Regulation of the expression of *prtW::gusA* fusions in *Erwinia carotovora* subsp. *carotovora*

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***Erwinia carotovora* subsp. *carotovora*, a Gram-negative phytopathogenic bacterium, secretes an extracellular metalloprotease, PrtW. Previous results demonstrated that protease activity is necessary for the normal progression of disease symptoms caused by this bacterium. The present study revealed that the *prtW* gene constitutes an independent transcriptional unit. It is demonstrated that introduction of the *prtW*⁺ plasmid *in trans* into the *prtW*[−] mutant restores the protease activity in this strain. Gene fusions to the *gusA* (β-glucuronidase) reporter were employed to analyse the transcription of *prtW*. The transcription of *prtW* is dependent on many environmental signals. When the bacteria were grown in the presence of potato extract, the expression of the protease gene was markedly higher at the beginning of the exponential phase of growth than that observed when cells were grown in the presence of polygalacturonate (PGA). Analysis of the promoter revealed that an essential regulatory region resided between 371 and 245 bp 5' of the translational start site. As this sequence showed no homology to the KdgR box it may be involved in the binding of an unknown negative regulator protein in *E. carotovora* subsp. *carotovora*. The differential responses of *prtW* expression to potato extract and to PGA appeared to be dependent on the KdgR repressor and the response regulator ExpA. According to the results presented here, it is conceivable that the multiple regulatory network allows flexibility in the expression of the *prtW* gene during different stages of infection.**

Keywords: phytopathogenic bacteria, protease, promoter fusions

INTRODUCTION

The phytopathogenicity of the soft-rot enterobacteria of the genus *Erwinia* is correlated with their ability to produce and secrete plant-cell-wall-degrading enzymes (PCWDEs) (Pérombelon & Kelman, 1980; Barras *et al.*, 1994). Many of these PCWDEs have been shown to trigger plant defence responses (probably by releasing cell wall fragments as active elicitors). The mechanism by which extracellular enzymes of *Erwinia carotovora* subsp. *carotovora* (*E. carotovora*) induce defence responses in plants has been proposed to involve the release of oligogalacturonide elicitors from plant cell wall pectin (Palva *et al.*, 1993; Vidal *et al.*, 1997).

Abbreviations: *E. carotovora*, *Erwinia carotovora* subsp. *carotovora*; PCWDE, plant-cell-wall-degrading enzyme; PGA, polygalacturonate; pNP, p-nitrophenol.

To be able to overcome a plant defence response and to survive in plant tissue, the invading pathogen produces toxins or substances called suppressors, which act as pathogenicity factors by suppressing the expression of the defence response(s) of the host plant (Basse *et al.*, 1993; Kato *et al.*, 1993; Bender *et al.*, 1999). As *E. carotovora* lacks these systems it has to find other means to escape the plant defence response(s). The expression of virulence in the soft-rot erwinias seems to depend on a fine balance between avoiding the plant defence reaction and rapid killing of the plant cells. To ensure that the balance is on the side of the pathogen, when the right environmental conditions prevail, members of the genus *Erwinia* have evolved multiple strategies to sense their environment and to modulate their gene expression both by positive *expA/expS*, *aepA*, *rdgA/rdgB*, *rpfA*, *rpoS* and *hor* regulators, and by negative *hexA* and *kdgR* regulators (Liu *et al.*, 1993, 1996; Mukherjee

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristics*	Source/reference
<i>Escherichia coli</i>		
DH5 α		BRL
M15	Km ^R ; pREP4	Qiagen
<i>E. carotovora</i>		
SCC3193	Wild-type	Pirhonen <i>et al.</i> (1991)
SCC3060	<i>expA</i> ::Km ^R	Pirhonen <i>et al.</i> (1991)
SCC6004	<i>prtW</i> ::Tn5- <i>gusA</i>	Marits <i>et al.</i> (1999)
SCC510	<i>kdgR</i> ::Cm ^R	Hyttiäinen <i>et al.</i> (2001)
SCC500	<i>expA</i> ::Km ^R ; <i>kdgR</i> ::Cm ^R	Hyttiäinen <i>et al.</i> (2001)
Plasmids		
pBluescript(+)	Ap ^R ; Cloning vector	Stratagene
pQE30	Ap ^R ; Expression vector	Qiagen
pMW119	Ap ^R ; Cloning vector	Eurogentec
pGUS102	Ap ^R ; Promoterless 1·8 kb <i>gusA</i> gene as an <i>EcoRI</i> fragment in pBR322	A. Eriksson†
pACYC184	Ap ^R , Km ^R ; Cloning vector	New England Biolabs
pMW119:: <i>gusA</i>	Ap ^R ; 1·8 kb <i>EcoRI</i> fragment containing <i>gusA</i> gene from pGUS102 cloned into <i>HindIII</i> site of pMW119	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(+)	Marits <i>et al.</i> (1999)
pROT3	Ap ^R ; <i>placZ</i> :: <i>prtW</i> PCR product of the ORF of <i>prtW</i> in pBluescript(+)	This study
pROT3A	Ap ^R ; Same as pROT3, but opposite insert orientation	This study
pROT4	Ap ^R ; pBluescript with the 2038 bp <i>KpnI</i> - <i>PaeI</i> fragment containing the <i>prtW</i> regulatory region and 43 bp of the ORF from SCC3193 inserted into the <i>KpnI</i> - <i>PaeI</i> sites	This study
pROT5	Ap ^R ; Same insert as pROT4, but cloned into the <i>SmaI</i> site of pMW118:: <i>gusA</i>	This study
pROT6	Ap ^R ; 1312 bp <i>KpnI</i> - <i>HindIII</i> fragment deleted from pROT5	This study
pROT7	Ap ^R ; PCR product of pROT5 using primers PROM5 and M13/pUC Reverse Primer (−24) in <i>SmaI</i> site of pMW119	This study
pROT8	Ap ^R , Cm ^R ; Same insert as pROT1, but cloned into <i>Clal</i> site of pACYC184	This study
pRT1	Ap ^R ; <i>prtW</i> PCR product of the ORF of <i>prtW</i> in expression vector pQE30	This study

* Km^R, Kanamycin-resistance; Cm^R, chloramphenicol-resistance; Ap^R, ampicillin-resistance.

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et al., 1996; Frederick *et al.*, 1997; Thompson *et al.*, 1997; Eriksson *et al.*, 1998; Harris *et al.*, 1998; Liu *et al.*, 1999). Some of these regulators respond to environmental stimuli by modulating the expression of the controlled genes (Eriksson *et al.*, 1998; Liu *et al.*, 1999).

Microbial resistance to plant defence(s) during infection can also be achieved by the direct degradation of defence proteins, or proteins involved in signal transduction, in the host plant (Heilbronn *et al.*, 1995). Plant response(s) triggered by *E. carotovora* have shown that the transcriptional activation of several defence genes is already achieved 4–6 h after infection (Vidal *et al.*, 1997). The expression of the protease gene *prtW* during the early exponential phase of growth and the observation that the *prtW* mutant exhibits reduced virulence on potato tubers suggest the possibility that protease might be necessary for the suppression of plant defence responses (Marits *et al.*, 1999).

Here we report the construction and analysis of promoter fusions between the *E. carotovora* protease gene

prtW and the reporter gene *gusA* (β -glucuronidase), to identify sequences 5' to the *prtW* coding region that might account for the expression of the gene. We have analysed the expression of each fusion in the wild-type strain (SCC3193) in response to the presence of potato extract or polygalacturonate (PGA), to verify the different effect(s) these inducers have on gene expression. We have also tested the expression of each of the fusions in the *kdgR*[−] mutant strain SCC510, in the *expA*[−] mutant strain SCC3060 and in the *expA*[−] *kdgR*[−] double mutant strain SCC500, to determine if these regulators modulate *prtW* expression in response to different physiological inducers.

METHODS

Bacterial strains, plasmids, media and culture conditions.

Bacterial strains and plasmids used or constructed in this study are described in Table 1 and Fig. 2(b). *E. carotovora* and *Escherichia coli* were grown at 28 or 37 °C, respectively, in L-broth (Miller, 1972) or in M9 minimal medium (Sambrook *et al.*, 1982) supplemented with 0·4% glycerol and trace elements

(Bauchop & Elsdén, 1960). When required, media were supplemented with 0.4% PGA, 16% potato extract and antibiotics at the following concentrations: 150 µg ampicillin ml⁻¹, 25 µg kanamycin ml⁻¹ and 25 µg chloramphenicol ml⁻¹. Crude potato extract was prepared according to Marits *et al.* (1999).

DNA manipulation, plasmid construction and determination of the nucleotide sequence. Unless otherwise stated, DNA cloning and gel analysis of plasmid DNA were done by established procedures (Sambrook *et al.*, 1982). Plasmid preparations and the isolation of restriction fragments from agarose gels were performed with the QIAprep Spin Miniprep Kit and the QIAquick Gel Extraction Kit (both from Qiagen). *Escherichia coli* cells were transformed by the CaCl₂ method (Sambrook *et al.*, 1982). Plasmids were introduced into *E. carotovora* cells by electroporation (Ausubel *et al.*, 1987; Py *et al.*, 1991).

DNA was sequenced by the dideoxy method of Sanger *et al.* (1977). The sequencing kit used was obtained from USB.

For the PrtW-His₆-tagged overexpression plasmid, the coding region of *prtW* was amplified by PCR with primers Qia1 (5'-GAGAAGGATCCATGGCTTTACGAGATGACG-3') and Qia2 (5'-TCCTCGTCTGACTCACACGATAAAATCGGTT-3'). The PCR product was digested with *Bam*HI and *Sal*I, and cloned into the vector pQE30 to yield pRT1. All PCR amplifications were performed with the proof-reading DNA polymerase *Pwo* (Boehringer Mannheim).

RNA isolation and Northern blot analysis. Total RNA was isolated with the RNeasy RNA isolation kit (Qiagen). Ten micrograms of total RNA was denatured in formamide, separated by electrophoresis through formaldehyde/agarose gels and blotted onto nylon filters (Sambrook *et al.*, 1982). To generate a *prtW*-specific probe, *prtW* was amplified from the wild-type strain (SCC3193) by PCR using primers Qia1 and Qia2. The probe was labelled by using the multiprimer DecaLabel DNA labelling Kit (MBI Fermentas). Prehybridization (1 h at 65 °C) and hybridization (12 h at 65 °C) were performed in prehybridization buffer (6× SSC, 2× Denhardt's, 0.1% SDS and 100 µg denatured salmon-sperm DNA ml⁻¹). After hybridization, the nylon membranes were washed twice for 20 min at 65 °C in 2× SSC plus 0.5% SDS, followed by 30 min at 65 °C in 0.5× SSC plus 0.5% SDS. The membranes were then examined by autoradiography.

Primer extension. The primer extension assay was performed according to the manufacturer's instructions (MBI Fermentas) with primer PROM1reverse (5'-GTCTCTTGCGGGGATA-3') and 10 µg RNA. The plasmid pROT4, primed with PROM1reverse, was used as a size marker.

Enzyme assays. β-Glucuronidase activity was assayed by using *p*-nitrophenyl β-D-glucuronide as substrate (Novel *et al.*, 1974). The degradation product, *p*-nitrophenol (*p*NP), was detected at an absorbance of 405 nm; the specific activity of β-glucuronidase was expressed as nmol *p*NP liberated min⁻¹ (OD₆₀₀ unit)⁻¹. The activity of protease was detected on L-agar plates containing 5% skim milk.

Expression of the *prtW* gene product in *E. coli*. *E. coli* M15(pRT1) was grown at 37 °C in L-broth containing kanamycin and ampicillin until the culture reached OD₆₀₀ 0.4. One hundred microlitre aliquots of cell suspensions were withdrawn from samples which had been incubated for 4 h with 1.0 mM IPTG or without IPTG. The cells were collected by centrifugation and solubilized in SDS sample buffer (20%

glycerol, w/v; 10% β-mercaptoethanol, w/v; 0.02% SDS, w/v; bromophenol blue in 0.25 M Tris/HCl, pH 6.8) to yield a preparation of total cellular proteins. The samples were analysed by SDS-PAGE according to Laemmli (1970) and visualized by Coomassie-blue staining.

RESULTS

Identification of the PrtW product

The small DNA fragment containing *prtW* was amplified by PCR (using primers Qia1 and Qia2) from strain SCC3193 and cloned into the *Bam*HI-*Sal*I site of the pBluescript(+) vector, yielding pROT3 and pROT3A, respectively. This produced both orientations of the gene with respect to the *lac* promoter. Only the orientation in pROT3, corresponding to the transcriptional direction of the predicted *prtW* ORF, allowed the complementation of the *prtW* mutant SCC6004 *in trans* (data not shown).

Cloning the *Bam*HI-*Sal*I restriction fragment containing the *prtW* gene into the compatible sites of a pQE30 vector allowed PrtW to be overproduced in *E. coli* M15. SDS-PAGE analysis of crude protein extracts of the *E. coli* M15(pRT1) strain showed, after IPTG induction, marked overproduction of a protein with an estimated molecular mass of 50 kDa (Fig. 1). This value is consistent with the 51 kDa protein predicted from the PrtW sequence (Marits *et al.*, 1999).

Mapping the transcriptional site of the *prtW* gene

RNA was extracted from SCC3193 cells and used in primer extension experiments. Primer extension terminated abruptly at an A base located 186 bp upstream from the translational start site of *prtW*; no other significant start sites were detected. Inspection of the DNA sequence of this *prtW* region disclosed the presence of a

Fig. 1. PrtW protein expression in *Escherichia coli*. Crude extracts were analysed by SDS-PAGE in a 12% (w/v) polyacrylamide gel. Lanes: M, molecular mass marker; 1, lysate of *E. coli* M15(pRT1) after 4 h growth without IPTG; 2, lysate of *E. coli* M15(pRT1) after 4 h growth with IPTG.

Fig. 2. (a) Summary of the promoter region of the *prtW* gene in *E. carotovora* SCC3193. The translational initiation sequence (ATG) is shown in bold. The transcriptional start site, identified by primer extension analysis, is indicated by an asterisk and bold-type A. Potential -35 and -10 promoter elements are bold and underlined, the potential Shine-Dalgarno (SD) sequence is underlined. *HindIII*, *ClaI* and *PaeI* restriction sites utilized for the construction of *gusA* fusions are also shown (bold and italic). The beginning of pROT7 is indicated by an arrow. (b) Endonuclease restriction site maps for the inserts of *prtW* in plasmids pROT5, pROT6, pROT7 and pROT8. The transcriptional start site of *prtW* is indicated by an arrow. The construction of the plasmids is described in Results and in Table 1.

possible σ^{70} promoter, with a -10 box 10 bp and a -35 box 33 bp upstream of the transcriptional start site (Fig. 2a).

In *Erwinia chrysanthemi* the secretion genes belong to one gene cluster along with the protease, the structural and the inhibitor genes (Létoffé *et al.*, 1990). The introduction of *prtW* (on pROT3) into the *prtW*⁻ mutant strain SCC6004 restored its proteolytic activity. This suggests that the secretion genes must be functional in SCC6004 and that they are transcribed separately from *prtW*. As a further test of the fidelity of this hypothesis, Northern blots of total RNA isolated from SCC3193 were hybridized with the *prtW* probe. The results indicate that when *prtW*-specific DNA was used as a probe only a single transcript with a size of 1500 bp was visible, which corresponds to the calculated size of the protease gene transcript (1421 bp; data not shown).

Deletions in the *prtW* promoter region

To define the DNA regions necessary for *prtW* promoter activity, restriction-site deletions and PCR constructs were made from the *prtW* promoter regions (Fig. 2b; Table 1). Expression of *prtW* was examined during bacterial growth on minimal medium (M9 + glycerol) in the presence and in the absence of PGA or potato extract using the *prtW*::*gusA* transcriptional fusions.

To determine the 5' extent of the *prtW* promoter, a 2038 bp *KpnI*-*PaeI* fragment, which had a 3' terminus at the 43 bp within the *prtW* coding region and a 5' terminus 1995 bp upstream from the first codon, was cloned from *E. carotovora* into the pBluescript(+) vector, yielding pROT4. The corresponding DNA fragment was then cloned into the low-copy vector pMW119::*gusA*, yielding pROT5.

In non-inducing conditions, pROT5 showed a low level of β -glucuronidase activity which remained constant

throughout the whole growth curve (data not shown). β -Glucuronidase expression clearly increased when potato extract or PGA was added to the minimal medium. When the bacteria were grown in the presence of potato extract the level of β -glucuronidase activity was markedly higher at the beginning of the exponential phase of growth compared to when the cells were grown in the presence of PGA. The maximum expression of β -glucuronidase occurred in a short period during which the cells were still in the mid-exponential phase of growth. The maximum expression of *gusA* was transient and was followed by a decline (Fig. 3). In the presence of PGA the timing of β -glucuronidase expression was somewhat delayed, relative to its expression in the presence of potato extract, and started to increase only after 6 h of growth. The plasmid pROT5 yielded β -glucuronidase activity data that were indistinguishable from those of SCC6004 under all conditions tested (Fig. 3). These results indicate that no essential promoter elements reside 3' to the *PaeI* site or 5' to the *KpnI* site.

As the rate of induction of the *prtW* promoter with the potato extract or the PGA is maximal after 8 h of growth, the expression of different promoter constructs was observed at this time point. In the first construct, a *HindIII* site located 683 bp upstream of the first codon in the *prtW* promoter region was exploited to delete the region upstream of this site in pROT5. The new construct was designated pROT6. *E. carotovora* SCC3193 cells carrying pROT6 showed the same basal level of β -glucuronidase activity when growing under non-inducing conditions as when carrying pROT5 (Fig. 4a). The induction rates of β -glucuronidase activity in the presence of PGA or potato extract were the same as in pROT5; however, the values attained were lower (Fig. 4a).

The promoter region of *prtW* from -371 to +43 nt (Fig. 2a) together with *gusA* was cloned from pROT5 by PCR, using primers PROM5 (5'-ATGTGAGCTCGT-

Fig. 3. Expression of the *prtW::gusA* fusion in strain SCC6004 and in strain SCC3193 pROT5 under various growth conditions. Cultures were grown in M9 minimal medium containing 0.4% glycerol and supplemented as indicated. (a) β -Glucuronidase activity of the bacterial cultures. (b) Growth of the bacterial cultures followed by measurement of OD₆₀₀. For both graphs: ■, SCC6004 in minimal medium plus 0.4% PGA; ▲, SCC3193(pROT5) in minimal medium plus 0.4% PGA; □, SCC6004 in minimal medium plus 16% potato extract; △, SCC3193(pROT5) in minimal medium plus 16% potato extract. Each value represents the mean of three experiments; the SD is indicated by the error bars.

AGCGCGGTTT-3') and M13/pUC Reverse Primer (-24), into the low-copy vector pMW119; the resulting plasmid was designated pROT7. The basal level of β -glucuronidase activity in pROT7 was low, but was comparable to that of pROT5. However, pROT7 was completely incapable of inducing β -glucuronidase activity, even when cells were grown in the presence of potato extract or PGA (data not shown). The results of these experiments refer to a hypothetical negative regulatory sequence spanning the region from -371 to -245 nt (Fig. 2a).

The *prtW* promoter region was also restricted at a unique *Clal* site located 245 bp upstream of the first

codon. The 2700 bp *Clal* fragment together with miniTn5Cm^R::*gusA* from pROT1 was cloned into the *Clal* site of the low-copy vector pACYC184 to produce plasmid pROT8. The β -glucuronidase activity was measured in the SCC3193(pROT8) cells under the same conditions as with pROT5 and pROT6. In the case of pROT8, β -glucuronidase expression increased eightfold relative to pROT5 in the absence of the inducer (Fig. 4a). It was interesting that β -glucuronidase activity was still stimulated about two- to threefold in the presence of potato extract or PGA (Fig. 4a).

Expression of *prtW::gusA* in various regulatory backgrounds

PCWDE synthesis is subjected to a wide range of plant signal molecules, including cell-wall fragments released by the action of PCWDEs and substances from the lysing plant cells. We compared *prtW::gusA* fusion activities in the wild-type strain and in *expA*⁻ and *kdgR*⁻ mutants under different induction conditions to see whether there were changes in regulation in response to different signals.

To analyse the effect of KdgR on protease expression, we analysed the expression of the transcriptional *prtW::gusA* fusions (pROT5, pROT6 and pROT8) in the *kdgR*⁻ mutant. In non-inducing conditions, *prtW::gusA* transcriptional fusions showed an approximately two- to fourfold increase in the expression of β -glucuronidase activity in comparison to the wild-type strain (Fig. 4a, b). The expression of pROT5, pROT6 and pROT8 clearly increased when the cells were grown in the presence of potato extract. The addition of PGA to the medium, however, did not result in further enhancement of promoter activity in comparison to the wild-type strain (Fig. 4a, b). In the case of pROT8 the induction rate of β -glucuronidase activity was lower, probably due to the high non-induced level of β -glucuronidase activity (Fig. 4b). To determine whether the promoter activity in pROT7 was affected by KdgR, pROT7 was introduced into SCC510; pROT7 was completely devoid of promoter activity on any medium tested (data not shown). These results indicate that expression of the *prtW::gusA* fusion in pROT7 appeared to be unaffected by KdgR.

We also studied the expression of the *prtW::gusA* transcriptional fusions (pROT5, pROT6 and pROT8) in the *expA*⁻ mutant strain SCC3060. When pROT5 and pROT6 were introduced into SCC3060, β -glucuronidase activity was almost undetectable on any medium tested (Fig. 4c). However, when pROT8 was introduced into SCC3060, β -glucuronidase activities in all conditions tested were on the same level as the non-induced activity in the wild-type strain (Fig. 4c).

When pROT5 and pROT6 were introduced into the *expA*⁻ *kdgR*⁻ double mutant, SCC500, the β -glucuronidase activities were higher than in the *expA*⁻ mutant, although the activities did not reach the levels observed in the wild-type strain (Fig. 4a, d). When pROT8 was

Fig. 4. Expression of pROT5, pROT6 and pROT8 in different strains of *E. carotovora*. β -Glucuronidase activity was measured 8 h after inoculation. Cultures were grown in M9 minimal medium containing 0.4% glycerol and supplemented as indicated. (a) Wild-type SCC3193; (b) SCC510 *kdgR*⁻; (c) SCC3060 *expA*⁻; (d) SCC500 *expA*⁻ *kdgR*⁻. Unsupplemented medium (open bars), medium plus 0.4% PGA (bricks) and medium plus 16% potato extract (solid bars). Each value represents the mean of three experiments; the SD is indicated by the error bars.

introduced into the *expA*⁻ *kdgR*⁻ double mutant, the β -glucuronidase activities remained at the same levels in both the presence and the absence of PGA. However, when medium with potato extract was used, the relative upregulation of pROT5, pROT6 and pROT8 activity was even greater than when strains carrying these plasmids were grown in the presence of medium with PGA (Fig. 4d).

As in the case of the wild-type and the *kdgR*⁻ mutant strains, pROT7 was completely devoid of promoter activity in the *expA*⁻ and *expA*⁻ *kdgR*⁻ mutants on any medium tested (data not shown).

DISCUSSION

In this study we have explored some aspects of the transcriptional regulation of the *E. carotovora* protease *prtW* gene. We analysed the effect of PGA and of potato extract on the different *prtW*::*gusA* fusions in the wild-type strain (SCC3193) and in different regulatory mutants.

Identification of the *prtW* transcriptional signal

Evidence from Northern analysis and from the complementation of the protease-negative phenotype in SCC6004 with the cloned *prtW* wild-type allele established that the protease gene is transcribed separately from the inhibitor and secretion genes. In spite of the extensive similarity of PrtW from *E. carotovora* with proteases of *Erwinia chrysanthemi*, the operon structure of the protease, the inhibitor and the secretions genes in these two species is different. These differences might result from the rearrangements that have occurred during the transfer of these genes between different *Erwinia* strains (Létoffé *et al.*, 1990; Ghigo & Wandersman, 1992; Marits *et al.*, 1999).

Effect of growth conditions on the expression of *prtW*::*gusA* fusions

Genes that encode the PCWDEs are often subject to coordinated regulation; these regulatory systems are able to respond to the various environmental signals

that may be encountered during the cycle of infection (Liu *et al.*, 1993, 1999; Eriksson *et al.*, 1998; Harris *et al.*, 1998). The most important signals originate from the infected plant tissues, as this is the main environment for the pathogen during the plant-microbe interaction. To mimic *in planta* conditions low nutrient medium (M9+glycerol) was used to culture the *E. carotovora* strains.

The expression of protease already during the early stages of infection correlates with the early expression of pathogenesis-related genes in infected plants, as shown by Vidal *et al.* (1997). Such a rapid response to the incoming signal, activating the expression of the protease, could facilitate the establishment of successful infection and makes the protease a possible candidate for the repression of plant defences. The signal to which *prtW* responds is, for the moment, unknown, but as the induction had already occurred by 4 h after infection this signal may be a plant protein(s), phenolic compounds or other factors that are released from the damaged plant cells. We also observed the activator effect of PGA on *prtW* expression; this function could result from the degradation products of PGA. These degradation products are the result of the enzymic activity of different pectinolytic enzymes whose expression is usually activated at the start of the stationary phase of growth (Pirhonen *et al.*, 1991; Eriksson *et al.*, 1998). This explains our results, which showed that expression of the *prtW* fusions reached its maximum in the presence of PGA only in the initial stages of the stationary phase of growth (Fig. 3).

Interestingly, although the induction rates were similar, we observed differences in the level of *prtW* expression in pROT5 and pROT6 in the wild-type (Fig. 4a). It is possible that these differences may be caused by the occurrence of regulatory elements in the upstream region of pROT5 that are necessary for the full expression of the *prtW* promoter. Huang *et al.* (1998) have described the occurrence of promoters in *Ralstonia solanacearum* that have distant *cis*-acting DNA sequences enhancing the expression of different virulence genes.

Role of the regulators KdgR and ExpA on the expression of *prtW*

The complex regulatory network controlling the production of virulence factors has been the subject of intensive studies in many Gram-negative pathogens (Liu *et al.*, 1993, 1996; Frederick *et al.*, 1997; Thompson *et al.*, 1997; Harris *et al.*, 1998). The data presented by Hyytiäinen *et al.* (2001) show that the global regulators ExpA and KdgR modulate extracellular enzyme gene expression through the RsmA-*rsmB* system. We now provide additional evidence for the role of KdgR and ExpA in the differential responses of *prtW* to potato extract and PGA. The interesting fact is that although the KdgR mutant shows increased *prtW* promoter activity in the absence of PGA, we could still observe the inducing effect of PGA on the *kdgR*⁻ mutant (Fig. 4b). This may indicate that KdgR on its own does not

mediate the induction of protease upon the addition of PGA, but that further levels of control are required for protease induction. The effect of potato extract seems to be more pronounced when used as an inducer than the effect of PGA on the expression of protease in the *kdgR*⁻ mutant SCC510 (Fig. 4a, b). These results might refer to the possibility that KdgR negatively regulates the expression of a regulator responding to the signal present in the potato extract.

The level of expression of the pROT5, pROT6 and pROT8 fusions in the *expA*⁻ mutant remained in all conditions tested at the same level as that in the wild-type strain under non-inducing conditions (Fig. 4a, c). In the *expA*⁻ *kdgR*⁻ double mutant the promoter activities of pROT5, pROT6 and pROT8 were similar or slightly increased when compared to those in the *expA*⁻ mutant (Fig. 4a, d). Similarly, Hyytiäinen *et al.* (2001) showed that under the non-induced conditions the production of protease was only partly restored in the *expA*⁻ *kdgR*⁻ mutant. Taken together, these data suggest that the presence of ExpA plays an important role in the expression of *prtW* under non-induced as well as under induced growth conditions.

Deletion analysis identified an essential regulatory element that resides between the nucleotides -371 and -245 relative to the *prtW* translational start site (Fig. 2a). The construct pROT7, which was lacking the region upstream from the -371 nt, was completely devoid of promoter activity in the wild-type strain as well as in the *kdgR*⁻ mutant (not shown). Furthermore, we searched for potential KdgR sites between nucleotides -371 and -245 and were unable to find any convincing matches to the *E. carotovora* consensus (Liu *et al.*, 1999). The deletion of this region restored the promoter activity of *prtW*, as was observed in the case of pROT8 (Fig. 4b). These results refer to the possibility that the corresponding DNA region may be involved in the binding of an unknown negative regulatory protein.

Differential responses of protease expression to physiological inducers, such as potato extract and PGA, probably allow flexibility in selectively expressing the *prtW* gene. Further studies of different regulatory systems, which must guarantee the coordinated expression of protease, may shed some light on the complex regulation of this virulence factor.

ACKNOWLEDGEMENTS

We thank Heidi Hyytiäinen (Dept of Biosciences, Division of Genetics, Viikki Biocenter, University of Helsinki, Finland) for the *E. carotovora* strains SCC500 and SCC510. We also thank Sulev Kuuse for help with the photography. Our work was supported by the Estonian Science Foundation (Grant TBGMR510) and the European Community (Contract ERBIC15-CT96-0908).

REFERENCES

- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Strahl, K. (1987). *Current Protocols in Molecular Biology*. New York: Wiley.

- Barras, F., van Gijsegem, F. & Chatterjee, A. K. (1994). Extracellular enzymes and pathogenesis of soft-rot *Erwinia*. *Annu Rev Phytopathol* 32, 201–234.
- Basse, C. W., Fath, A. & Boller, T. (1993). High affinity binding of a glycopeptide elicitor to tomato cells and microsomal membranes and displacement by specific glycan suppressors. *J Biol Chem* 268, 14724–14731.
- Bauchop, T. & Elsdén, S. R. (1960). The growth of microorganisms in relation to their energy supply. *J Gen Microbiol* 23, 457–469.
- Bender, C. L., Alarcon-Chaidez, F. & Gross, D. C. (1999). *Pseudomonas syringae* phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. *Microbiol Mol Biol Rev* 63, 266–292.
- Eriksson, A. R. B., Andersson, R. A., Pirhonen, M. & Palva, E. T. (1998). Two-component regulators involved in the global control of virulence in *Erwinia carotovora* subsp. *carotovora*. *Mol Plant–Microbe Interact* 11, 743–752.
- Frederick, R. D., Chiu, J., Bennetzen, J. L. & Handa, A. K. (1997). Identification of a pathogenicity locus, *rpfA*, in *Erwinia carotovora* subsp. *carotovora* that encodes a two-component sensor-regulator protein. *Mol Plant–Microbe Interact* 6, 407–415.
- Ghigo, J.-M. & Wandersman, C. (1992). A fourth metalloprotease gene in *Erwinia chrysanthemi*. *Res Microbiol* 143, 857–867.
- Guzzo, J., Pages, J. M., Duong, F., Lazdunski, A. & Murgier, M. (1991). *Pseudomonas aeruginosa* alkaline protease: evidence for secretion genes and study of secretion mechanisms. *J Bacteriol* 173, 5290–5297.
- Harris, S. J., Shih, Y.-L., Bentley, S. D. & Salmond, G. P. C. (1998). The *hexA* gene of *Erwinia carotovora* encodes a LysR homologue and regulates motility and the expression of multiple virulence determinants. *Mol Microbiol* 29, 705–717.
- Heilbronn, J., Johnston, D. J., Dunbar, B. & Lyon, G. (1995). Purification of a metalloprotease produced by *Erwinia carotovora* spp. and the degradation of potato lectin *in vitro*. *Physiol Mol Plant Physiol* 47, 285–292.
- Huang, J., Yindeeyoungyeon, W. Y., Garg, R. P., Denny, T. P. & Schell, M. A. (1998). Joint transcriptional control of *xpsR*, the unusual signal integrator of the *Ralstonia solanacearum* virulence gene regulatory network, by a response regulator and a LysR-type transcriptional activator. *J Bacteriol* 180, 2736–2743.
- Hyttiäinen, H., Montesano, M. & Palva, E. T. (2001). Global regulators ExpA (GacA) and KdgR modulate extracellular enzyme gene expression through the RsmA–*rsmB* system in *Erwinia carotovora* subsp. *carotovora*. *Mol Plant–Microbe Interact* 14, 931–938.
- Kato, T., Shiraishi, T., Toyoda, K., Saitoh, K., Satoh, Y., Tahara, M., Yamada, T. & Oku, H. (1993). Inhibition of ATPase activity in pea plasma membranes by fungal suppressors from *Mycosphaerella pinoides* and their peptide moieties. *Plant Cell Physiol* 34, 439–445.
- Laemmli, U. K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Létoffé, S., Delepelaire, P. & Wandersman, C. (1990). Protease secretion by *Erwinia chrysanthemi*: the specific secretion functions are analogous to those of *Escherichia coli* alpha-haemolysin EMBO J 9, 1375–1382.
- Liu, Y., Murata, H., Chatterjee, A. & Chatterjee, A. K. (1993). Characterization of a novel regulatory gene *aepA* that controls extracellular enzyme production in the phytopathogenic bacterium *Erwinia carotovora* subsp. *carotovora*. *Mol Plant–Microbe Interact* 6, 299–308.
- Liu, Y., Wang, A., Mukherjee, A. & Chatterjee, A. K. (1996). RecA relieves negative autoregulation of *rdgA*, which specifies a component of the RecA–Rdg regulatory circuit controlling pectin lyase production in *Erwinia carotovora* subsp. *carotovora*. *Mol Microbiol* 22, 909–918.
- Liu, Y., Jiang, G., Cui, Y., Mukherjee, A., Ma, W. L. & Chatterjee, A. K. (1999). *kdgR_{Ecc}* negatively regulates genes for pectinases, cellulases, protease, harpin_{Ecc}, and a global RNA regulator in *Erwinia carotovora* subsp. *carotovora*. *J Bacteriol* 181, 2411–2422.
- Marits, R., Kõiv, V., Laasik, E. & Mäe, A. (1999). Isolation of an extracellular protease gene of *Erwinia carotovora* subsp. *carotovora* strain SCC3193 by transposon mutagenesis and the role of protease in phytopathogenicity. *Microbiology* 145, 1959–1966.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory, NY: Cold Spring Harbor.
- Mukherjee, A., Cui, W., Liu, Y., Domenyo, C. K. & Chatterjee, A. K. (1996). Global regulation in *Erwinia* species by *Erwinia carotovora* *rsmA*, a homologue of *Escherichia coli* *csrA*: repression of secondary metabolites, pathogenicity and hypersensitive reaction. *Microbiology* 142, 427–434.
- Novel, G., Didier-Fichet, M. L. & Stoeber, F. (1974). Inducibility of β -glucuronidase in wild-type and hexuronate-negative mutants of *Escherichia coli* K-12. *J Bacteriol* 120, 89–95.
- Palva, T. K., Holmström, K.-O., Heino, P. & Palva, E. T. (1993). Induction of plant defense response by exoenzymes of *Erwinia carotovora* subsp. *carotovora*. *Mol Plant–Microbe Interact* 6, 190–196.
- Pérombelon, M. C. M. & Kelman, A. (1980). Ecology of the soft rot *Erwinias*. *Annu Rev Phytopathol* 18, 361–387.
- Pirhonen, M., Saari-lahti, H., Karlsson, M.-B. & Palva, E. T. (1991). Identification of pathogenicity determinants of *Erwinia carotovora* subspecies *carotovora* by transposon mutagenesis. *Mol Plant–Microbe Interact* 4, 276–283.
- Py, B., Bortoli-German, I., Haiech, I., Chippaux, M. & Barras, F. (1991). Cellulase EG2 of *Erwinia chrysanthemi*: structural organization and importance of His 98 and Glu 133 residues for catalysis. *Protein Eng* 4, 325–333.
- Sambrook, J., Maniatis, T. & Fritsch, E. F. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory, NY: Cold Spring Harbor.
- Sanger, F., Nicklen, S. & Coulson, A. (1977). DNA sequencing with chain terminating inhibitors. *Proc Natl Acad Sci USA* 74, 5463–5467.
- Thompson, N. R., Cox, A., Bycroft, B. W., Stewart, G. S. A. B., Williams, P. & Salmond, G. P. C. (1997). The Rap and Hor proteins of *Erwinia*, *Serratia* and *Yersinia*: a novel subgroup in a growing superfamily of proteins regulating diverse physiological processes in bacterial pathogens. *Mol Microbiol* 26, 531–544.
- Vidal, S., Ponce de León, I., Denecke, J. & Palva, E. T. (1997). Salicylic acid and the plant pathogen *Erwinia carotovora* induce defence genes via antagonistic pathways. *Plant J* 11, 115–123.

Received 6 August 2001; revised 17 October 2001; accepted 25 October 2001.