Isolation of an extracellular protease gene of *Erwinia carotovora* subsp. *carotovora* strain SCC3193 by transposon mutagenesis and the role of protease in phytopathogenicity

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Using mini-Tn5Cm^R::*gusA*, a transposon that allows transcriptional fusions to a promoterless β -glucuronidase gene, a mutant of *Erwinia carotovora* subsp. *carotovora* SCC3193 deficient in extracellular protease production and soft-rot pathogenicity in plants was isolated. The mutant, designated SCC6004, produced normal levels of pectate lyase, polygalacturonase and cellulase. The region of the transposon insertion was partially sequenced to permit the design of specific oligonucleotide primers to amplify a 2.7 kb *Cl*I fragment from *E. carotovora* subsp. *carotovora* SCC3193. The DNA sequence of the cloned fragment contained two complete and one partial ORFs. One of the complete ORFs (ORF1) was designated *prtW* and encodes a secreted protease. The deduced amino acid sequence of PrtW showed a high overall identity of 60–66% to the previously described *Erwinia chrysanthemi* proteases, but no homology to other proteases isolated from different *E. carotovora* strains. Downstream from ORF1, a further complete ORF (ORF2) and a partial ORF (ORF3) were found, with deduced peptide sequences that have significant similarity to the Inh and PrtD proteins, respectively, from *E. chrysanthemi*, which are involved in protease secretion. Gene fusion to the *gusA* reporter was employed to characterize the regulation of *prtW*. The *prtW* gene was found to be strongly induced in the presence of plant extracts. The mutant exhibited reduced virulence, suggesting that PrtW enhances the ability of strain SCC3193 to macerate plant tissue.

Keywords: *Erwinia*, transposon mutagenesis, protease gene, pathogenicity

INTRODUCTION

The interaction between plant and pathogen is a dynamic process involving signal exchange between interacting organisms. It is conceivable that the different extracellular enzymes produced by certain plant pathogens have different roles in pathogenesis, for example are required at different stages of infection or in different host plant tissues. The central role of pectolytic enzymes in *Erwinia* soft rot is well documented. This has been demonstrated by isolation of avirulent mutants

of *Erwinia carotovora* that are defective either in enzyme production (Murata *et al.*, 1991; Pirhonen *et al.*, 1991, 1993; Jones *et al.*, 1993; Liu *et al.*, 1993) or secretion (Pirhonen *et al.*, 1991). In *Erwinia chrysanthemi*, differential expression of genes for pectate lyases has been observed in different hosts (Beaulieu *et al.*, 1993) and even in different tissues of the same host (Lojkowska *et al.*, 1993). Some evidence suggests that in soft-rot *erwinias* some genes which are involved in virulence are activated only by compounds of plant origin. Kelemu & Collmer (1993) and McMillan *et al.* (1994) have shown that several novel isoenzymes of Pel (pectate lyase) and Peh (polygalacturonase) were induced when soft-rot *erwinias* were grown in the presence of methoxylated pectin or cell-wall extract. In *E. carotovora* subsp. *carotovora*, induction of pectic enzymes involves cell-wall degradation products (Yang *et al.*, 1992). Similarly,

Abbreviations: pNP, *p*-nitrophenol; X-GlcA, 5-bromo-4-chloro-3-indolyl β -D-glucuronic acid.

The GenBank accession number for the sequence determined in this work is AF141295.

Mäe *et al.* (1995) suggested that two distinct cellulases of *E. carotovora* are differentially controlled by plant-derived factors.

In addition to pectolytic enzymes and cellulases, several erwinias and pseudomonads causing soft rot secrete proteases. Among soft-rot pseudomonads, extracellular protease production correlates more strongly with the ability to macerate plant tissue than does pectolytic enzyme production (Sands & Hankin, 1975). Relatively little work has previously been done to characterize the role of proteases in bacterial soft-rot disease caused by erwinias. However, several studies have been conducted to investigate the biochemical properties and secretion of proteases in plant-pathogenic bacteria (Delepelaire & Wandersman, 1989; Wandersman, 1989; Letoffe *et al.*, 1989, 1990; Dahler *et al.*, 1990). Extracellular proteases produced by plant pathogens have been postulated to provide either amino acids for the biosynthesis of microbial proteins or to degrade host proteins associated with resistance mechanisms (Heilbronn & Lyon, 1990; Kyöstiö *et al.*, 1991). Although there is little experimental evidence concerning either of these possible functions, Heilbronn *et al.* (1995) showed *in vitro* degradation of potato lectins by a protease from the potato pathogen *E. carotovora*.

As plant molecules can act as recognition factors for invading bacteria, we attempted to identify host-inducible genes, expecting that these genes would be involved in virulence functions. Mutagenesis was done with a mini-Tn5Cm^R::*gusA* transposon which carries a promoterless selectable reporter gene, the β -glucuronidase gene. Upon insertion, the truncated gene can fuse to *E. carotovora* subsp. *carotovora* promoters. Mutants containing insertions in plant-inducible genes were selected on minimal medium plates containing plant extract. This method permits both the identification of *E. carotovora* subsp. *carotovora* promoters inducible by host factors present in plant extracts and the isolation in a single step of mutants which can be directly tested for virulence on plants.

Using this method, we identified several plant-inducible genes and showed that some of them are involved in virulence functions. One of the mutants produced undetectable levels of protease under all conditions tested. Plant infection studies showed that the protease-deficient mutant was less virulent than the wild-type parent on tobacco and potato tubers. The insertion in this mutant mapped in a genomic cluster involved in protease production. The deduced translated product of this ORF was designated PrtW. This report describes the isolation and characterization of the *prtW* gene.

METHODS

Bacterial strains, plasmids, media and culture conditions. Bacterial strains and plasmids used or constructed in this study are described in Table 1. Unless otherwise stated, *Escherichia coli* strains were grown at 37 °C and *E. carotovora* was grown at 28 °C in L broth (Miller, 1972) or in M9 minimal

medium (Maniatis *et al.*, 1982) supplemented with trace elements (Bauchop & Elsdon, 1960). When required, media were supplemented with 0.4% (w/v) glycerol, X-GlcA (5-bromo-4-chloro-3-indolyl β -D-glucuronic acid) at a concentration of 50 μ g ml⁻¹ and with antibiotics at the following concentrations (μ g ml⁻¹): ampicillin, 150; kanamycin, 25; and chloramphenicol, 25. Celery extract was prepared according to Murata *et al.* (1991). Crude potato extract was obtained by crushing potato tubers. The juice was clarified by centrifugation at 10000 *g* and subsequent filtration on membranes of decreasing porosity. The extract was sterilized by filtration through a 0.22 μ m porosity membrane and stored at -70 °C in aliquots for several months. Protease activity was detected on L agar plates containing 5% skim milk.

DNA transformation, isolation, analysis and manipulation. *Escherichia coli* cells were transformed by the CaCl₂ method (Maniatis *et al.*, 1982). Plasmids were introduced into *E. carotovora* by electroporation (Ausubel *et al.*, 1987; Py *et al.*, 1991). Plasmid preparations and isolation of restriction fragments from low-melting-point agarose gels were performed with Qiagen plasmid kits. DNA cleavage by restriction endonucleases and agarose gel electrophoresis were performed as described by Maniatis *et al.* (1982).

Construction of a promoter-probe transposon. The promoterless *gusA* gene from pGUS102 was inserted as a 1.8 kb *EcoRI* fragment into pUT/mini-Tn5Cm^R. The plasmid in which the *gusA* gene was oriented so that it would be transcribed from the outside end of pUT/mini-Tn5Cm^R was designated pPRG (Table 1).

Introduction of transposons into *E. carotovora* subsp. *carotovora* strain SCC3193. *E. carotovora* subsp. *carotovora* strain SCC3193 was used as a recipient. Biparental matings were carried out as described by de Lorenzo *et al.* (1990) on M9 minimal medium at 28 °C using *Escherichia coli* S17-1 λ -pir containing a suicide vector pUT/mini-Tn5Cm^R::*gusA* as the donor strain. Transconjugants were selected on M9 minimal medium (using 0.4% glycerol as carbon source for the recipient strain) plus chloramphenicol to select for insertion of the transposon. S17-1 λ -pir cannot grow on this medium because it is auxotrophic for proline.

DNA sequence determination. The mutated *prtW* gene was cloned by cutting chromosomal DNA from the protease transposon mutant SCC6004 with *Clal*, ligating this DNA to *Clal*-digested pBluescript SK(+), followed by transformation into *Escherichia coli* strain DH5 α . The plasmid obtained was named pROT1. The DNA flanking the transposon was then partially sequenced by the dideoxy method of Sanger *et al.* (1977), using the Sequenase System version II of US Biochemicals and [³⁵S]dATP α S with oligonucleotide primers annealing to the vector DNA and synthetic oligonucleotide primers designed from the sequences obtained. The corresponding genomic fragment was amplified from the wild-type strain SCC3193 by PCR using as primers: ProS, 5'-AATCGATAATAAAAATTAGTCATCATTACC-3' and Pro2, 5'-GCTACATCACACGATAAAATCGGTTG-3'. All PCR amplifications were performed with the proof-reading DNA polymerase *Pwo* (Boehringer Mannheim). The resulting 2.7 kb PCR fragment containing the *prtW* gene and flanking regions was ligated to *SmaI*-digested pBluescript SK(+) to create plasmid pROT2. The complete sequence of both strands was determined. The data were analysed by the PC/GENE program.

Enzyme assays. β -Glucuronidase activity was assayed by using *p*-nitrophenyl β -D-glucuronide as substrate (Novel *et al.*,

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Bacterial strains		
<i>Escherichia coli</i>		
DH5 α		BRL
S17-1 λ -pir	Km ^R Sm ^R , <i>recA thi pro hsdR</i> ⁻ R ⁺ Rp4:2-Tc ^R :Mu:Km ^R Tn7 λ -pir	de Lorenzo <i>et al.</i> (1990)
<i>Erwinia carotovora</i> subsp. <i>carotovora</i>		
SCC3193	Wild-type	Pirhonen <i>et al.</i> (1991)
SCC3009	<i>expS</i> ::Km ^R	Pirhonen <i>et al.</i> (1991)
SCC3060	<i>expA</i> ::Km ^R	Pirhonen <i>et al.</i> (1991)
SCC3065	<i>expI</i> ::Tn903	Pirhonen <i>et al.</i> (1991)
SCC6004	<i>prtW</i> ::Tn5- <i>gusA</i>	This study
Plasmids		
pBluescript SK(+)	Cloning vector (Ap ^R)	Stratagene
pUT/mini-Tn5Cm ^R		de Lorenzo <i>et al.</i> (1990)
pGUS102	Ap ^R Tc ^R , promoterless 1.8 kb <i>gusA</i> gene as an <i>EcoRI</i> fragment in pBR322	A. Eriksson, Swedish University of Agricultural Sciences, Uppsala, Sweden
pPRG	Ap ^R Cm ^R ; miniTn5Cm ^R :: <i>gusA</i> (promoterless <i>gusA</i> for transcriptional fusions) in pUT/mini-Tn5Cm ^R	This study
pROT1	Ap ^R , 2.7 kb <i>Clal</i> fragment from SCC6004 containing <i>prtW</i> with a miniTn5Cm ^R :: <i>gusA</i> cloned into pBluescript SK(+)	This study
pROT2	Ap ^R , 2.7 kb PCR fragment from SCC3193 containing <i>prtW</i> cloned into pBluescript SK(+)	This study

1974). The degradation product, *p*-nitrophenol (pNP), was detected at 405 nm and β -glucuronidase-specific activities were expressed as nmol pNP liberated min⁻¹ (OD₆₀₀ unit)⁻¹.

Protease was assayed by the azocasein method (Ji *et al.*, 1987), and one unit was defined as the amount of enzyme that produced an increase in *A*₄₃₆ of 1.0 h⁻¹ at 30 °C.

The activities of polygalacturonase, pectate lyase and cellulase were assayed as described previously (Pirhonen *et al.*, 1991).

Pathogenicity test. Tubers were washed and immersed twice for 20 min in 5% sodium hypochlorite, rinsed in sterile deionized water and air-dried under a laminar hood. Maceration of potato was tested by injection by pipette tip of 5 μ l containing 10⁶ c.f.u. bacteria from an overnight culture into the tubers. The inoculated tubers were incubated at 28 °C under 100% humidity and the development of symptoms was evaluated after 48 h by measuring the weight of the macerated tissue. Each value is the mean of five inoculations.

The virulence of the *E. carotovora* subsp. *carotovora* strains to tobacco (*Nicotiana tabacum* cv. Samsun) seedlings was tested. Two- to four-week-old tobacco seedlings grown on 24-well tissue culture plates were locally inoculated with 10⁶ c.f.u. bacteria from an overnight culture. The leaves were punctured by needle and 1 μ l of bacterial suspension was applied to the leaf. One leaf per well was inoculated. The inoculated plants were incubated at 28 °C under 100% humidity. The development of disease symptoms (tissue maceration) was followed for 12–66 h.

RESULTS

Isolation of plant-inducible mutants by mini-Tn5Cm^R::*gusA* mutagenesis

E. carotovora subsp. *carotovora* SCC3193 was mutagenized by random insertion of the mini-Tn5Cm^R::*gusA* transposon. A pool of insertion mutants was tested in parallel on X-GlcA-containing plates with and without plant extract added. Mutants containing insertions in plant-inducible genes were identified by a blue colour on M9 minimal medium/glycerol plates supplemented with X-GlcA in the presence of plant extract. Colonies which were blue on both plates were considered to have insertions in constitutively expressed genes. Of 6000 colonies tested only 12 showed induction, at levels between 1.3- and 17-fold. One of the isolated mutants, designated SCC6004, did not show the normal halo around the colony on skimmed milk agar but produced normal levels of pectate lyase, polygalacturonase and cellulase and was selected for further study.

Isolation of the *prtW* gene

To characterize the mutated locus and gain an insight into its function, the entire nucleotide sequence of the 2.7 kb genomic fragment originating from *E. carotovora*

Fig. 1. Localization of the mini-transposon Tn5Cm^R::*gusA* in *prtW* mutant SCC6004; the 2.7 kb insert in pBluescript SK(+) is shown. The site of insertion of the transposon leading to the fusion with the protease gene *prtW* is marked with a filled circle. The map also shows the localization and transcription direction of ORF2 whose deduced polypeptide sequence shows similarity to the inhibitory gene *inh* from *E. chrysanthemi*, and the 5' part of ORF3 whose deduced polypeptide sequence shows similarity to *prtD* from *E. chrysanthemi*. Arrows indicate the location of *prtW*, ORF2 and ORF3 coding regions and the direction of transcription.

subsp. *carotovora* SCC3193 in pROT2 was determined. This fragment was shown to contain three ORFs, two complete and one partial (Fig. 1). The first ORF, corresponding to the *prtW* gene, consists of 1421 nucleotides. The deduced amino acid sequence of PrtW would give a polypeptide with calculated molecular mass of 51 kDa. A second ORF was found downstream, apparently organized in the same operon. The deduced polypeptide sequence shows similarity to the *inh* gene product from *E. chrysanthemi* (Letoffe *et al.*, 1989). Further downstream of these two ORFs, we found the 5' part of a third ORF. The deduced polypeptide sequence has high homology to protease secretion protein *prtD* from *E. chrysanthemi* (Letoffe *et al.*, 1990).

Sequence analysis of the *prtW* gene

A search of the protein databases with the deduced amino acid sequence of the PrtW polypeptide showed significant similarity to proteases PrtB, PrtC, PrtA and PrtG from *E. chrysanthemi* (Delepelaire & Wandersman, 1989; Dahler *et al.*, 1990; Ghigo & Wandersman, 1992a, b). The predicted sequence of PrtW is 66.2% identical to PrtC, 65.5% identical to PrtA and 65.8% identical to PrtG. The alignment between these polypeptides indicates that the similarity between the deduced protein sequences is not restricted to certain domains but is found throughout the entire length of the molecules. Multiple sequence alignment of PrtW, PrtA, PrtB, PrtC and PrtG revealed two domains that are probably associated with Ca²⁺- and Zn²⁺-binding (Baumann *et al.*, 1993). The Zn²⁺-binding domain was characterized by a well-defined signature HEXXH-XGXXH (aa 182–192), whereas the Ca²⁺-binding domain was characterized by the presence of four glycine-rich repeats GGXGXD (aa 339–344, 357–362, 366–371 and 375–380). In contrast, alignment of the amino acid sequence of PrtW with known proteases from other *E. carotovora* strains showed that PrtW is clearly distinct: we could not find amino acid sequence similarity between any of these proteases and PrtW (not shown).

Fig. 2. Expression of the *prtW*::*gusA* fusion in strain SCC6004 under various growth conditions. Cultures were grown in M9 minimal media containing 0.4% glycerol and supplemented as indicated. (a) β -Glucuronidase activity measured as a function of time (h) after inoculation; (b) growth of the bacterial cultures followed by measurement of OD₆₀₀. ◆, Unsupplemented medium; ■, medium plus 16% celery extract; ▲, medium plus 16% potato extract.

Expression of the *prtW* gene

To obtain clues regarding the role of protease in phytopathogenicity, we constructed a *gusA* transcriptional fusion to the promoter of the *prtW* gene in the mutant strain SCC6004. The *prtW*::*gusA* fusion was analysed for β -glucuronidase synthesis in the absence and presence of plant extract as inducer to test possible induction by plant molecules.

The data shown in Fig. 2 are for cells grown in minimal medium supplemented with celery or potato extract. The effects of potato and celery extracts on *prtW*::*gusA* fusion expression were maximal with an extract concentration of 16%. Extracts from both celery and potato showed inducing effects, suggesting that the potential coinducing factor(s) is (are) widely distributed among plant species and tissues. Expression of the *prtW*::*gusA*

fusion was induced 3- to 4-fold in the presence of celery extract and 17-fold in the presence of potato extract in comparison to the expression of the fusion under uninduced conditions. In these studies, the possibility exists that the difference between potato- and celery-extract-mediated induction rates could result from the different methods used for the preparation of the extracts.

Pirhonen *et al.* (1991) reported that the level of extracellular enzyme synthesis varies during bacterial growth, being low at the beginning of bacterial growth but starting to accumulate during the exponential growth phase. We found that the expression of *prtW*, estimated by β -glucuronidase activity, was also growth-phase-dependent. The *prtW::gusA* fusion was induced maximally during the early exponential growth phase and fell back to the basal levels at the beginning of stationary phase. A similar growth-phase-dependent increase was observed in the protease activity of the wild-type strain SCC3193 (Fig. 3). In conclusion, the maximum synthesis of protease in *E. carotovora* subsp. *carotovora* occurs earlier than synthesis of other extracellular enzymes, which usually reach their maximum in the beginning of early stationary phase (Pirhonen *et al.*, 1993).

PrtW expression in different genetic backgrounds

In order to identify the regulatory genes controlling protease synthesis, we characterized *prtW* expression in different regulatory mutants of *E. carotovora* subsp. *carotovora* strain SCC3193. The results show that the *prtW* is not expressed in an *expl* mutant (Fig. 3), demonstrating global regulation of *prtW* expression as previously described for *pehA*, *pelB*, *pelC* and *celV1* (Pirhonen *et al.*, 1991; Mäe *et al.*, 1995). The protease activity was inhibited by 92 % after addition of 90 mM EDTA, confirming that it was a metalloprotease.

Recently Eriksson *et al.* (1998) described the global two-component regulatory system in *E. carotovora* subsp. *carotovora* strain SCC3193. To define the possible role of the two-component regulatory system *expA-expS* in protease expression, we studied protease activity in *expA* and *expS* mutants. A mutation in either of these genes drastically reduced the production of protease (Fig. 3). Taken together, these results show that the *prtW* gene expression is regulated by the same global regulatory systems as the other extracellular enzymes involved in virulence. As the expression of the *prtW* gene is induced in the presence of plant extracts, the two-component regulatory system *expA-expS* might be responsible for sensing this signal derived from the host plant extract.

Role of PrtW in phytopathogenicity

The virulence of the protease mutant was tested in two different plant systems: in isolated plant organs (potato tubers) and in *in-vitro*-grown tobacco plants (*Nicotiana tabacum* cv. Samsun). To determine the ability of the

Fig. 3. Growth and protease activity in wild-type and mutant strains. Cultures were grown in M9 minimal media containing 0.4% glycerol and supplemented with 16% potato extract. (a) Protease activity measured as a function of time (h) after inoculation; (b) growth of the bacterial cultures followed by measurement of OD_{600} . ♦, SCC3193 (wild-type); ■, SCC3065 (*expA* mutant); ▲, SCC3009 (*expS* mutant); ×, SCC3060 (*expA* mutant); and ★, SCC6004 (*prtW* mutant).

prtW mutant to induce soft rot, potato tuber maceration experiments were carried out. As the isolated mutant produced normal levels of pectate lyase, polygalacturonase and cellulase (data not shown) any reduction in virulence is a result of loss of protease activity caused by mini-Tn5 insertion into the *prtW* gene (the *prtW* mutant did not show any residual protease activity).

The virulence of the *prtW* mutant and of the *expl* mutant (defective in quorum sensing) was compared with that of the wild-type parent, SCC3193. The results showed that the *expl* mutant had a markedly reduced capacity to macerate potato tuber tissue, retaining only 10% of its maceration capacity. The *prtW* strain still retained about 60% of its maceration capacity.

The virulence of the *prtW* mutant was tested by monitoring soft rot symptom development using 2-

week-old tobacco seedlings. This analysis indicated that 37 % of the plants infected with the *prtW* mutant did not show any maceration. The remaining 63 % of the infected plants exhibited the normal spreading of soft-rot symptoms associated with the wild-type strain SCC3193. Approximately 98 % of plants infected with SCC3193 showed normal disease symptoms. These results suggest that although *PrtW* is not essential for pathogenesis, it enhances disease development during the infection process.

DISCUSSION

PrtW shows similarity to the proteases from *E. chrysanthemi*

We employed mini-Tn5-mediated mutagenesis to isolate new genes of *E. carotovora* subsp. *carotovora* that are involved in virulence. The central role of pectolytic enzymes in *Erwinia* soft rot is well documented (Pirhonen *et al.*, 1991, 1993; Liu *et al.*, 1993). Here, we report the isolation and study of a new gene, *prtW*, encoding an extracellular protease. The *PrtW* protease does not show similarity to the previously described proteases from *E. carotovora* subsp. *carotovora* (Kyöstiö *et al.*, 1991; Heilbronn *et al.*, 1995). Comparison of the sequence of *PrtW* with a range of proteases showed over 60 % identity to *PrtB*, *PrtC*, *PrtA* and *PrtG* from *E. chrysanthemi* (Delepelaire & Wandersman, 1989; Dahler *et al.*, 1990; Ghigo & Wandersman, 1992a, b). The extensive similarity of *PrtW* with proteases from *E. chrysanthemi* indicates a possible common origin of these proteins. Comparison of the amino acid sequence of protease *PrtW* and proteases produced by *E. chrysanthemi* allowed the tentative identification of sequences essential for Ca^{2+} -binding (aa 182–192) and Zn^{2+} -binding (aa 339–344, 357–362, 366–371 and 375–380) in *PrtW*. The presence of these specific sequences in *PrtW* allowed us to categorize the protease from *E. carotovora* subsp. *carotovora* strain SCC3193 as a metalloprotease and this was confirmed by its inhibition by EDTA.

Expression of *prtW* is growth-phase dependent and regulated by global regulatory systems

Assay of β -glucuronidase activity from cells harbouring a *prtW::gusA* fusion demonstrated the growth-phase-dependent expression of *prtW*. The level of *prtW::gusA* activity increased rapidly during the early growth stages and reached peak levels within 6–8 h, during early exponential growth phase (Fig. 2). These findings suggest that the protease gene is responsive to coordinate control by autoinducer and are in agreement with our results showing that *prtW* is not expressed in an autoinducer-defective *expI* mutant. The result is in agreement with the recent work of Chatterjee *et al.* (1995) and Cui *et al.* (1996). In addition to coordinate control by autoinducer, *PrtW* synthesis is also controlled by a two-component regulatory system, *expA-expS*, described by Eriksson *et al.* (1998). The other extracellular enzymes produced by SCC3193 were also

expressed in a growth-phase-dependent manner, reaching their maxima only in the beginning of stationary phase (Pirhonen *et al.*, 1993). Sequential production of cell-wall-degrading enzymes also occurs in fungal pathogens (Cooper, 1983).

PrtW is involved in virulence in *E. carotovora* subsp. *carotovora* SCC3193

The importance of proteases in virulence also seems to differ between various bacterial species. Marker exchange mutants of *E. chrysanthemi* EC16 defective in production of one or all of the extracellular proteases were not impaired in virulence on plant tissue (Dahler *et al.*, 1990). Among soft-rot pseudomonads, extracellular protease production correlates more strongly with the ability to macerate plant tissue than does pectolytic enzyme production (Sands & Hankin, 1975). Tang *et al.* (1987) reported only slight differences in the virulence of protease-deficient mutants of *Xanthomonas campestris* pv. *campestris* on turnip leaves when relatively high inoculum levels were used; at lower inoculum levels, a pronounced reduction in virulence of the protease mutant was observed relative to the wild-type strain. Recently, Shevchik *et al.* (1998) demonstrated that *PrtA* and *PrtC* from *E. chrysanthemi* were very efficient in processing pectate lyase Peli-2 into Peli-3.

Our results demonstrate that protease activity in *E. carotovora* subsp. *carotovora* is necessary for normal progression of disease symptoms. Mutants defective in protease *PrtW* production exhibited reduced virulence in a potato tuber maceration test. It has been suggested that the expression of virulence by *Erwinia* spp. involves a fine balance between avoiding the induction of plant defence mechanisms and the rapid killing of plant cells by pectolytic enzymes. Vidal *et al.* (1997) showed the rapid local induction of pathogenesis-related (PR) genes during the early stages of infection (4–8 h after infection). Suppression of these resistance mechanisms is important during the initial stages of infection when the synthesis of virulence factors is not sufficient for the establishment of infection. The expression of the *prtW* gene during the early growth phase suggests the possibility that protease might be necessary for suppressing the plant defence response. This effect may be achieved by degradation of host proteins associated with resistance mechanisms. This hypothesis is supported by the work of Heilbronn *et al.* (1995) who presented data demonstrating *in vitro* degradation of potato lectin by protease purified from a potato-pathogenic *E. carotovora* strain.

From the data presented in this report we conclude that we have characterized a new protease from *E. carotovora* subsp. *carotovora* with potential importance in plant tissue maceration. This conclusion is supported by the observation that mutation of *prtW* in strain SCC3193 reduced maceration virulence by 40%. However it remains unclear what role *PrtW* plays in disease development. The presence of protease(s) in many phytopathogenic bacteria suggests a more important

role in relation to the plant host than previously supposed. These results emphasize the importance of extending the studies on proteases in order to determine their role in virulence.

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