., Hugouvieux-Cotte-Pattat, N., Robert-Baudouy, J., and Lojowska, E. 1996. Production of *Erwinia chrysanthemi* pectinases in potato tubers showing high or low level of resistance to soft rot. *Eur. J. Plant Pathol.* 102:511-517.
- Henrissat, B. S., Heffron, S. E., Yoder, M. D., Lietzke, S. E., and Jurnak, F. 1995. Functional implication of structure-based sequence alignment of proteins in the extracellular pectate lyase superfamily. *Plant Physiol.* 107:963-976.
- Hugouvieux-Cotte-Pattat, N., Condemine, G., Nasser, W., and Reverchon, S. 1996. Regulation of pectinolysis in *Erwinia chrysanthemi*. *Annu. Rev. Microbiol.* 50:213-257.
- Hugouvieux-Cotte-Pattat, N., Dominguez, H., and Robert-Baudouy, J. 1992. Environmental conditions affect the transcription of the pectinase genes of *Erwinia chrysanthemi* 3937. *J. Bacteriol.* 174: 7807-7818.
- Hugouvieux-Cotte-Pattat, N., Reverchon, S., and Robert-Baudouy, J. 1989. Expanded linkage map of *Erwinia chrysanthemi* strain 3937. *Mol. Microbiol.* 3:573-581.
- Jafra, S., Figura, I., Hugouvieux-Cotte-Pattat, N., and Lojowska, E. 1997. Characterization and role in the pathogenesis of potatoes of a novel pectate lyase from *Erwinia chrysanthemi* 3937. Pages 511-514 in: *Developments in Plant Pathology: Diagnosis and Identification of Plant Pathogens*. Vol. 11. E. Dehne, ed. Kluwer Academic Pub., Dordrecht, The Netherlands.
- Kelemu, S., and Collmer, A. 1993. *Erwinia chrysanthemi* EC16 produces a second set of plant-inducible pectate lyase isozymes. *Appl. Environ. Microbiol.* 59:1756-1761.
- Laurent, F., Kotoujansky, A., Labesse, G., and Bertheau, Y. 1993. Characterization and overexpression of the *pem* gene encoding pectin methylsterase of *Erwinia chrysanthemi* strain 3937. *Gene* 131:17-25.
- Lojowska, E., Dorel, C., Reignault, P., Hugouvieux-Cotte-Pattat, N., and Robert-Baudouy, J. 1993. Use of GUS fusion to study the expression of *Erwinia chrysanthemi* pectinase genes during infection of potato tubers. *Mol. Plant-Microbe Interact.* 6:488-494.
- Lojowska, E., Masclaux, C., Boccara, M., Robert-Baudouy, J., and Hugouvieux-Cotte-Pattat, N. 1995. Characterization of the gene encoding a secondary pectate lyase of *Erwinia chrysanthemi* 3937. *Mol. Microbiol.* 16:1183-1195.
- Maher, E. A., and Kelman, A. 1983. Oxygen status of potato tuber tissue in relation to maceration by pectic enzymes of *Erwinia carotovora*. *Phytopathology* 73:536-539.
- Masclaux, C., Hugouvieux-Cotte-Pattat, N., and Expert, D. 1996. Iron is a triggering factor for differential expression of *Erwinia chrysanthemi* strain 3937 pectate lyases in pathogenesis of African violets. *Mol. Plant-Microbe Interact.* 9:198-205.
- Miller J. H. 1972. *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Moran, F., Nasuno, S., and Starr, M. P. 1968. Extracellular and intracellular polygalacturonic acids *trans*-eliminases of *Erwinia carotovora*. *Arch. Biochem. Biophys.* 123:293-306.
- Perombelon, M. C. M., and Kelman, A. 1980. Ecology of the soft rot *Erwinia*. *Annu. Rev. Phytopathol.* 18:361-367.
- Pissavin, C., Robert-Baudouy, J., and Hugouvieux-Cotte-Pattat, N. 1996. Regulation of *pelZ*, a gene of the *pelB-pelC* cluster encoding a new pectate lyase of *Erwinia chrysanthemi* 3937. *J. Bacteriol.* 178: 7187-7196.
- Pissavin, C., Robert-Baudouy, J., and Hugouvieux-Cotte-Pattat, N. 1998. Biochemical characterization of *pelZ* of *Erwinia chrysanthemi* 3937. *Biochem. Biophys. Acta* 1383:188-196.
- Resibois, A., Colet, M., Faelen, M., Schoonejans, E., and Toussaint, A. 1984. Phi-EC2 a new generalized transducing phage of *Erwinia chrysanthemi*. *Virology* 137:102-112.
- Ried, J. L., and Collmer, A. 1988. Construction and characterization of an *Erwinia chrysanthemi* mutant with directed deletions in all of the pectate lyase structural genes. *Mol. Plant-Microbe Interact.* 1:32-38.
- Sauvage, C., and Expert, D. 1994. Differential regulation by iron of *Erwinia chrysanthemi* pectate lyases: Pathogenicity of iron transport regulatory (*cbr*) mutants. *Mol. Plant-Microbe Interact.* 7:71-77.
- Shevchik, V. E., and Hugouvieux-Cotte-Pattat, N. 1997. Identification of bacterial pectin acetyl esterase in *Erwinia chrysanthemi* 3937. *Mol. Microbiol.* 24:1285-1301.
- Shevchik, V. E., Robert-Baudouy, J., and Hugouvieux-Cotte-Pattat, N. 1997. Pectate lyase *pelI* of *Erwinia chrysanthemi* 3937 belongs to a new family. *J. Bacteriol.* 179:7321-7330.
- Tardy, F., Nasser, W., Robert-Baudouy, J., and Hugouvieux-Cotte-Pattat, N. 1997. Comparative analysis of the five major *Erwinia chrysanthemi* pectate lyases: Enzyme characteristic and potential inhibitors. *J. Bacteriol.* 179:2503-2511.