Regulation of the expression of \( \textit{prtW}::gusA \) fusions in \textit{Erwinia carotovora} subsp. \textit{carotovora}

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\textit{Erwinia carotovora} subsp. \textit{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 \( \textit{prtW} \) gene constitutes an independent transcriptional unit. It is demonstrated that introduction of the \( \textit{prtW}^+ \) plasmid \textit{in trans} into the \( \textit{prtW}^- \) mutant restores the protease activity in this strain. Gene fusions to the \( gusA \) (\( \beta \)-glucuronidase) reporter were employed to analyse the transcription of \( \textit{prtW} \). The transcription of \( \textit{prtW} \) 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 \textit{E. carotovora} subsp. \textit{carotovora}. The differential responses of \( \textit{prtW} \) expression to potato extract and to PGA appeared to be dependent on the KdgR repressor and the response regulator \textit{ExpA}. According to the results presented here, it is conceivable that the multiple regulatory network allows flexibility in the expression of the \( \textit{prtW} \) gene during different stages of infection.

Keywords: phytopathogenic bacteria, protease, promoter fusions

INTRODUCTION

The phytopathogenicity of the soft-rot enterobacteria of the genus \textit{Erwinia} is correlated with their ability to produce and secrete plant-cell-wall-degrading enzymes (PCWDEs) (Pérombelon & Kelman, 1980; Barras \textit{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 \textit{Erwinia carotovora} subsp. \textit{carotovora} (\textit{E. carotovora}) induce defence responses in plants has been proposed to involve the release of oligogalacturonide elicitors from plant cell wall pectin (Palva \textit{et al}., 1993; Vidal \textit{et al}., 1997).

\textbf{Abbreviations}: \textit{E. carotovora}, \textit{Erwinia carotovora} subsp. \textit{carotovora}; PCWDE, plant-cell-wall-degrading enzyme; PGA, polygalacturonate; \( \textit{pNP}, \) \( p \)-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 \textit{et al}., 1993; Kato \textit{et al}., 1993; Bender \textit{et al}., 1999). As \textit{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 \textit{Erwinia} have evolved multiple strategies to sense their environment and to modulate their gene expression both by positive \textit{expA/expS}, \textit{aepA}, \textit{rdgA}–\textit{rdgB}, \textit{rpfA}, \textit{rpoS} and \textit{hor} regulators, and by negative \textit{hexA} and \textit{kdgR} regulators (Liu \textit{et al}., 1993, 1996; Mukherjee...
Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant characteristics*</th>
<th>Source/reference</th>
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<tr>
<td><em>Escherichia coli</em></td>
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<tr>
<td>DH5α</td>
<td>Km*R; pREP4</td>
<td>BRL</td>
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<tr>
<td>M15</td>
<td></td>
<td>Qiagen</td>
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<td><em>E. carotovora</em></td>
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<tr>
<td>SCC3193</td>
<td>Wild-type</td>
<td>Pirhonen et al. (1991)</td>
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<tr>
<td>SCC3060</td>
<td>expA::Km*R</td>
<td>Pirhonen et al. (1991)</td>
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<tr>
<td>SCC6004</td>
<td>prtW::Tn5–gusA</td>
<td>Marits et al. (1999)</td>
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<tr>
<td>SCC510</td>
<td>kdgR::Cm*R</td>
<td>Hyytiäinen et al. (2001)</td>
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<tr>
<td>SCC500</td>
<td>expA::Km<em>R; kdgR::Cm</em>R</td>
<td>Hyytiäinen et al. (2001)</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pBluescript(+)</td>
<td>Ap*R; Cloning vector</td>
<td>Stratagene</td>
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<tr>
<td>pQE30</td>
<td>Ap*R; Expression vector</td>
<td>Qiagen</td>
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<tr>
<td>pMW119</td>
<td>Ap*R; Cloning vector</td>
<td>Eurogentec</td>
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<tr>
<td>pGUS102</td>
<td>Ap*R; Promoterless 1·8 kb gusA gene as an EcoRI fragment in pBR322</td>
<td>A. Eriksson†</td>
</tr>
<tr>
<td>pACYC184</td>
<td>Ap<em>R; Km</em>R; Cloning vector</td>
<td>New England Biolabs</td>
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<tr>
<td>pMW119::gusA</td>
<td>Ap*R; 1·8 kb EcoRI fragment containing gusA gene from pGUS102 cloned into HindIII site of pMW119</td>
<td>This study</td>
</tr>
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<td>pROT1</td>
<td>Ap<em>R; 2·7 kb Clal fragment from SCC6004 containing prtW with a miniTn5Cm</em>R::gusA cloned into pBluescript(+)</td>
<td>Marits et al. (1999)</td>
</tr>
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<td>pROT3</td>
<td>Ap*R; placZ::prtW PCR product of the ORF of prtW in pBluescript(+)</td>
<td>This study</td>
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<tr>
<td>pROT3A</td>
<td>Ap*R; Same as pROT3, but opposite insert orientation</td>
<td>This study</td>
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<td>pROT4</td>
<td>Ap*R; pBluescript with the 2038 bp KpnI–Pael fragment containing the prtW regulatory region and 43 bp of the ORF from SCC3193 inserted into the KpnI–Pael sites</td>
<td>This study</td>
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<td>Ap*R; Same as insert as pROT4, but cloned into the SmaI site of pMW118::gusA</td>
<td>This study</td>
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<td>pROT6</td>
<td>Ap*R; 1312 bp KpnI–HindIII fragment deleted from pROT5</td>
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<td>pROT7</td>
<td>Ap*R; PCR product of pROT3 using primers PROM5 and M13/pUC Reverse Primer (–24) in SmaI site of pMW119</td>
<td>This study</td>
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<td>pROT8</td>
<td>Ap<em>R; Cm</em>R; Same insert as pROT1, but cloned into Clal site of pACYC184</td>
<td>This study</td>
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<td>pRT1</td>
<td>Ap*R; prtW PCR product of the ORF of prtW in expression vector pQE30</td>
<td>This study</td>
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† Swedish University of Agricultural Sciences, Uppsala, Sweden.

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.
**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 BamHI–SalI 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 BamHI–SalI 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...
To determine the 5′ bp (Table 1). Expression of were made from the activity, restriction-site deletions and PCR constructs SCC3193 were hybridized with the 43 bp within the To define the DNA regions necessary for prtW Deletions in the were examined during planktonic growth, with a 5′ 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 (Létolle 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 PaeI site or 5′ to the KpnI site. The 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-3′) and PROM6 (5′-GGCTGGCTTTCTTTCATGAC-3′). The construction of the plasmids is described in Results and in Table 1.
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− kdgR− double mutant strain SCC500. When pROT5 and pROT6 were 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). 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 pROT8 was introduced into the expA− kdgR− double mutant, SCC500, the β-glucuronidase activity was almost undetectable 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.
introduced into the \( \text{expA}^- \text{kdgR}^- \) double mutant, the \( \beta \) -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 \( \text{kdgR}^- \) mutant strains, pROT7 was completely devoid of promoter activity in the \( \text{expA}^- \) and \( \text{expA}^- \text{kdgR}^- \) 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* transcripational 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

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**Fig. 4.** Expression of pROT5, pROT6 and pROT8 in different strains of *E. carotovora*. \( \beta \)-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.
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 the 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.

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