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ROLE OF TWO-COMPONENT REGULATOR SYSTEM PehR-PehS AND EXTRACELLULAR PROTEASE PrtW IN VIRULENCE OF *ERWINIA CAROTOVORA* subsp. *CAROTOVORA*

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TARTU UNIVERSITY
PRESS

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers which will be referred to by their Roman numerals in the text.

I Flego, D., **Marits, R.**, Eriksson, A. R. B., Kõiv, V., Karlsson, M.-B., Heikinheimo, R. and Palva, E. T. 2000. A two component regulatory system, *pehR-pehS*, controls endopolygalacturonase production and virulence in the plant pathogen *Erwinia carotovora* subsp. *carotovora*. Mol. Plant-Microbe Interact. **13**, 447–455.

II **Marits, R.**, Kõiv, V., Laasik, E. and Mäe, A. 1999. Isolation of an extracellular protease gene of *Erwinia carotovora* susbsp. *carotovora* strain SCC3193 by transposon mutagenesis and the role of protease in phytopathogenicity. Microbiology, **145**, 1959–1966.

III **Marits, R.**, Tshuikina, M., Pirhonen, M., Laasik, E. and Mäe, A. 2001. Regulation of *prtW*::*gusA* fusion in *Erwinia carotovora* subsp. *carotovora*. Microbiology, in press.

LIST OF ABBREVATIONS

aa	amino acids
ABC protein	for ATP-binding cassette
Aep	activator of extracellular protein production
avr	avirulence genes
bp	base pairs
cAMP	cyclic AMP
Cel	Cellulase
CF	culture filtrate
CRP	cyclic AMP receptor protein
Csr	carbon storage regulator
Da	daltons
Ecc	*Erwinia carotovora* subsp. *carotovora*
Ech	*Erwinia chrysanthemi*
Eco	*Escherichia coli*
EDTA	N,nN;N',N'-ethylenedinitrilo tetraacetic acid
EPS	extracellular polysaccharide
exp	extracellular enzyme production
gac	global antibiotic and cyanide control
gusA	β-glucuronidase gene
hex	hyperinduction of exoenzymes
hor	homologue of *rap*
HR	hypersensitive response
hrp	hypersensitive response and pathogenicity
HSL	N-acyl-homoserine lactone
inh	inhibitor
IPTG	isopropyl-β-D-thiogalactopyranoside
Kb	kilo base pairs
kDa	kilodaltons
KdgR	general repressor of pectin and calacturonate catabolism
LB	Luria broth
LPS	lipopolysaccharide
MFP	membrane fusion protein
NSAP	nonspecific acid phosphatase
OHL	N-(3-oxohexanoyl)-L-homoserine lactone
OMP	outer membrane protein
ORF	open reading frame
out	general cecretory genes in *Erwinia* species
PCWDEs	plant cell wall-degrading enzymes
PGA	polygalacturonic acid
Peh	polygalacturonase
Pel	pectate lyase

Pme	pectin methylesterase
pmr	polymyxin resistance
Pnl	pectin lyase
PR	pathogenesis related proteins
prg	PhoP-repressed genes
Prt	protease
R	resistance genes
rap	regulation of antibiotic and pigment
rex	regulator of exoenzymes
rpf	regulator of pathogenicity factors
rsm	repressor of secondary metabolites
SAR	systemic aquired resistance
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
X-GlcA	5-bromo-4-chloro-3-indolyl β-D-glucuronic acid

INTRODUCTION

Plants make up the majority of the living environment on Earth and, directly or indirectly, plants also make up all the food on which human beings and all animals depend. Plant diseases have been feared as much as human diseases and war, they have been considered to be a curse and a punishment of people by God for the wrongs and sins they had committed. The only way to prevent such diseases were festivals and sacrifices to thank, please, or appease a god. The invention of the compound microscope in the mid-1600s resulted in the discovery of microorganisms, however, scientists believed that microorganisms and their spores were the result rather than the cause of diseases. The devastating epidemics of late blight of potato in Northern Europe in the 1840s, which resulted in the death of hundreds of thousands of people, greatly stimulated an interest in the causes of the disease and disease control. It was as late as in 1861 that German deBary experimentally established that a fungus (*Phytophthora infestans*) was the cause of the plant disease known as late blight of potato (Agrios, 1997). Today the protection of plants against diseases has been improved mainly by the use of chemical pesticides, which, however, can contaminate the environment and also the crop. And even now, about one third of potential world crop production is lost due to diseases, insects and weeds. Together with post-harvest losses this accounts for about 48% of all food crops (Agrios, 1997).

About 100 species of bacteria cause plant diseases, which are usually very difficult to control. The use of chemicals is much less successful with bacterial diseases than with fungal diseases. The use of crop varieties resistant to certain bacterial diseases is one of the best ways to avoid heavy losses. Molecular biology is expected to contribute greatly and to provide tremendous benefits in the area of gene detection, identification, isolation, modification, transfer, and expression for the purpose of increasing resistance to transmittable plant diseases. For this we have to know what happens when bacteria and plant interact, how does a plant recognise the pathogen and how does it defend itself. How is it that in some cases bacteria can overcome plant defence, but not always. The bacterial virulence determinants and their regulators have been under extensive study. Now the main goal is to study the plant-microbe interaction processes and signals, which are exchanged.

1. REVIEW OF LITERATURE

1.1. Bacterial soft-rot and genus *Erwinia*

The soft-rot phytopathogens cause greater total loss of agricultural produce than any other bacterial plant disease. Bacterial soft-rots occur most commonly on fleshy storage tissues of vegetables and annual ornamentals such as potatoes, carrots, onions, fleshy fruits such as cucumber and tomato, or succulent stems, stalks, or leaves such as cabbage, lettuce, and spinach. They occur worldwide and cause serious diseases of crops in the fields, in transit, and especially in storage. Several bacteria as *Erwinia*, *Pseudomonas*, *Bacillus* and *Clostridium* spp. attack living plant tissues and cause soft-rot. The soft-rot bacteria may survive in infected tissues, in the soil, and in contaminated equipment and containers, some of them also over winter in insects. They enter plants or plant tissue primarily through wounds. Within tissue they multiply profusely in the intercellular spaces, where they produce enzymes that dissolve the middle lamella and separate cells from one another causing maceration and softening of affected tissues. At first plant cells start losing water, which leads to a loss of turgor and finally to the collapse and death of the cells.

The genus *Erwinia* belongs to the family *Enterobacteriacea*, which are Gram-negative straight rods, motile with peritrichous flagella, and they are also facultative anaerobs. Several *Erwinia* spp., so called soft-rot erwinias, are highly pectolytic and cause soft-rot in plants. From this group three species *Erwinia carotovora* subsp. *carotovora*, *Erwinia carotovora* subsp. *atroseptica* and *Erwinia chrysanthemi* have been most frequently characterized. *E. carotovora* subsp. *atroseptica* is most common in temperate climate where it attacks mostly potato, whereas *E. chrysanthemi* is a pathogen on a wide range of tropical and subtropical crops. *E. carotovora* subsp. *carotovora* has a wide distribution in both temperate and tropical climates, and shows a much wider host range than *E. chrysanthemi*. Some *Erwinia* do not produce pectic enzymes but extracellular polysaccharides and cause necrotic or wilt disease: example *E. stewartii* causes Stewart's wilt of corn and *E. amylovora* causes fire blight of apples and pears (Pérombelon and Kelman, 1980; Agrios, 1997).

1.2. Virulence determinants of *Erwinia*

The soft-rot erwinias usually enter the plant through wounds or natural openings such as stomata or hydrophores. Inside the plant, they multiply in the intercellular space and degrade the tissue by secreting large amounts of plant cell wall-degrading enzymes (PCWDEs) (Barras *et al.*, 1994; Pérombelon and Salmond, 1995). The crucial role of the PCWDEs in virulence has been

demonstrated by isolation of avirulent mutants that are defective either in the enzyme production (*exp* mutants; Pirhonen *et al.*, 1991; 1993) (*aep* mutants; Murata *et al.*, 1991; 1994; Liu *et al.*, 1993) (*rex* mutants; Jones *et al*; 1993) or in the secretion of the enzymes (*out* mutants; Andro *et al.*, 1984; Ji *et al.*, 1987; Pirhonen *et al.*, 1991; Thurn and Chatterjee, 1985).

The PCWDEs are able to degrade and modify the structural constituents of the primary and secondary cell walls and middle lamella of higher plants: pectin, cellulose and hemicellulose. These enzymes can be divided into several subgroups depending on their depolymerizing or modifying activities.

Pectin is a heteropolysaccharide with a backbone consisting of partially methylesterified polygalacturonate (PGA). Pectin methylesterase (Pme) removes methoxyl groups linked to galacturonate residues, which transform pectin into polygalacturonic acid (pectate). Pectate lyases (Pel) cleavage the α-1,4-glycosidic bonds of pectate by β-elimination, producing unsaturated products, whereas polygalactouronases (Peh) cleave these bonds by hydrolysis, producing saturated products (Barras *et al.*, 1994). Pectin lyases (Pnl) cleave pectin or methylesterified polygalacturonate.

The β-1,4-linkage of the polysaccharide chain of cellulose is hydrolyzed by cellulases (Cel).

The peptide bonds in polypeptides from plant cell wall or plant cell are hydrolyzed by proteases.

Inactivation of one extracellular enzyme coding gene or subgroup of genes (*peh*; Willis *et al.*, 1987; Saarilahti *et al.*, 1992; Nasser *et al.*, 1999) (*pel*; Roeder and Collmer, 1985; Trollinger *et al.*, 1989) (*pme*; Boccara and Chatain, 1989) (*prt*; Dahler *et al.*, 1990) has little effect on virulence. Hence it is not one individual extracellular enzyme or isoenzyme that is essential for virulence, but all the enzymes together. However, the pectinases (Pel, Peh) are the major cell wall degrading enzymes, the role of Cel and Prt is to support and complement the reaction.

The PCWDEs have a dual role in the plant-microbe interaction. They do not only break down the plant cell wall to release nutrients for bacterial growth, but they also form shorter oligogalacturonic fragments, which can trigger plant defence responses (Palva *et al.*, 1993; Vidal *et al.*, 1998; Norman, *et al.*, 1999; Norman-Setterblad *et al.*, 2000). The oligogalacturonic fragments, generated by the basal level of enzymes, also serve as the inducers for enhanced synthesis and release of pectolytic enzymes (Hugouvieux-Cotte-Pattat *et al.*, 1996).

The Pel, Peh, Pme and Cel proteins have N-terminal signal peptides and are secreted by type II or Sec-dependent pathway crossing the inner membrane via the Sec-machinery and the outer membrane by an auxiliary secretion system composed of 13 to 15 proteins (*out* genes). The proteases are secreted by type I secretion (or ABC-transporter) pathway in a single step mediated by transmembrane complex made up of three proteins (Pugsley, 1993; Delepelaire and Wandersman, 1991; Barras *et al.*, 1994; Lindeberg *et al.*, 1996).

In *E. carotovora* subsp. *carotovora* strain SCC3193 has been characterized so far as: an endopolygalacturonase (encoded by *pehA*), at least four Pels (encoded by *pelA* to *pelD*) and two Cels (encoded by *celC*, *celVI*) (Saarilahti *et al.*, 1990; Heikinheimo *et al.*, 1995; Mäe *et al.*, 1995).

1.2.1. Polygalacturonases

There are two types of polygalacturonases (Peh), endo-Peh and exo-Peh. Both types cleave pectate by hydrolysis but they release different products: endo-Pehs cleave the polymer at random, releasing large fragments, while exo-Pehs release either galacturonate monomers (in the case of exoplygalacturonase) or dimers (in the case of exo-poly-α-D-galacturonosidase) (Cooper, 1983). The Pehs from different bacteria are quite homologous and have a number of regions conserved in both, endo- and exo-Pehs. Moreover, the polygalacturonases from bacteria and plants are quite similar, suggesting their common origin (Saarilahti *et al.*, 1990; Laing and Pretorius, 1993; Huang and Allen, 1997).

In *E. carotovora* subsp. *carotovora* strain SCC3193 the endopolygalacturonase PehA is one of the major secreted virulence factors. The purified PehA protein is able to cause extensive maceration of plant tissue. A single gene, *pehA*, is responsible for all detectable Peh activity produced by the bacterium (Saarilahti *et al.*, 1990; 1992). The PehA is synthesized with a 26-aa cleavable signal peptide, the proenzyme has a calculated molecular mass of 42 849 and the mature enzyme of 40 064 Da. The PehA does not require Ca^{2+} for activity.

The *pehA* gene is expressed in SCC3193 at a fairly high constitutive level in LB (Luria broth) medium, but can be further induced about twofold by PGA. The *pehA* transcription is positively controlled by PehR regulatory locus. The control is specific as mutation in *pehR* regulatory region has no effect on the production of Pel and Cel, but production of PehA was 1–8% compared with the wild-type level. The $PehR^-$ mutant strains exhibited a reduced virulence and seemed to have a problem establishing the infection. However, in plants that became infected with the $PehR^-$ mutant, the bacteria appeared to spread as well as in plants infected with the wild-type strain. Similar results were obtained by $PehA^-$ mutant. According to these results was supposed that production of PehA is important in the initial phase of infection by *E. carotovora* subsp. *carotovora*. According to the hypothesis, PehA is important in the establishment and Pels in the subsequent spread of the infection. This is also reflected in the regulatory properties: Peh is produced at a fairly high level under non-induced conditions, is not repressed by glucose and is only moderately induced by PGA (about twofold). At the same time, the basal level of Pel activity is rather low, subject to catabolic repression and is effectively induced by PGA (about tenfold) (Saarilahti *et al.*, 1992). Similarly high is the basal expression of *peh-1* in *E. carotovora* subsp. *carotovora* 71, which is inducible by celery extract (twofold) (Liu *et al.*, 1994). The exo-poly-α-D galacturonidase (*pehX*) of

E. chrysanthemi EC16 is necessary for pectate utilization under conditions of pectate lyase deficiency, and has a role in the induction of Pel when bacteria is cultured in media containing high-molecular-weight pectic polymers by releasing of assimilable oligogalacturonates. The induction of Pel by the PehX⁻ mutant was significantly (2h) reduced when compared with the wild type (He and Collmer, 1990). The polugalacturonases have been shown to contribute to primary infection with *Ralstonia* (*Pseudomonas*) *solanacearum* involving the entry of the bacterium from wounds into the vascular systems, which causes wilt disease of many plant species (Huang and Allen, 1997).

The expression of *pehA* in *E. carotovora* subsp. *carotovora* is Ca^{2+} repressed, while accumulation of *pelB* mRNA is slightly stimulated. This Ca^{2+} dependent expression is mediated by PehR regulatory locus. Moreover, the calcium-mediated repression of *pehA* expression appears to be the main cause for the observed resistance increase in higher calcium content plants against *E. carotovora* subsp. *carotovor*a infection (Flego *et al.*, 1997). The production of three polygalacturonases (*pehV*, *pehW* and *pehX*) from *E. chrysanthemi* 3939 are also affected by Ca^{2+}, but the expression of the corresponding genes were repressed by Ca^{2+} only when bacteria were grown in medium containing PGA but not other carbon sources (glycerol, glucose or galacturonate). However, the effect was visible at very low $CaCl_2$ concentrations but was maximal for concentration higher than 0.2 mM (Nasser *et al.*, 1999).

The *pehA* gene in *E. carotovora* subsp. *carotovora* 3193 is transcribed from control region common with *pelB* gene, the *peh-1* and *pel-3* of *E. carotovora* subsp. *carotovora* have a similar gene organization (Heikinheimo *et al.*, 1995; Liu *et al.*, 1994). The promoter region of *pehA* contains several regions sharing homology with the binding site of the negative regulatory protein KdgR and putative binding site for the cAMP receptor protein (Saarilahti *et al.*, 1990).

1.2.2. Extracellular proteases

Several erwinias and pseudomonads cause soft-rot secrete proteases. In *E. carotovora* subsp. *carotovora*, large amounts of extracellular protease are produced when the bacterium is grown in rich broth, on bean (*Phaseolus vulgaris*) hypocotyls, or on sliced cucumber (*Cucumis sativus*) fruit (Tseng and Mount, 1973) but the physiological role of the proteases is unknown. Extracellular proteases have been suggested to provide amino acids for bacterial protein synthesis, degrade proteins involved in plant defence, or post-translational modification of endopectate lyase (Kyöstiö *et al.*, 1991; Heilbronn *et al.*, 1995; Shevchik, *et al.*, 1998).

1.2.2.1. Exoprotease of *E. carotovora* subsp. *carotovora*

E. carotovora subsp. *carotovora* EC14 produces an extracellular metalloprotease Prt1 with calculated molecular mass of 38,826 Da. The corresponding transcript was detected during the late stationary growth phase. The Prt1 mutant L-957 showed approximately 60 to 80% reduced protease activity with the wild type. These results and the Southern analysis indicated the possible existence of an additional protease gene. The deduced amino acid sequence of Prt1 showed significant sequence identity to several bacterial metalloproteases. It was most similar to *Bacillus thermoproteolyticus* (thermolysin), less to *B. subtilis* (neutral protease), *Legionella pneumophila* (metalloprotease), and *Pseudomonas aeruginosa* (elastase). Prt1 shows little sequence relatedness to metalloproteases of *Erwinia crysanthemi*, a closely related soft-rot pathogen. Amino acid sequence comparison of Prt1 with thermolysin showed high conservation of active sites. The calcium-binding sites were not conserved in Prt1, which may suggest that Prt1 does not require calcium for its activity, which was supported by the slight inhibition of Prt1 activity by EDTA, a chelator with high affinity for calcium. Prt1 has the N-terminal 20 amino acid signal sequence typical to the *E. coli* signal sequence (Kyöstiö *et al.*, 1991).

The high degree of amino acid identity between Prt1 and thermolysin in the substrate-binding site suggests similarities in the substrate specificity. Thermolysin has been shown to cleave plant proteins such as the heme free horseradish (*Armoracia rusticana*) peroxidase and hydroxyprolinerich glycoproteins (Welinder and Smillie, 1971; Adair and Appel, 1989), which are located in the plant cell wall and serve structural and defence functions against microbial attack (Showalter *et al.*, 1985). Degradation of potato cell wall hydroxyproline-rich glycoproteins by an *E. carotovora* subsp. *carotovora* extracellular protease has been demonstrated (Lewosc *et al.*, 1989). Heilbronn *et al.* (1995) showed *in vitro* degradation of potato lectins by a protease from the *E. carotovora* subsp. *carotovora*. Lectins are proteins that bind specifically to certain sugars and occur in large concentrations in many types of seeds, and cause lysis and growth inhibition of many fungi.

1.2.2.2. Exoproteases of *E. chrysanthemi*

Different strains of *E. chrysanthemi* have been reported to produce several extracellular proteases. *E. chrysanthemi* 3937JS2 produces at least two proteases of apparent molecular masses of 50 and 55 kDa, respectively. The proteolytic activity was expressed during the exponential growth phase but the proteases reached their highest specific activities only in the early stationary phase. The activity appeared when cells were grown in the presence of peptides, but not in the presence of a mixture of amino acids (Wandersman *et al.*, 1986).

Erwinia chrysanthemi B374 secretes three distinct proteases: PrtA (50 kDa), PrtB (53 kDa) and PrtC (55 kDa), which, introduced as a plasmid into *E. coli*, are secreted during the exponential phase of growth. The respective activities were sensitive to EDTA suggesting that PrtA, PrtB and PrtC are metalloproteases. Preliminary results indicated that these three enzymes could have different substrate specificities (Wandersman *et al.*, 1987; Delepelaire and Wandersman, 1989; Ghigo and Wandersman, 1992a). The fourth, minor metalloprotease PrtG (52kDa) shows only weak activity on the tested media (LB) and is not activated in the presence of other proteases (Ghigo and Wandersman, 1992b). The PrtA and PrtC of *E. chrysanthemi* 3937 efficiently process the endopectate lyase PelI-2 to the PelI-3 by cleaving the 97 N-terminal amino acids after secretion. The PelI-3 form is able to elicit necrosis on tobacco leaves, while the PelI-2 form is unable to give such effect. From eight endopectate lyases, secreted by *E. crysanthemi* 3937, the PelI-2 was the only one processed proteolytically (Shevchick *et al.*, 1998).

E. crysanthemi EC16 secretes also three proteases, called PrtA (51,5 kDa), PrtB (52,5 kDa) and PrtC (50 kDa), which became active when cells were grown in LB medium or in M9 glycerol medium supplemented with 0,5% tryptone. Similarly to the strain B374, their activities were sensitive to EDTA, indicating that those proteases belong to the family of metalloproteases. The EC16 marker exchange protease mutants all exhibited virulence indistinguishable from that of the parent strain on potato tuber tissue and chrysanthemum stems (Dahler *et al.*, 1990).

Bacteria, producing proteases, have also developed protective mechanisms to avoid proteolytic activity inside the cells. Most secreted proteases are synthesized as inactive precursors (zymogens), containing C-terminal or N-terminal extensions, which are subsequently cleaved to give fully active mature proteases. These bacterial zymogens are usually converted to active forms by autocatalytic processing during secretion, and not inside the cell (Ikemura *et al.*, 1987).

The *E. chrysanthemi* strain B374 proteases B, C, A and G are synthesized and initially secreted as inactive proenzymes. These propeptides are subsequently cleaved after secretion to yield active mature proteases. The activation of proteases is apparently not coupled to secretion and occurs autocatalytically after the precursor has been released into the medium. No specific cell function appears to be required for maturation. The maturation of proenzyme occurred in the presence of divalent cation Ca^{2+}. Besides, Ca^{2+}, Zn^{2+} and Mg^{2+} are partly interchangeable in the activation process. In the presence of EDTA the maturation of proenzyme is inhibited (Delepelaire and Wandersman, 1989; Ghigo and Wandersman, 1992a,b). The activity of proteases of *E. chrysanthemi* strain EC16 increased significantly in the presence of Ca^{2+} and Mg^{2+} when Zn^{2+} and Rb^{2+} were not effective (Dahler *et al.*, 1990).

1.2.2.3. Protease inhibitors

In addition to the extracellular proteases, the *E. crysanthemi* B374 produces a protease inhibitor specifically active against PrtA, PrtB, PrtC and 50 kDa metalloprotease of *Serratia marcescens*. The inhibitor protein is a low-molecular-weight (10 429 Da), heat-stable, active monomer. It has been shown that inhibitor protein form specific non-covalent complexes with protease B of *E. chrysanthemi* and with 50 kDa metalloprotease of *S. marcescens* (Wandersman *et al.*, 1987; Létoffé *et al.*, 1989). The protease inhibitor activity was observed also in *E. crysanthemi* strain EC16 (Dahler *et al.*, 1990).

The protease inhibitor is not required in order to protect the cell against intracellular protease activity as *inh* gene mutation does not affect protease synthesis or secretion, although its structural gene is located in an operon or closed to the genes involved in these steps (Wandersman *et al.*, 1987; Dahler *et al.*, 1990). The inhibitor might prevent autocatalytic cleavage of the zymogen in the periplasm since preliminary results indicated that the inhibitor interacts with purified PrtB precursor (Létoffé *et al.*, 1989).

1.2.2.4. Secretion of exoproteases

Previously described proteases, except the Prt1 of *E. carotovora* subsp. *carotovora*, lack an N-terminal signal sequence and their secretion mechanism is independent of the sec gene-mediated pathway (Delepelaire and Wandersman, 1989; Dahler *et al.*, 1990; Schatz and Beckwith, 1990; Kyöstiö *et al.*, 1991; Ghigo and Wandersman, 1992a,b). The transport of these type proteins requires three specific components; mutations in any of the three components abolish secretion (Létoffé *et al.*, 1990). The first protein is situated in the inner membrane and belongs to the well-characterized ATP-binding cassette (ABC) protein superfamily of transporters, which includes eukaryotic and prokaryotic proteins involved in the import or export of a wide variety of substrates, such as antibiotics, sugars, amino acids, peptides and proteins (Higgins, 1992). The second protein is associated with the inner membrane and is a member of a novel family of transport accessory proteins, found mostly in gram-negative bacteria. These proteins function in conjunction with membrane transporters such as the ABC proteins or the drug resistance proton-linked antiporters. They are involved in the export of peptides, proteins, drugs, metallic cations, and oligosaccharides. This protein family is termed as the membrane fusion protein (MFP) family (Dinh *et al.*, 1994). The third component is an outer membrane protein (OMP) with a typical N-terminal signal sequence. The export system consisting of these three proteins is categorized as the exporter subclass of the ABC transporter family, named as type I secretion pathway (Létoffé *et al.*, 1990; Delepelaire and Wandersman, 1991; Binet *et al.*, 1997; Schneider and Hunke, 1998).

The ABC exporters of gram-negative bacteria are often encoded by genes closely linked to the structural gene for the secreted polypeptide. The four highly homologous metalloproteases of *E. chrysanthemi* are encoded by contiguous genes clustered with three genes coding for their common ABC exporter (*prtDEG*), in the order PrtD (an ABC protein), PrtE (a MFP), and PrtF (an OMP). (Létoffé *et al.*, 1990; Delepelaire and Wandersman, 1991).

The ABC protein is responsible for the substrate specificity. The specific interaction between the ABC protein and the MFP, also between the MFP and OMP is required for formation of active exporters but "crosstalk" between the ABC and MFP proteins or MFP and OMP proteins is possible (Binet and Wandersman, 1995; Akatsuka *et al.*, 1997).

Ghigo and Wandersman (1994) demonstrated that the extreme C-terminal sequence Dxxx of proteases, where x-s are hydrophobic residues, is a conserved motif in all constructs that are secreted through the *E. chrysanthemi* type I secretion pathway. They suggested that this motif might be a key feature of the secretion signal recognized by the *E. chrysanthemi* secretion apparatus (PrtDEF). The proteins without this motif were secretion defective, also the addition of extra residues to the C-terminus have the blocking effect to the secretion.

The proteases of *E. chrysanthemi* and their homologues have in the N-terminal proteolytic domain a well conserved Zn^{2+}- binding domain HEXXHXUGUXH, in which X represents an arbitrary amino acid, U is a bulky hydrophobic residue and the three histidines are zinc ligands. The C-terminal domain contains multiple tandem repeats of a nine residues sequence which includes a GGXGXD consensus motif (X is an arbitrary amino acid) and binds Ca^{2+} ions (Baumann *et al.*, 1993; Delepelaire and Wandersman, 1989; Dahler *et al.*, 1990; Ghigo and Wandersman, 1992a; Liao and McCallus, 1998). The functional significance of those Ca^{2+} binding motifs is still unclear. The same motifs are conserved also in hemolysin of *E. coli*, and metalloproteases of *Pseudomonas aeruginosa* and *S. marcescens*. Its presence in a group of proteins which differ significantly in their biochemical functions, but which employ similar translocation systems for secretion, would suggest a role related to the secretion and the subsequent extracellular (re)folding processes. The deletion analysis demonstrated that the secretion signal is located in the C-terminus downstream of a domain that contains Ca^{2+} binding motifs (in the last 48 residues of hemolysin *E. coli*, in the last 39 residues of PrtB *E. chrysanthemi*, in the last 29 residues of PrtG *E. chrysanthemi*, in the last 80 residues of PrtSM *S. marcescens*). Furthermore, the hybrid proteins carrying the secretion signals fused to different polypeptides showed that they are able to promote the specific secretion of chimeric proteins. But the secretion of high-molecular-weight fusion proteins also required an addition of the Ca^{2+} binding motifs to the C-terminal signal part (Delepelaire and Wandersman, 1990; Stanley *et al.*, 1991; Létoffé and Wandersman, 1992; Ghigo and Wandersman, 1994). Baumann *et al.* (1993) proposed that Ca^{2+} binding region may have a role in the folding of

the molecule after transmembrane translocation and that the structure will be unstable in the absence of Ca^{2+} ions. Such instability could facilitate membrane translocation of the polypeptide in a presumably unfolded form. Presence of Ca^{2+} in the extracellular medium could induce the polypeptide to condense into a well-defined tertiary structure. Ludwig *et al.* (1988) suggested that in the case of *E. coli* hemolysin, the Ca^{2+} binding region might be responsible for receptors binding on erythrocytes.

1.2.2.5. Exoproteases of *Pseudomonas*

The opportunistic pathogen *Pseudomonas aeruginosa* produces a battery of extracellular proteases: elastolytic proteases (LasA protease and elastase), a lysine-specific protease and alkaline protease (AprA). Alkaline protease, encoded by gene *aprA*, is a protein of 50.4 kDa, the amino acid sequence of which is homologous with the PrtB and PrtC proteases of *E. chrysanthemi* (52%) and with the metalloprotease of *Serratia marcescens* (53%). It has conserved domains specific for Ca^{2+} and Zn^{2+} binding. The genes *aprDEF*, necessary for AprA secretion, are functionally and structurally related with *prtDEF* encoded secretion system of *E. chrysanthemi* (Duong *et al.*, 1992). Alkaline protease appears to be involved in the extracellular processing of proteases, such as the LasA protease. It has a fairly broad substrate range and is thought to act synergistically with other proteases. *P. aeruginosa* can be isolated from soil and water and the skin of healthy human beings. Its infections usually involve someone who is immunocompromised. *P. aeruginosa* has been implicated in a variety of nosocomial infections, including infections of burned tissue and colonization of indwelling medical devices, and is the primary infectious agent in the lungs of cystic fibrosis patients (Parsek and Greenberg, 2000).

The induction of fruit and vegetable spoilage by soft-rot strains *Pseudomonas fluorescens* (sometimes referred as *P. marginalis*) is attributed mainly to the production of pectate lyases (Liao, 1989). Addition to the Pels, the strain CY091 produces an extracellular protease, AprX, with an estimated molecular mass 50 kDa. The AprX protease is heat stable and requires Ca^{2+} and Zn^{2+} for activity and/or stability. It has conserved domains specific to Ca^{2+} and Zn^{2+} binding, indicating that AprX is a metalloprotease. The AprX is the primary factor responsible for the spoilage in milk and possibly in other dairy products. The amino acid sequence showed a homology (50 to 60%) with AprA of *P. aeruginosa* and the PrtC, PrtB and PrtA proteases of *E. chrysanthemi*. Like PrtC and AprA, AprX is predicted to be synthesised as a proenzyme with a prosequence consisting of 12 aa, which is assumed to be removed autoproteolytically. The AprX protease is secreted by *aprDEF* transporter genes, which are homologous to the ABC transporter *prtDEF* and *aprDEF*. The production of AprX was not induced by the substrates (skim milk, gelatin), however, it required Ca^{2+} for optimal activity. The requirement of Ca^{2+} for AprX production

by strain CY091 is specific and concentration dependent. It is unclear whether Ca^{2+} is directly involved in the regulation of the AprX or whether it is simply required for stabilising the enzyme after synthesis. The other enzyme, pectate lyase, secreted by strain CY091 also requires the Ca^{2+} (Liao *et al.*, 1993). A two-component global regulator GacA-LemA (Liao *et al.*, 1997; Liao and McCallus, 1998) mediates the production of pectate lyase and AprX protease.

Although the proteases AprA (*P. aeruginosa*), AprX (*P. fluorescenc*) PrtG, PrtB, PrtC, PrtA (*E. chrysanthemi*), respective ABC exporters AprDEF, AprDEF and PrtDEF, share a high homology, the organisation of those gene operons are different. In all cases the protease structural genes and ABC exporter genes are closely linked. In *E. chrysanthemi* B374 the *prtG* together with *inh* gene and secretory genes *prtDEF* form one operon, which is followed by *prtBCA* genes (or *prtABC* genes in the case of *E. chrysanthemi* strain EC16). Each respective protease gene, *prtA*, *prtB* and *prtC* has its own promoter. The *inh* gene and secretory genes *aprDEF* follow the *P. fluorescens* protease gene *aprX* similarly to the *prtG* operon of *E. chrysanthemi*. In *P. aeruginosa* the protease gene *aprA* and *inh* gene are located downstream of the transport genes *aprDEF* (Dahler *et al.*, 1990; Létoffé *et al.*, 1990; Ghigo and Wandersman, 1992a, b; Duong *et al.*, 1992; Liao and McCallus, 1998).

1.2.3. Harpins and exopolysaccharides

Many plant-pathogenic bacteria elicit in nonhost plants a rapid localised response, generally known as the hypersensitive response (HR). A typical HR in tobacco leaves is characterised by a rapid collapse of leaf tissue followed by necrosis (cell death) of the collapsed area. The affected areas are limited to the region infiltrated with bacterial cells. This response makes plants disease resistant (Dangl *et al.*, 1996; Alfano and Collmer, 1996)

Among gram-negative bacteria, *Agrobacterium* spp. and soft-rotting *Erwinia* spp. are unusual in that they do not elicit a typical HR when infiltrated into the leaves of nonhost plants. However, *E. carotovora* subsp. *carotovora* $RsmA^-$ mutants (RsmA = repressor of secondary metabolites) cause typical HR in tobacco leaves (Cui *et al.*, 1996). The *E. carotovora* subsp. *carotovora* strain Ecc71 possesses a gene $hrpN_{Ecc}$, which encodes the homologue of the HR elicitors Harpins. The Harpins lack cysteine and are rich in glycine, they do not have an N-terminal signal, however, they do contain a putative membrane-spanning domain and are secreted by *hrp*-encoded (hypersensitive response and pathogenicity) secretion system (type III secretion). Mukherjee *et al.* (1997) demonstrated that the inability of the wild-type *E. carotovora* subsp. *carotovora* to elicit the HR is due the lack of significant level of $Harpin_{Ecc}$ production.

Extracellular polysaccharides (EPS) are bacterial products causing wilting during infection by several bacterial species including *E. stewartii* and *E. amylovora* (Billing, 1996). EPS can form an organised capsule surrounding the

bacterial cell or be spread as slime into the environment (Coplin and Cook, 1990). The release of EPS in the xylem is believed to cause mechanical blockage in the vascular system of the plant and thus initiate wilting (Agrios, 1997).

1.3. Regulation of the virulence genes

The success of soft-rot pathogens depends on a fine balance between avoiding plant defence reactions while killing the plant cells rapidly. The virulence determinants in genus *Erwinia* are very tightly controlled and their expression is dependent of environmental conditions (temperature, availability of oxygen and nutrition, humidity etc.) and plant hosts or types of plant tissue, which are attacked. In *E. chrysanthemi* differential expression of genes for Pels has been observed in different hosts (Beaulieu *et al.*, 1993) and even in different tissues of the same host (Lojkowska *et al.*, 1993). Some Pel isoforms are produced only during infection *in planta* (Kelemu and Collmer, 1993). Hence, the virulence determinants are regulated by global regulators, which co-ordinately control the expression of all extracellular enzymes and by specific regulators which, in turn, modulate the expression of one enzyme or a group of isoenzymes. Several regulatory systems have been described in *E. carotovora* subsp. *carotovora* and in other soft-rot erwinias.

1.3.1. The Rsm system

In addition to the transcriptional control, the virulence determinants and other processes are also controlled post-transcriptionally in bacteria. The post-transcriptional regulation mediated by the Rsm (repressor of secondary metabolites) system is thought to be the most critical factor in soft-rot causing *Erwinia*.

RsmA is a small RNA binding protein, which acts by reducing the half-life of mRNA species. The RsmA suppresses the production of PCWDEs, Harpin, N- acyl-homoserine lactone (HSL), antibiotics, pigments, extracellular polysaccharides (EPS) and motility in many *Erwinia* spp. by decay of the respective mRNAs. The $RsmA^-$ mutant is hypervirulent. Besides, the $RsmA^-$ mutant produces PCWDEs and macerates plant tissue in an HSL-independent manner. While the production of the Pel, Cel and Prt is still inducible in $RsmA^-$ mutant with celery extract, the Peh production is constitutive in *E. carotovora* subsp. *carotovora* strain Ecc71 (Chatterjee *et al.*, 1995; Cui *et al.*, 1995; Mukherjee *et al.*, 1996).

While the RsmA is a repressor, the *rsmB* regulates PCWDEs, HSL, Harpin, EPS and pigments production, motility and pathogenicity positively. *rsmB*

(previously *aepH*) specifies an untranslated regulatory RNA, which neutralises the effect of RsmA by forming an inactive ribonucleprotein complex. *E. carotovora* subsp. *carotovora* strain Ecc71 produces two *rsmB* RNA species: a primary RNA of 479 bases, which is processed to yield a 259-base RNA, designated *rsmB´* RNA. The *rsmB´* RNA negatively controls RsmA levels by affecting transcription and/or translation of *rsmA* or the turnover of RsmA. Some of *Erwinia* spp. have two type of *rsmB*, others only one type *rsmB*, which is not subject to processing (Liu *et al.*, 1998; Ma *et al.*, 2001; Hyytiäinen *et al.*, 2001).

The RsmC in turn activates RsmA production and represses *rsmB* transcription, hence causing the repression of PCWDEs and $Harpin_{Ecc}$ production and virulence in *E. carotovora* subsp. *carotovora* (Ecc71). The hypothesis is that RsmC interacts with RNA polymerase holoenzyme and this ternary complex modulates transcription. It is possible that RsmC acts directly or indirectly both as a positive regulator of *rsmA* and a negative regulator of *rsmB* transcription. The PCWDEs production by the $RsmC^-$ mutant is partially dependent on the quorum signal HSL (Cui *et al.*, 1999). These results are in accordance with Kõiv and Mäe (2001) hypothesis that *rsmA* expression is modulated also by HSL and this regulation is independent of RsmC.

The *Erwinia* RsmA/*rsmB* system is homologous with CsrA/*csrB* (carbon storage regulator) system of *E. coli* (for review Romeo, 1998).

1.3.2. Quorum sensing

Gene expression in many gram-negative bacteria is regulated through a cell density-dependent process known as quorum sensing. The spectrum of phenotypes regulated in this manner includes the production of exoenzymes in the opportunistic pathogen *Pseudomonas aeruginosa*, the conjugal transfer of Ti plasmids in the plant pathogen *Agrobacterium tumefaciens*, and the production of antibiotics and PCWDEs in *E. carotovora* ssp. (for review Whitehead *et al.*, 2001). The paradigm for this system is the regulation of bioluminescence in the marine bacterium *Photobacterium fischeri* (Kaplan and Greenberg, 1985). Expression of the structural genes (*luxCDABEG*) required for light production is dependent on *luxI*-directed synthesis of diffusible compound N-acyl-homoserine lactone (HSL). Accumulation of the HSL, also referred as the autoinducer (AI), in the growth medium results in complex formation with the HSL receptor/transcriptional regulator of the *lux* genes, LuxR. The LuxR/HSL complex activates expression of the *luxICDABEG* operon, creating a positive feedback loop that results in both enhanced production of HSL and light emission. Hence, *P. fischeri* is able to sense its own cell density and light is produced only when high enough density is reached (for review Meighen, 1991).

In *E. carotovora* subsp. *carotovora* strain SSC3193, the production of PCWDEs is co-ordinately activated by HSL in a population density-dependent manner. The HSL is synthesised by the action of ExpI. The ExpI$^-$ mutants that are defective in HSL production are avirulent, and the production of all types of extracellular enzymes is affected, indicating that HSL is involved in the global control of virulence (Pirhonen *et al.*, 1993). Expression of the virulence genes is only activated when a sufficient amount of the signal molecule has accumulated and the cell density is enough to attack the plant and overcame its defence reactions.

The *expI* gene is linked to the gene encoding the putative regulator of the LuxR-family. The gene *expR*$_{Ecc}$ is transcribed convergently to the *expI* gene and the two open reading frames are partially overlapping. Inactivation of *expR*$_{Ecc}$ caused no decrease in virulence or production of PCWDEs, but increased HSL levels during early logarithmic growth phase, which probably was the reason of slight increase in the maceration capacity of the mutant strain. The ExpR$_{Ecc}$ does not regulate the *expI* transcription and its role is unknown. It was also shown that *expI* is not autoregulated (Andersson *et al.*, 2000).

1.3.3. The KdgR repressor

The KdgR$_{Ecc}$ of *E. carotovora* subsp. *carotovora* (Kdg, 2-keto-3-deoxyglyconate; KdgR, general repressor of genes involved in pectin and galacturonate catabolism), a homologue of the *E. chrysanthemi* repressor KdgR$_{Ech}$ (Reverchon *et al.*, 1989) and *E. coli* repressor KdgR$_{Eco}$, controls negatively Pel, Peh, Cel, Prt and Harpin$_{Ecc}$ production. The KdgR$_{Ecc}^-$ mutant is more virulent than the wild type. Addition, the KdgR$_{Ecc}$ negatively controls the transcription of *rsmB* RNA regulator. KdgR$_{Ecc}$ binds to the operator DNAs of *pel*-1 and *peh-1* genes, also to the three binding sites within the transcriptional unit of *rsmB*. The hypothesis is that KdgR$_{Ecc}$ affects extracellular enzyme productions in two ways: (i) directly, by inhibiting the transcription of exoenzyme genes; and (ii) indirectly, by preventing the production of a global RNA regulator. So, KdgR$_{Ecc}$ prevent the initiation of transcription of *pel-1* and *peh-1*, and affect the elongation of transcription of *rsmB*. In *E. chrysanthemi*, the KdgR$_{Ech}$ has been found to affect the expression of at least 13 operons involved in pectin catabolism and enzyme export via the type II secretion pathway (Hugouvieux-Cotte-Pattat *et al.*, 1996). The negative regulation of Cel, Prt and Harpin$_{Ecc}$ by KdgR has been described only in *E. carotovora* subsp. *carotovora*. There is no knowledge if the promoter region of Prt structural gene contains the KdgR$_{Ecc}$ binding site. It is known that KdgR$_{Ecc}$ binding sites (5'-G/AA/TA/TGAAA[N$_6$]TTTCAG/TG/TA-3') are not present within 475 bp upstream of the translational start site of *hrpN*$_{Ecc}$ (Mukherjee *et al.*, 1997) and within 490 bp upstream of the translational start site of *celV*. It is supposed that the KdgR$_{Ecc}$ effect on Cel, Prt and Harpin$_{Ecc}$ production is due to the regulation of *rsmB* level (Liu *et al.*, 1999). Hyytiäinen

et al. (2001) demonstrated that KdgR also positively regulates the expression of *rsmA*; this supports the hypothesis that KdgR effect is partly due to a modulation in the stability of transcripts.

1.3.4. The HexA repressor

The HexA regulator (Harris *et al.*, 1998; Mukherjee *et al.*, 2000), described in *E. carotovora* subsp. *carotovora* (strains SCRI193 and Ecc71) and subsp. *atroseptica* strain (SCRI1043), is a LysR-type regulator, which negatively controls the production of N-(3-oxohexanoyl)-L-homoserine lactone (OHL), exoenzymes (Pel, Peh, Cel and Prt), $Harpin_{Ecc}$, and motility. HexA shares a significant homology with PecT of *E. chrysanthemi* (80% identity) (Surgey *et al.*, 1996) and LrhA of *E. coli* (64% identity) (Bongaerts *et al.*, 1995). Generally the phenotypes of $HexA^-$ mutants of *E. carotovora* subsp. *carotovora* strains Ecc71 and SCRI193 are similar except for the following: (i) the Peh production was not affected by HexA in SCRI193 but was affected in Ecc71; and (ii) the accumulation of OHL occurs in a growth phase dependent manner in the $HexA^-$ mutant and in the parent strain Ecc71, whereas in the $HexA^-$ mutant of SCRI193, the production of OHL occurred somewhat independently of the growth phase. The *hexA* expression is positively autoregulated but the regulation mechanism is not known. It has been shown that HexA protein binds directly to the *pelC* promoter (Harris *et al.*, 1998).

According to Mukherjee *et al.* (2000), the HexA regulates the production of exoenzymes and $Harpin_{Ecc}$ and consequently the virulence by modulating the levels of at least three global regulators: (i) it negatively controls the levels of the stationary phase sigma factor RpoS required for the expression of many genes in bacteria during this growth phase; (ii) HexA negatively regulates transcription of *ohlI* (*expI* homologue) and, thus, OHL production; and (iii) HexA inhibits the expression of the RNA regulator (*rsmB*) that positively controls PCWDEs and $Harpin_{Ecc}$ production as well as various secondary metabolites.

The first pathway depicts the fact that HexA regulates RpoS stability by SprE and ClpX/ClpP components and that RpoS, in turn affects the production of exoenzymes and $Harpin_{Ecc}$ by positively regulating *rsmA* expression. HexA could positively control *sprE* expression directly or indirectly. The active SprE probably functions as a response regulator to activate ClpX/ClpP proteases, the enzymes specifically responsible for the degradation of RpoS, as proposed in *E. coli* (Pratt and Silhavy, 1996). Andersson *et al.* (1999a) have identified ExpM, a global regulator of *E. carotovora* subsp. *carotovora* strain SCC3193, which shares high homology with SprE and negatively affects the levels of RpoS. They also suggested that a functional *rpoS* gene is needed mainly for survival in a competitive environment and under stress conditions, and not for effective infection of plants (Andersson *et al.*, 1999b).

High levels of OHL are required for activation of the production of exoenzymes (Pirhonen *et al.*, 1993; Jones *et al.*, 1993), an increase in the OHL level in the HexA$^-$ mutant could contribute to their overproduction. It is possible that HexA regulates OHL production by modulating the levels of *ohlI* transcripts via the *rsmB* RNA regulator, which neutralizes the RsmA. In the HexA$^-$ mutant the *rsmB* expression is higher and therefore the *ohlI* transcripts are more stable (Liu *et al.*, 1998). It is supposed that high levels of *rsmB* RNA regulators are most critical in the production of exoproteins, Harpin$_{Ecc}$ and secondary metabolites observed in the HexA$^-$ mutants.

The HexA occurs in soft rotting (*E. carotovora* subsp. *carotovora, E. carotovora* subsp. *atroseptica, E. chrysanthemi, E. betavasculorum*) and also in non-soft-rotting *Erwinia* species (*E. amylovora, E. stewartii* etc.). The multiple copies of *hexA*$_{193}$ affect also various phenotypes of *Serratia marcescens* (Harris *et al.*, 1998; Mukherjee *et al.*, 2000).

1.3.5. The HexY repressor

Mutations located in *hexY* (hyperproduction of exoenzymes) gene of *E. carotovora* subsp. *carotovora* SCRI193 and *atroseptica* SCRI1043 cause strong co-ordinated upregulation of PCWDEs and motility. The HexY$^-$ mutants were hyperflagellated, had reduced growth rates but enhanced maceration capacity on potato tubers as a result of overproduction of exoenzymes and an enhanced swimming speed. For Prt and Cel the overproduction is independent of the presence of PGA but the pectate lyase (*pel*) activity in the mutant is still inducible by PGA addition. This may indicate that the induction of Cel and Prt seen upon addition of PGA is mediated solely by *hexY* derepression, whereas other regulator(s) are required for *pel* induction. The *hexY* gene encodes a 14.4 kDa protein with no known homologues and conserved motifs. The *hexY* transcript has an unusually long (525 bp) 5'untranslated region containing several pairs of perfect/imperfect, direct/indirect repeats, which are potentially able to form secondary structures and may be involved in post-transcriptional regulation. The *hexY* expression is negatively autoregulated and is not greatly affected by PGA addition. It is possible that HexY is a transcriptional regulator, acting either directly or indirectly as a DNA-binding protein or a cofactor for other regulators. It has been shown that HexY modulates the transcription of *pelC* and *celV* genes, but the experiments to bind the HexY to the *pelC* or *celV* promoters were unsuccessful. Differently to the many other regulators, no homologues of the *hexY* in other *Erwinia* spp. were found by Southern analysis (Shih *et al.*, 1999).

1.3.6. The AepA activator

A new type of regulator has been isolated from *E. carotovora* subsp. *carotovora* strain 71. The AepA (activator of extracellular protein production) protein has a putative N-terminal signal sequence and several hydrophobic domains suggesting its membrane localisation. The sequence of *aepA* lacks the helix-turn-helix motif and significant homology with other prokaryotic regulatory genes. The AepA controls the production of Pel, Peh, Cel and Prt: in the absence of functional *aepA*, the levels of enzymatic activities and *pel-1* expression remained low under conditions that would normally induce the expression of respective genes. The *aepA* is itself negatively regulated. In the upstream of *aepA* translational start site are two putative KdgR-binding sites but their significance is not known yet. The *aepA* expression is induced by pectate and celery extract. The AepA regulation mechanism is not known. It is possible that AepA interacts with another regulator protein and the resulting complex then binds to regulatory sequences thus activating the transcription of target genes. Alternatively, AepA may acts as a sensor of environmental signals, transmitting them to other regulatory components. The homologues of Ecc71 *aepA* were found in several *E. carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica* strains (Murata *et al.*, 1991; Liu *et al.*, 1993).

1.3.7. The RexZ activator

Erwinia carotovora subsp. *carotovora* (ATTn10; SCRI193) possess a gene *rexZ* (regulator of exoenzymes) encoding a homologue of the major repressor of pectinolysis (KdgR) in *E. chrysanthemi*. The RexZ$^-$ mutant exhibited reduced level of Pel (fourfold), Cel (86%) and Prt (63%) production compared with the wild type and therefore RexZ, in contrast to the KdgR, is likely to be an activator, the activity of which is not modulated by pectic compounds and HSL. The promoter region of *rexZ* contains consensus binding sites for KdgR and CRP (cyclic AMP receptor protein). Moreover, the cAMP-CRP and KdgR bind strongly to the *rexZ* promoter region. Although the respective sites are partly overlapped, both proteins could occupy their binding sites simultaneously. While the catabolic repression of *rexZ* expression was verified by quantification of the RexZ protein in the presence and absence of glucose, the KdgR role in the expression of *rexZ* is unknown. The immunoblotting experiments indicated that RexZ protein is well conserved among *Erwinia* spp. (Thomson *et al.*, 1999).

1.3.8. The Hor activator

Different *Erwinia* sp strains have regulator protein Hor_{Er} (homologue of rap) which share homology with a highly conserved family of bacterial regulatory proteins controlling diverse physiological processes in plant, human and animal pathogens. The Hor_{Er} together with Rap (regulation of antibiotic and pigment) of *Serratia marcescens*, Hor_{Ye} of *Yersinia* and SlyA of *Salmonella typhimurium* form a highly conserved subgroup of this family. The Hor^- mutant of *E. carotovora* subsp. *carotovora* strain GS101 has reduced Pel (52%), Cel (31%) and Prt (20%) production compared with wild type and it was defective in antibiotic carbapenem production. The reduced exoenzyme phenotype also caused reduced virulence ability (about 50%). The Hor protein lacks an obvious DNA-binding motif, so it is unclear how it might act. Although several *Erwinia* spp. have a *hor* homologues, Thomson *et al.* (1997) could not detect this homologue by Southern analysis in all tested *E. carotovora* subsp. *carotovora* strains, including strain SCC3193.

1.3.9. Two-component regulator systems

Cells must sense and respond to their environment, a process that requires signal transduction across biological membranes. A major mechanism of signal transduction, widespread in bacteria, is the so-called two-component system that has adopted phosphorylation as a means of information transfer. Pathogenesis requires two-component modification of cellular physiology as well. It is unlikely that any pathogen can survive the varied and changing environments of the host without involving at least one two-component pathway. These systems are involved in chemotaxis, sporulation, motility, osmoregulation, nitrogen assimilation, virulence and so on (for reviews Stock *et al.*, 1989; Parkinson and Kofoid, 1992; Falke *et al.*, 1995).

The simplest two component system has two protein components — a sensor or histidine kinase often located in the cytoplasmic membrane, that monitors certain extracellular (environmental) or intracellular parameters; and a cytoplasmic response regulator that mediates an adaptive response, usually a change in gene expression (Figure 1.) (for reviews Parkinson and Kofoid, 1992; Parkinson, 1995). Sensors typically contain a C-terminal transmitter module coupled to an N-terminal input domain. The input domain contains usually two membrane-spanning segments, the domain between them is located in periplasm. The periplasmic segments are structurally unrelated and have diverse receptor functions. Response regulators typically contain an N-terminal receiver module coupled to one or more C-terminal output domains. On detecting a stimulus, the input domain of a sensor modulates the signalling activity of its associated transmitter to communicate with its response regulator partner. The receiver domain of the response regulator detects the incoming sensor signal

and then alters the activity of its associated output domain to trigger the response.

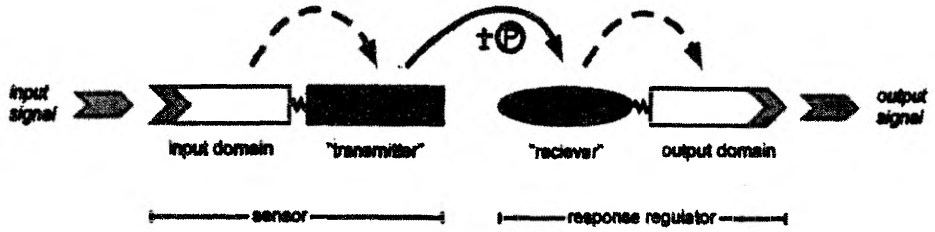

Figure 1. "Two-component" paradigm for sensory signalling via communication modules. Sensory information flows through noncovalent controls exerted by one domain on another (dashed arrows) and through phosphorylation reactions between transmitter and receiver domains (Parkinson, 1995).

The only demonstrated mechanism of transmitter-receiver communication involves phosphorylation and dephosphorylation reactions. Transmitters have an autokinase activity that attaches phosphate groups from ATP to histidine residue. This autophosphorylation reaction is readily reversible. The product phosphohistidine serves as a high-energy intermediate for the subsequent transfer of the phosphate group to an aspartate residue in receiver. Receivers also catalyze hydrolytic loss of their phosphate groups, with half-lives ranging from a few seconds to many minutes. Transmitters can also have an apparent phosphatase activity toward their cognate receivers. In some cases an accessory protein is required for dephosphorylation. In principle, therefore, the regulation of any of the reactions, the autophosphorylation of sensor, the phosphorylation and dephosphorylation of response regulator, can contribute to the control of an adaptive response (Parkinson and Kofoid, 1992; Parkinson, 1995)

Transmitter- and receiver-containing proteins exhibit a variety of module arrangements, and the number of domains can be modified. Example, receivers can occur alone, in tandem, or in combination with different output domains (Parkinson and Kofoid, 1992).

1.3.9.1. Two-component system ExpA-ExpS

In *E. carotovora* subsp. *carotovora* a two component regulatory system ExpA-ExpS has been described. The ExpA$^-$ mutant of *E. carotovora* subsp. *carotovora* SCC3193 has an avirulent phenotype: the production of exoenzymes Pel, Peh and Cel is drastically reduced. However, the residual expression of respective genes is still responsive to the growth phase regulation (Eriksson *et al.*, 1998). The amino acid sequence of ExpA is homologous to the UvrY of *E. coli*

(82.6% identical) (Sharma *et al.*, 1986) and GacA of *Pseudomonas fluorescens* (57.3% identical) (Laville *et al.*, 1992), both are two-component response regulators. Moreover, the UvrY of *E. coli* is a functional analogue of ExpA. The *expA* gene is apparently organised in the same operon with gene homologues to *uvrC* gene of *E. coli* and *P. fluorescens*. The *uvrC* gene encodes one component of the UV induced DNA damage repairs system, however the ExpA is not directly involved in the UV repair. Part of the host defence against invading bacteria could involve production of DNA-damaging substances and then it would be useful to have a gene of the DNA repair system linked to the gene controlling virulence. The similar genomic organisation is also described in *E. coli* (*uvrY*), *P. fluorescens* (*gacA*-global antibiotic and cyanide control), *P. syringae* (*gacA*) and *Salmonella enterica* (*sirA-Salmonella* invasion regulator) (Sharma *et al.*, 1986, Laville *et al.*, 1992; Rich *et al.*, 1994; Johnston *et al.*, 1996; Eriksson *et al.*, 1998).

The ExpS (extracellular enzyme production) of *E. carotovora* subsp. *carotovora* strain SCC3193 has a 92.2% amino acid identity with RpfA. The $ExpS^-$ mutant has reduced virulence but to a lesser extent than $RpfA^-$ mutant (about 70% of wild type level), and in addition to the Cel also the production of Pel and Peh were reduced and delayed. It is not known whether this is a result of different insertion location in respective genes or different enzyme composition (Eriksson *et al.*, 1998). Based on the structural similarity of ExpA and GacA, it is supposed that response regulator ExpA together with sensor kinase ExpS compose a two-component system similarly to the GacA/LemA system in *P. syringae* (Rich *et al.*, 1994; Eriksson *et al.*, 1998).

In *E. carotovora* subsp. *carotovora* strain AH2 a regulator RpfA (regulator of pathogenicity factors) has been described, which belongs to the subclass of two-component regulators, which in a single protein contain both a histidine kinase sensor and a response regulator domain. The $RpfA^-$ mutant was found to be reduced in plant pathogenicity (about 10% of wild type level) and deficient in extracellular protease (Prt) and cellulase (Cel) activity, although it produced normal levels of pectate lyase (Pel) and polygalacturonase (Peh). The deduced amino acid sequence of RpfA shares 37.7% identity and 55.3% similarity with LemA (lesion manifestation) from *Pseudomonas syringae* pv. *syringae* (Hrabak and Willis, 1992), which also regulates the extracellular protease production. Both regulators lack a helix-turn-helix motif in the putative response regulator region that would make a direct recognition of the target promoter region unlikely. In the case of *P. syringae* pv. *syringae* was shown that *gacA* encode the response regulator for LemA (Rich *et al.*, 1994). The RpfA is supposed to be a sensor kinase, which is involved in extracellular protease and cellulase production and the pathogenicity of *E. carotovora* subsp. *carotovora* on potato tubers. (Frederick *et al.*, 1997).

Cui *et al.* (2001) showed that both, GacA (homologue of ExpA) and GacS (homologue of ExpS) of *E. carotovora* subsp. *carotovora* strains 71 and AH2 positively control the expression of PCWDEs and $Harpin_{Ecc}$ via *rsmB*

expression. While the GacA and GacS positively regulate the expression of *rsmB* then *rsmA* expression and RsmA levels were unaffected. These results are contrary to the studies of Hyytiäinen *et al.* (2001). They demonstrated that KdgR mutation partly restored the extracellular enzyme production and virulence in ExpA$^-$ mutant. It appeared that ExpA controls positively the expression of *rsmB* and negatively the expression of *rsmA*. They also noted that *pehA* expression was less affected in ExpA$^-$ mutant compared with *celVI* and *prtW* expression.

1.3.9.2. Two-component system PhoP-PhoQ of *Salmonella enterica*

The PhoP/PhoQ regulon of *Salmonella enterica* serovar Typhimurium is one of the best-characterized two-component systems in enteric bacteria (for review Groisman, 2001), first identified as regulator of expression of a nonspecific acid phosphatase (Kier *et al.*, 1979). The PhoP is a response regulator and PhoQ is a sensor. This system governs virulence, mediates the adaptation to Mg^{2+}-limiting environments and the response to other stress conditions by regulating expression of as much as 1% of the genes in certain gram-negative species, hence it is a major regulator of virulence in *Salmonella*.

The PhoP-PhoQ regulated genes have been termed as PhoP-activated (*pag*) and PhoP-repressed (*prg*) genes. All PhoP-regulated genes mediating *Salmonella* virulence appear to have been acquired by horizontal gene transfer. The hypothesis is that these genes have been put under PhoP-PhoQ control to insure that *Salmonella* expresses its virulence genes at the right time and in the right place. According to this hypothesis, *Salmonella* determines its subcellular location (at least in part) by examining the Mg^{2+} levels in its surroundings via the PhoQ protein: a low Mg^{2+} concentration is an indication of an intracellular environment, whereas a high Mg^{2+} concentration denotes an extracellular environment. In addition to Mg^{2+}, Ca^{2+} and Mn^{2+} can also repress the transcription of PhoP-activated genes *in vitro*, whereas Ni^{2+}, Cu^{2+}, Co^{2+}, and Ba^{2+} have no effect (García Véscovi *et al.*, 1996). It is supposed that concentrations of Ca^{2+} and Mn^{2+} in host tissues are too low to control PhoP-PhoQ *in vivo*. Although transcription of PhoP-regulated genes can be regulated by signals other than Mg^{2+} and Ca^{2+}, these signals are sensed typically by sensors other than PhoQ, activate regulators other than PhoP, and they affect only a subset of genes belonging to the PhoP-PhoQ regulon. For example mild acidic pH promotes transcription of subset PhoP-activated genes, but this activation still takes place in *phoQ* null mutant (García Véscovi *et al.*, 1996; Soncini *et al.*, 1996).

PhoP-PhoQ also mediates its effects indirectly: via activation of other regulatory systems such as the PmrA-PmrB two component system, where PmrA (polymyxin resistance) is the response regulator and PmrB is a sensor kinase that responds to extracytoplasmic ferric ion. Thus, either low Mg^{2+} or

high Fe^{3+} concentrations promote transcription of PhoP-activated PmrA-dependent genes. Transcription of PhoP-activated genes, which are PmrA-independent does not respond to the Fe^{3+} (Gunn and Miller 1996; Wösten *et al.*, 2000). Kox *et al.* (2000) demonstrated that *pmrD* encoded protein mediates the transcriptional induction of PmrA-activated genes during growth in low Mg^{2+} by controlling the activity of the PmrA-PmrB system at a post-transcriptional level. The PmrA-PmrB two component system of *Salmonella* controls resistance to the peptide antibiotic polymyxin B and to other cationic antimicrobial peptides (CAMP) and genes required for growth in low Mg^{2+} solid media (Groisman *et al.*, 1997; Gunn *et al.*, 1998; Kox *et al.*, 2000). Resistance is connected with the transcription activation of genes that results in outer membrane alterations that include modifications of lipid A, a component of lipopolysaccharide (LPS), the major cell surface molecule of Gram-negative bacteria (Guo *et al.*, 1998; Gunn *et al.*, 1998). The PhoP-regulated genes are required for intramacrophage survival (Blanc-Potard and Groisman, 1997) and are probably involved in peptidoglycan remodeling. At least 26 species of outer membrane proteins (OMP) were identified as being regulated by PhoP-PhoQ activation (Guina *et al.*, 2000).

PhoP-PhoQ negatively regulates epithelial invasion by repressing the transcription of genes, which encode type III secretion apparatus proteins (*prgHIJK*) and several of the Ssp (*Salmonella* secreted protein(s)) (Behlau and Miller, 1993; Pegues *et al.*, 1995; Groisman, 2001). The positive regulation of invasion is mediated by regulatory cascade. A two-component response regulator SirA (*Salmonella* invasion regulator) positively regulates the production of HilA (via putative SirB and /or SirC), which positively regulates the expression of *prgHIJK* and Ssp secretion. The amino acid sequence of SirA shows a 96% identity to UvrY (*E. coli*) and 58% identity to GacA (*Pseudomonas fluorescens*) (Johnston *et al.*, 1996), which are homologues of ExpA (*E. carotovora* subsp. *carotovora*) (Eriksson *et al.*, 1998).

The *phoPQ* operon is positively autoregulated by the PhoP and PhoQ proteins. The *phoPQ* operon is transcribed from two promoters, one is active only during growth in low Mg^{2+} and dependent on the PhoP and PhoQ proteins and the weaker one is constitutive (Soncini *et al.*, 1995).

1.4. Plant defence

Plants have the ability to defend themselves against pathogen attacks by activating a series of both local and systemic defence responses. The interaction between a virulent pathogen and a susceptible host plant is said to be compatible, as opposed to the interaction between an avirulent pathogen and a resistant host plant, which is said to be incompatible. These responses, triggered

by a race/cultivar-specific recognition event, are based on the gene-for-gene type of interaction (Flor, 1971), where resistance depends on matching of a dominant avirulence (*avr*) gene from the pathogen and a corresponding resistance gene (R) from the host. In the absence of the R gene or the corresponding *avr* gene, recognition does not occur and the pathogen is able to infect the plant. When R and *avr* gene products recognise each other, a chain of defence reactions is launched, starting with a hypersensitive response (HR) which in turn leads to the activation of systemic acquired resistance (SAR) (Dangl *et al.*, 1996; Alfano and Collmer, 1996). *E. carotovora* subsp. *carotovora* do not elicit a typical HR in nonhost plants.

In addition to such specific resistance, a range of unspecific or abiotic elicitors can activate the plant defence responses. In both cases, the responses appear to involve transcriptional activation of defence genes and *de novo* synthesis of proteins, including enzymes involved in the biosynthesis of phytoalexins, defensins and pathogenesis-related (PR) proteins. In many plant species, local infection by incompatible pathogen leads to the induction of a broad-spectrum disease resistance in uninfected parts of the plant termed systemic acquired resistance (SAR). The PR proteins are a structurally diverse group of proteins widely distributed in plant cells intracellularly and also in the intercellular spaces. Varying types of PR proteins have been isolated from each of several crop plants. The significance of PR proteins lies in the fact that they show strong antipathogenic activity (Agrios, 1997; van Loon and van Strien, 1999)

The pathogenicity of *E. carotovora* subsp. *carotovora* is correlated with its ability to produce and secrete a large arsenal of plant cell wall-degrading enzymes (PCWDEs). These enzymes, in addition to being the main virulence determinants of the pathogen they also trigger plant defence. The treatment of tobacco plants with acellular preparations of cell wall-degrading enzymes (culture filtrate-CF) from *E. carotovora* subsp. *carotovora* induces both local and systemic expression of several genes encoding PR proteins (Palva *et al.*, 1993; Vidal *et al.*, 1997; 1998). Moreover, these enzymes induce both local and systemic resistance responses to this pathogen in tobacco (Palva *et al.*, 1993; Vidal *et al.*, 1998). The pectic enzymes (PehA, PelA and PelD) and at least one cellulase (CelVI) induced basic β-1,3-glucanase (one member of PR-s) gene expression. However, pectic enzymes induced defence gene expression to a much higher extent than the cellulase. The results showed that pectinases but not cellulases elicit systemic induction of β-1,3-glucanase gene expression. However, the best results are achieved with treatment of all enzymes (CF), so the pectic enzymes and cellulases cooperate in the induction of defence gene expression and enhanced resistance to the pathogen (Vidal *et al.*, 1998).

The kinetics of the β-1,3-glucanase mRNA accumulation was markedly different, when plants were treated alone with PehA or with the different Pels. Treatment by Pels resulted in a very rapid accumulation and also a very rapid decrease of β-1,3-glucanase mRNA, while PehA treatment resulted in a slow

reaching of the maximum, which persisted much longer. When plants were inoculated with a wild-type *E. carotovora* subsp. *carotovora*, only a weak and transient response of the plant was observed. These results suggested that the wild-type pathogen is capable of suppressing the plant response caused by the action of its own exoenzymes. The mechanism of this suppression is not known (Palva *et al.*, 1993).

The cell wall fragments released by the action of PCWDEs have been shown to be elicitors for defense reactions of plants (Palva *et al.*, 1993; Vidal *et al.*, 1998; Norman *et al.*, 1999; Norman-Setterblad *et al.*, 2000). However, their limited mobility within plant tissue restricts their function as a signal for systemic responses (Baydoun and Fry, 1985). The synergistic action of plant hormones ethylene and jasmonates signal pathways have a central role in regulating defence gene induction and resistance triggered by PCWDEs from *E. carotovora* subsp. *carotovora*. This induction is enhanced by the presence of salicylic acid (Norman-Setterblad *et al.*, 2000). The allene oxide synthase production in *Arabidopsis thaliana*, an enzyme involved in the biosynthesis of jasmonates, is induced by the PCWDEs of *E. carotovora* subsp. *carotovora* as well as by treatment with short oligogalacturonides (Norman *et al.*, 1999). Jasmonic acid is synthesized from linolenic acid, and activation of a lipase activity is involved in the production of substrates for jasmonic acid synthesis. Spraying *A. thaliana* plants with *Erwinia* culture filtrate or with a mixture of extracellular enzymes of this pathogen, leads to about four-fold induction of a phospholipase activity compared to the background activity in untreated plants. The timing of phospholipase activity upon *Erwinia* CF treatment corresponds with the time course of *Erwinia* CF-induced transcript accumulation of defence genes (Norman *et al.*, 1999; Norman-Setterblad, 2000).

AIMS OF THE STUDY

Each plant species is affected by approximately 100 different kinds of pathogens. A plant pathogen can successfully infect only a limited number of plants, defined as susceptible hosts. Plants are resistant to pathogens either, because they belong to the species, which is resistant to these pathogens, or because they belong to the species, which have developed a resistance mechanism for the pathogen. Hence, pathogens must overcome the plant defence reactions and produce the virulence factors at the right time and in the right place for successful infection. The pathogenicity is a complex interaction between bacteria and plant, where signals are exchanged between them. For a full understanding of the bacterial infection we need to study the interactions between bacteria and plant.

1. The first goal of the present study was to characterize the *pehR* regulatory locus of *Erwinia carotovora* subsp. *carotovora* and its role in the regulation of endopolygalacturonase. Endopolygalacturonase PehA is one of the main virulence factors, the role of which is supposed to be important in the establishment of infection and induction of other extracellular enzymes. The *pehR* locus may be one of the factors indicating to the bacteria whether they are inside or outside a plant.

2. The second goal was to isolate *Erwinia carotovora* subsp. *carotovora* genes, which are induced in the presence of plant extracts and are involved in virulence, especially in the initial phase of infection.

3. The third goal was to study the expression of plant inducible exoprotease of *Erwinia carotovora* subsp. *carotovora*, encoded by gene *prtW*, in the presence of plant extracts in different regulatory backgrounds in order to define which regulatory systems are involved in responses to plant derived signals.

2. PRESENT INVESTIGATION AND DISCUSSION

2.1. *pehR-pehS* regulatory system of *Erwinia carotovora* (paper I)

2.1.1. Endopolygalacturonase regulatory locus *pehR* contains an operon of two genes

The production of plant cell wall degrading enzymes PCWDEs, the main virulence determinants of *E. carotovora* subsp. *carotovora*, is tightly controlled by several global regulative systems, including both positive and negative factors. In addition to global regulators, specific regulators, which can modify the expression of one gene or a subset of genes, are also involved. The endopolygalacturonase is one of the major exoenzymes secreted by *E. carotovora* subsp. *carotovora* SCC3193. The hypothesis is that in soft-rot bacteria the Pehs have an important role in the initial phase of infection and induction of Pels for the spread of the infection (Saarilahti, *et al.*, 1992; He and Collmer, 1990; Huang and Allen, 1997).

Previously mutants of *E. carotovora* subsp. *carotovora* SCC3193 that are impaired in the production of Peh but that have produced and secreted pectate lyases (Pel) and cellulases (Cel) at the wild-type level, have been characterized (Saarilahti *et al.*, 1992). The mutation was shown to affect transcriptional activation of the endopolygalacturonase gene, *pehA*. This suggested that the mutated locus, designated *pehR*, could encode a specific regulator of *pehA*. To characterize the function of this locus, the mutant allele was cloned and sequenced. The structural characterization of *pehR* locus revealed two complete open reading frames (ORFs), apparently present as an operon. The first 684 bp ORF was identified as *pehR* and second 1,455 bp ORF, which overlapped the *pehR* 3' end by two nucleotides, was designated *pehS*. To assess the role of the individual genes, *pehR* and *pehS*, on exoenzyme production and virulence, the *pehS* insertion mutant (SCC4194) was constructed by marker exchange.

2.1.2. Characterization of *pehR-pehS* regulatory system

The results demonstrated that the extracellular enzyme phenotypes of *pehR* and *pehS* mutants were similar. Neither of them produced measurable amounts of PehA but produced wild type level of Pel and Cel. Moreover, both mutants are not able to accumulate detectable amounts of *pehA* mRNA. The contribution of PehR-PehS in virulence was tested on *in vitro* grown tobacco seedlings. The results demonstrated that the *pehR* mutants exhibited reduced virulence at the same level as a *pehA* mutant (SCC4080; Saarilahti *et al.*, 1992), but the *pehS* mutant appeared to be even less virulent. The plasmid, harbouring the wild type

allele of *pehR-pehS* (pREM2000), complemented PehA activity and expression of *pehA* in both, *pehR* and *pehS* mutants. The Peh activity obtained in both backgrounds by the complementing clone was about 35% of the wild-type level. Furthermore, the pREM200 was able to restore the virulence of *pehR* mutant to wild-type level, the virulence of *pehS* was restored only partially. The plasmid pREM201 (*pehR* mutant-*pehS*$^+$), carrying frameshift-mutated *pehR* gene but intact *pehS* gene, could not complement the *pehR* mutant, but restored the Peh$^+$ phenotype to the *pehS* mutant. The plasmid pREM202 (*pehR*$^+$-*pehS* mutant), carrying an intact *pehR* gene and a deletion of the *pehS* gene, failed to restore the Peh$^+$ phenotype to the *pehS* mutant, but unexpectedly complemented the *pehR* mutant, which having transposon insertion should be polar. Taken together, these results indicate that both, *pehR* and *pehS*, are required for *pehA* expression and that the *pehR-pehS* operon controls polygalacturonase production and virulence in *E. carotovora* subsp. *carotovora*.

The transcriptional β-glucuronidase fusion, inserted by marker exchange into the *pehS* gene in *pehR*$^-$ transposon mutant, express β-glucuronidase activity at low level: the colonies colored when incubated (about 24h) on plates containing substrate (X-GlcA) for β-glucuronidase (data not published). The control, where β-glucuronidase gene is inserted opposite to the transcriptional direction, incubated under the same conditions did not give any color. These data indicated that PehS is still produced at low level in *pehR*$^-$ mutant and explain why the plasmid pREM202 (*pehR*$^+$-*pehS* mutant) restored the Peh activity to the *pehR*$^-$ mutant.

2.1.3. PehR and PehS are structurally similar to two-component regulatory system PhoP-PhoQ of *E. coli* and *S. typhimurium*

Searches in databases with the deduced amino acid sequences of PehR and PehS revealed that they are similar to the two-component regulatory system PhoP-PhoQ of *Escherichia coli* (Kasahara *et al.*, 1992) and *Salmonella typhimurium* (*Salmonella enterica* serovar Typhimurium) (Miller *et al.*, 1989). The response regulators, PehR and PhoP, share identity throughout the whole amino acid sequence. The crucial regions for phosphorylation and putative DNA binding region are very well conserved. In sensor kinase PehS the two hydrophobic transmembrane segments are very well conserved but sequence between them, corresponding to the periplasmic loop, is the most variable part. Periplasmic domain is the region, which has the signal-recognizing receptor function (Parkinson, 1995; García Véscovi *et al.*, 1996; 1997). Together, these comparisons demonstrate the close structural similarity of the PehR and PehS polypeptides to the two-component regulator PhoP-PhoQ of *E. coli* and *S. typhimurium* and indicate that *pehR-pehS* encodes a two-component regulator system.

2.1.4. PehR-PehS is functionally related to PhoP-PhoQ two-component regulatory system of *E. coli* and *S. typhimurium*

The high structural similarity of PehR-PehS of *E. carotovora* and PhoP-PhoQ system of *E. coli* and *S. typhimurium* prompted us to test whether they are also functionally related. For this purpose we transferred a plasmid (pDFL2) harboring a *pehA-lacZ* transcriptional fusion to the *E. coli* wild type (with respect to PhoP-PhoQ) strain MC4100 and to its *phoP* mutant FS1002. As a control, the β-galactosidase activity from pDFL2 was measured in the *E. carotovora* subsp. *carotovora* wild-type strain, the *pehR*⁻ and *pehS*⁻ mutant. As expected, β-galactosidase activity was almost abolished in the *pehR*⁻ and *pehS*⁻ mutant compared with the activity in wild-type strain. The β-galactosidase activity in the *E. coli* wild-type strain MC4100 was about 42% of the activity in *E. carotovora* subsp. *carotovora* wild type, showing that *E. coli* can support the expression of *pehA* gene. This activity was abolished in the *phoP*⁻ mutant, indicating that intact *phoP* is required for *pehA* expression in *E. coli*. Previous work (Flego *et al.*, 1997) has indicated that *pehA* expression is calcium dependent and that this regulation is mediated by *pehR*. Similarly, the PhoP-PhoQ system is involved in Mg^{2+} and Ca^{2+} controlled expression of a number of genes in *E. coli* and *S. typhimurium* (García Véscovi *et al.*, 1996). To test whether PhoP-PhoQ system of *E. coli* is also involved in Ca^{2+} control of *pehA* expression, we measured β-galactosidase activity from pDFL2 in both wild-type (MC4100) and *phoP*⁻ mutant (FS1002) strains of *E. coli* in the presence and absence of Ca^{2+}. The results demonstrated that *pehA* expression was repressed in the wild-type strain in the presence of Ca^{2+}, and this regulation is abolished in the *phoP*⁻ mutant, suggesting that PhoP is a functional analogue of PehR also in this respect.

PhoP of *S. typhimurium* was originally discovered as a transcriptional regulator of nonspecific acid phosphatase (NSAP). Both *phoP*⁻ and *phoQ*⁻ mutants lack NSAP activity. To assess whether *pehR-pehS* is able to complement the NSAP-negative phenotype of the *phoP*⁻ and *phoQ*⁻ mutants, we transferred pREM200, pREM201 and pREM202 to the *phoP*⁻ mutant (MS7953s) and *phoQ*⁻ mutant (MS5996s) of *S. typhimurium*. The wild-type strain (ATCC14028s) containing the cloning vector was used as a control. The analysis of phosphatase activity was performed on plates stained for NSAP activity. The plasmids carrying intact *pehR* gene (pREM200 and pREM202) restored the NSAP activity in *phoP* mutant, but the plasmid carrying frameshift-mutated *pehR* and intact *pehS* did not complement the NSAP-negative phenotype. The sensor kinase mutant *phoQ*⁻ was complemented by all three plasmids. This differs from the results in *E. carotovora* subsp. *carotovora*, where the sensor kinase *pehS*⁻ mutant was complemented only by plasmids containing the intact *pehS* gene. These data demonstrate that PehR-PehS is functionally similar to the two-component regulator system PhoP-PhoQ of *E. coli* and *S. typhimurium*. The extensive structural and functional similarity

between PehR-PehS and PhoP-PhoQ suggests that PehR-PehS could control expression of several genes. The PhoP-PhoQ of *Salmonella* regulates as much as 1% of the genes and is a major regulator of virulence (Groisman, 2001). Calcium is physiologically very important and virulence determinants pectate lyases require calcium for their activity. Also the metalloproteases need calcium for their activity and/or stability (Barras *et al.*, 1994; Delepelaire and Wandersman, 1989; Dahler *et al.*, 1990; Liao and McCallus, 1998). It is possible that the lack of extracellular calcium has feedback regulation effect and this may be mediated by PehR-PehS system.

2.1.5. Role of *pehR* and *pehS* in virulence

The observed effect of *pehR*$^-$ or *pehS*$^-$ mutants on virulence on the *in vitro* grown tobacco seedlings could be explained by their effect on PehA production. The reduced virulence phenotypes of *pehA*$^-$, *pehR*$^-$ and *pehS*$^-$ mutants are similar and the phenotypes of *pehA*$^-$ and *pehR*$^-$ mutants could not be distinguished. Interestingly, the *pehS*$^-$ mutant appeared to be even less virulent. Introduction of *pehR-pehS* clone restored the Peh activity for both mutants and this activity was sufficient for *pehR*$^-$ mutant to reach the virulence level of wild-type strain. The virulence of *pehS*$^-$ mutant was only partially restored. This phenomenon and the fact that Peh activity was restored to about 35% from wild-type level, is possible to explain with the effects of allele copy number. To explain why *pehS*$^-$ mutant is less virulent than *pehR*$^-$ mutant we need more information about the functioning of PehR-PehS regulatory system. The two-component systems have very many different levels of regulation. Example, the response regulator may be also phosphorylated without sensor kinase and may or may not need the sensor kinase for dephosphorylation etc. (McCleary *et al.*, 1993; Parkinson, 1995; Stock *et al.*, 1995). The response regulator without phosphorylation may also function and regulate the expression of other genes, which are directly or indirectly involved in virulence.

2.2. Metalloprotease of *Erwinia carotovora* (paper II, III)

2.2.1. Isolation of plant-inducible mutant by mini-Tn5Cm^R:: *gusA* mutagenesis

The bacterial soft rotting of plants is a process involving the complicated plant-microbe interaction events. Both, plant and bacteria exchange signals. The plants recognise the intruders and signal to trigger the defence mechanisms, while bacteria have to express the most suitable complex of exoenzymes for this

particular host plant or plant tissue, and simultaneously prevent the defense reactions of the host. Many of the virulence determinants of bacteria are induced by plant extracts or cell wall degradation products (Kelemu and Collmer, 1993, McMillan *et al.*, 1994; Yang *et al.*, 1992; Mäe *et al.*, 1995). Plant molecules can act as recognition factors for invading bacteria and therefore we attempted to identify host-inducible genes, expecting these genes to be involved in virulence functions. For this purpose we used mutagenesis with a mini-Tn5Cm^R::*gusA* transposon, which carries a promoterless selectable reporter gene, the β-glucuronidase gene (*gusA*). 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.

E. carotovora subsp. *carotovora* SCC3193 was mutagenized by random insertion of the mini- Tn5Cm^R::*gusA* transposon and a pool of insertion mutants were tested parallelly on plates containing substrate for *gusA* (X-GlcA) with or without plant extract added. Mutants containing insertions in plant-inducible genes were identified by blue color on the plates supplemented with X-GlcA in the presence of plant extract. 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 Pel, Peh and Cel, and was selected for further study.

2.2.2. Isolation of the *prtW* gene, sequence analysis and identification of transcriptional signal

The mutated region was cloned and sequenced. The 2.7 kb genomic fragment appeared to contain two complete and one partial ORF. The first 1421 bp ORF, which had original transposon insertion, encoded a polypeptide with a calculated molecular mass of 51 kDa. A search in 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 and Wandersman, 1989; Dahler *et al.*, 1990; Ghigo and Wandersman, 1992a,b). A similarity between these polypeptides was found throughout the entire length of the sequence. A Zn^{2+}-binding domain and four Ca^{2+}-binding sites were well conserved. In contrast, we could not find amino acid sequence similarity between PrtW and other known proteases from *E. carotovora* strains. The extensive similarity of PrtW with proteases from *E. chrysanthemi* indicates a possible common origin of these proteins. The deduced polypeptide sequence of the second ORF, found downstream of *prtW*, showed similarity to the *inh* gene product from *E. chrysanthemi*. Further downstream of these two ORFs, we found the 5`part of the third ORF, which deduced polypeptide sequence has a

high homology to protease secretion protein *prtD* from *E. chrysanthemi* (Letoffe *et al.*, 1990). The transcription of *prtW* is mediated by putative sigma 70 promoter and transcriptional start site was mapped 186 bp upstream from the translational start of *prtW*. The structural similarity to the metalloproteases secreted by *E. chrysanthemi*, suggests that PrtW is an extracellular metalloprotease. This was confirmed by the inhibition of the activity in the presence of EDTA.

Using Northern blot analysis we demonstrated that the protease PrtW structural gene, similarly to the *E. chrysanthemi* proteases PrtA, PrtB and PrtC is transcribed separately from the inhibitor and secretion genes. The PrtG of *E. chrysanthemi* belongs to the same opreon with *inh* and secretion genes. Although the proteases of *E. chrysanthemi* (PrtA-G), *Pseudomonas aeruginosa* (AprA) and *P. fluorescens* (AprX) and respective secretion genes share a high homology, the organization of these gene clusters is different. In respect of genomic organization, the *prtW* is most similar with *prtG* of *E. chrysanthemi* and *aprX* of *P. fluorescens*. (Dahler *et al.*, 1990; Létoffé *et al.*, 1990; Liao and McCallus, 1998; Ghigo and Wandersman, 1992a,b). The differences in the operon structure might result from the rearrangements that have occurred during the transfer of these genes between different strains.

2.2.3. Identification of PrtW product

The ORF encoding the polypeptide of PrtW was amplified from the wild-type strain SCC3193 by PCR. Only the orientation, which corresponds to the transcription direction of the predicted *prtW* ORF from *lac* promoter in the vector of pBluescript, allowed the complementation of the *prtW*⁻ mutant *in trans*. The same fragment, cloned into the expression vector, was used to overproduce the PrtW in *E. coli*. SDS-PAGE analysis of crude protein extract of *E. coli* harboring *prtW*-expression plasmid showed, after IPTG induction, a major overproducing protein with an estimated molecular mass of 50 kDa. This value is consistent with the 51 kDA molecular mass of PrtW protein predicted from the sequence.

2.2.4. Expression of *prtW* gene is induced by plant extracts and is controlled by global regulators

To obtain clues regarding the role of protease in phytopathogenicity, we analysed β-glucuronidase activity expressed by *prtW*::*gusA* fusion in transposon mutant. The β-glucuronidase activity was measured in minimal medium supplemented with celery or potato extract. The maximal effects of plant extracts were obtained with a 16% concentration. Extracts from both, celery stems and potato tubers, 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 in the preparation of extracts. The crude potato extract was sterilised by filtration but the celery extract was autoclaved.

It has been reported earlier that the expression of extracellular enzymes is growth phase dependent, being low at the beginning of bacterial growth but starting to accumulate during the exponential growth phase (Pirhonen, *et al.*, 1993). We found that expression of *prtW*, estimated by β-glucuronidase activity and protease activity of wild-type strain, was also growth-phase dependent. The PrtW activity was induced maximally during the early exponential growth phase and fell back to the basal levels at the beginning of stationary phase. In conclusion, the maximum synthesis of protease in *E. carotovora* subsp. *carotovora* occurs earlier than the synthesis of other extracellular enzymes, which usually reach their maximum in the beginning of early stationary phase (Pirhonen, *et al.*, 1993).

In order to identify the regulatory genes controlling protease synthesis, we measured PrtW activity in different regulatory mutants of *E. carotovora* subsp. *carotovora* strain SCC3193. The results showed that protease production was drastically reduced in *expI*$^-$ mutant, which is defective of the HSL production and also in the two-component response regulatory mutant *expA*$^-$ and sensor kinase mutant *expS*$^-$ (Pirhonen *et al.*, 1991; Eriksson *et al.*, 1998). 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.

2.2.5. *prtW*::*gusA* fusion expression in presence of PGA or potato extract

Expression of transcriptional *prtW*::*gusA* fusions in *E. carotovora* subsp *carotovora* wild-type SCC3193 background was examined during the bacterial growth in minimal medium (M9+glycerol as carbon source), in the presence and absence of PGA or potato extract. The plasmid pROT5 containing the longest promoter region, 1995 bp, yielded indistinguishable β-glucuronidase activity from original *prtW*::*gusA* transposon mutant (SCC6004). Both, pROT5 and SCC6004, showed in non-induced conditions a low level of β-glucuronidase activity, which remained on the same level throughout the whole growth curve. The expression was clearly increased in the presence of PGA or potato extract. However, the potato extract had an inducing effect already in the

beginning of the logarithmic phase and maximum activity occurred in the short period during which the cells were still in the middle of the logarithmic growth phase. The *gusA* maximum expression was transient and was followed by a decline. The signal, to which *prtW* responds in plant extract, is unknown, this may be a plant protein(s), phenolic compounds etc., which are released from damaged plant cells. The inducing effect of PGA was delayed compared with potato extract. It has been shown earlier that proteases are mainly induced by presence of polypeptides. The *E. chrysanthemi* 3937 protease activity toward PelI-2 appeared in the presence of polypeptides or casamino acids, but not when PGA or galacturonate were used as inducers (Shevchick *et al.*, 1998). The activator function of PGA could result from its degradation products, which are produced by enzymatic activity of pectinases. The expression of pectinases is usually activated in the beginning of stationary growth phase (Pirhonen *et al.*, 1991), which could explain the delayed inducing effect of PGA compared with potato extract.

2.2.6. Deletion analysis of the *prtW* promoter region

For further study of *prtW* expression and to define the DNA regions necessary for *prtW* promoter activity, we constructed transcriptional *prtW*::*gus* fusions with different promoter length. The pROT6, 1312 bp deletion derivative of pROT5, showed the same basal activity and the same induction rate in the presence of PGA and potato extract as pROT5, however the magnitude was lower. Hence, the main regulatory elements are located in the region up to 683 bp from translational codon, but for the full expression of *prtW* promoter, the regulatory elements from further upstream region are necessary. Huang *et al.* (1998) have described the occurrence of promoters in *Ralstonia solanacearum* which have distant cis-acting DNA sequences enhancing the expression of different virulence genes. The pROT7, harboring 371 bp of promoter region, showed basal activity, but was completely devoid of induction of β-glucuronidase activity in the presence of PGA or potato extract. These results refer to a hypothetical negative regulatory sequence spanning region from –371 to –245 nt. In the case of pROT8, which includes only 245 bp of promoter region, the β-glucuronidase activity was increased eightfold in comparison with the pROT5 in the absence of the inducer, but was still induced about two- to threefold in the presence of potato extract or PGA.

2.2.7. Role of regulators KdgR and ExpA on expression of *prtW*

The protease PrtW activity appeared to be controlled by two-component regulatory system ExpA-ExpS, which is the one candidate for mediating the plant signal(s) dependent regulation of PCWDEs in *Erwinia*. It has been

demonstrated earlier that PGA effect in the regulation of PCWDEs is mediated through the interaction of its degradation products with the KdgR repressor (Nasser *et al.*, 1991; 1992). To assess the role of ExpA and KdgR regulators in the expression of *prtW* to response different inducers, the β-glucuronidase activity was measured in the presence of PGA and potato extract.

To study the effect of KdgR on protease expression, we analyzed the expression of the transcriptional *prtW*::*gusA* fusions (pROT5, pROT6, pROT7 and pROT8) in *kdgR*$^-$ mutant (SCC510). The pROT7 was completely devoid of promoter activity on any medium tested. The region between –371 and –245 nt does not contain sequences homologous to the KdgR binding site and the deletion of this region restored the promoter activity of *prtW* as it was observed in the case of pROT8. These results indicate that a corresponding region may be involved in the binding of an unknown negative regulatory protein. In non-inducing conditions *prtW*::*gusA* fusions (pROT5, pROT6 and pROT8) showed an approximately two- to fourfold increase in the expression of β-glucuronidase activity in comparison to the wild-type strain. The expression was clearly increased by the presence of potato extract, but addition of PGA did not result in the additional enhancement of activity compared to the wild-type strain. In the case of pROT8 the induction rate of activity decreased due to the raise of uninduced level of β-glucuronidase activity. Our results suggest that KdgR may not be the only regulator mediating the effect of PGA on *prtW* expression. The effect of potato extract as an inducer of *prtW* expression is much more pronounced in *kdgR*$^-$ mutant compared with the inducing effect of PGA. There is a possibility that KdgR negatively regulates the expression of regulator responding to the signal present in the potato extract.

We also studied the expression of the *prtW*::*gusA* fusions (pROT5, pROT6 and pROT8) in the *expA*$^-$ mutant strain (SCC3060) and in the *expA*$^-$ *kdgR*$^-$ double mutant (SCC500). The β-glucuronidase activities in *expA*$^-$ mutant remained unchanged under all conditions tested and on the same levels as in the wild-type strain under non-inducing conditions. In the *expA*$^-$-*kdgR*$^-$ double mutant the promoter activity of pROT5 and pROT6 was restored to about 10–20% of the wild-type level. In the case of pROT8, the expression remained almost to the same level as in the *expA*$^-$ mutant. These results refer to the possibility that the effect of KdgR on the protease expression is mediated through ExpA. The activity from pROT7 was completely devoid in all tested strains. According to the present results it is conceivable that the multiple regulatory network controls the expression of *prtW*, which allows flexibility in selective production of the PrtW suitable for present conditions.

2.2.8. Role of PrtW in phytopathogenicity

The virulence of the protease mutant was tested on two different plant systems: on isolated plant organs (potato tubers) and on *in-vitro*-grown tobacco plants

(*Nicotiana tabacum* cv. Samsun). The maceration ability of *prtW*⁻ mutant was assayed on potato tubers and was compared with the wild type and *expI*⁻ mutant. The results showed that *expI*⁻ mutant had only 10% and *prtW* mutant 60% of the maceration capacity compared to wild-type strain. The virulence ability was tested by monitoring soft-rot symptoms development on the 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 a normal spreading of symptoms associated with the wild-type strain SCC3193. Approximately 98% of plants infected with wild-type showed normal disease symptoms. These results suggest that although PrtW is not essential for pathogenesis, it enhances disease development during infection process. The virulence results are quite similar to the previously represented virulence assays with *pehA*⁻ mutant (Saarilahti *et al.*, 1992). The *pehA*⁻ and its positive regulator *pehR*⁻ mutants, defective in Peh production, have reduced virulence, but in plants that became infected the symptoms developed similarly to wild-type infected plants. The Peh is supposed to be expressed before Pel activity and it is important in the initiation and establishment of infection.

The role of exoproteases in virulence seems to differ between various bacterial species. The *E. chrysanthemi* EC16 defective in production of extracellular proteases were not impaired in virulence on plant tissue (Dahler etal., 1990). However, Shevchik *et al.* (1998) demonstrated that PrtA and PrtC of *E. chrysanthemi* 3937 were very efficient in processing pectate lyase PelI-2 into PelI-3, the latter is able to elicit a necrotic response on tobacco plants. The Prt1 of *E. carotovora* subsp. *carotovora* EC14 is presumed to cleave plant proteins, which are located in the plant cell wall and serve structural and defense functions against microbial attack (Kyöstiö *et al.*, 1991). In contrast, the Prt1 is not structurally related to PrtW and proteases of *E. chrysanthemi*, and it is expressed during the late stationary growth phase. The metalloprotease AprX secreted by soft-rot *Pseudomonas fluorescens* is the primary factor responsible for the spoilage in milk and possibly in dairy products (Liao *et al.*, 1993). The alkaline protease AprA of opportunistic pathogen *P. aeruginosa* is involved in the extracellular processing of proteases involved in pathogenesis (Parsek and Greenberg, 2000).

Our results indicated that PrtW is required in the initial phase of infection, when the suppression of the plant defence reactions is very important. Vidal *et al.* (1997) showed that pathogenesis related (PR) genes, involved in plant defence mechanisms, are locally induced already during the early stages of infection (4–8 h after infection). Moreover, the infection of plants with the wild-type pathogen induced plant response only weakly and transiently, compared with acellular culture filtrates, suggesting that the wild-type bacteria are able to suppress plant response (Palva *et al.*, 1993). The expression of *prtW* in response to plant signals already during the early growth phase and a reduced virulence of *prtW*⁻ mutant suggested the possibility that protease might be necessary for suppressing plant defence response. This effect may be achieved by a degradation of host proteins associated with resistance mechanisms or signalling pathways.

CONCLUSIONS

The present work can be summarized as follows.

1.1. The regulatory locus *pehR* of *Erwinia carotovora* subsp. *carotovora* contains an operon of two genes, *pehR* and *pehS*. Mutations in either *pehR* or *pehS* caused an endopolygalacturonase negative phenotype and resulted in reduced virulence on tobacco seedlings but they still produced wild-type levels of pectate lyases and cellulases.

1.2. According to the complementation experiments both genes, *pehR* and *pehS*, are required for transcriptional activation of the endopolygalacturonase gene, *pehA*, as well as for restoration of virulence.

1.3. The PehR-PehS regulatory system is structurally and functionally similar to the two-component system PhoP-PhoQ of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium. Moreover, the PhoP of *E. coli* mediates the Ca^{2+} repression of PehA production.

2.1. We isolated exoprotease encoding gene *prtW* of *Erwinia carotovora* subsp. *carotovora*, which is strongly induced by celery and potato extract. The expression of protease PrtW reaches its maximum already during early exponential growth phase, which occurs earlier than other extracellular enzymes involved in virulence.

2.2. The *prtW* mutant, defective in protease PrtW production, resulted reduced virulence on tobacco seedlings and reduced maceration ability on potato tubers regardless of the production of wild-type level pectate lyase, endopolygalacturonase and cellulase activity.

3.1. The expression of *prtW* is N-acyl-homoserine lactone dependent (growth phase dependent), regulated by two-component system ExpA-ExpS and repressor KdgR. In addition to the plant extracts, the *prtW* expression is induced in the presence of polygalacturonic acid. The differential responses of *prtW* expression to potato extract and polygalacturonate appeared to be dependent on the KdgR and ExpA regulators. The expression of *prtW* in response to plant signals already during the early growth phase and a reduced virulence of *prtW*$^-$ mutant suggested the possibility that protease might be necessary for suppressing plant defence response.

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SUMMARY IN ESTONIAN

Kahekomponendilise regulaatorsüsteemi PehR-PehS ja ekstratsellulaarse proteaasi PrtW roll *Erwinia carotovora* alamliik *carotovora* virulentsuses

Taimepatogeen *Erwinia carotovora* alamliik *carotovora* kuulub märgmädanikku põhjustavate bakterite hulka. Nimetatud bakter on laialt levinud nii mõõdukas kui ka subtroopilises kliimas ning on võimeline infekteerima väga palju taimeliike, sealhulgas majanduslikult tähtsaid kultuure. Märgmädanikku põhjustavad erwiniad kasutavad taimerakke toitainete allikana, produtseerides ja sekreteerides suurt hulka ensüüme (polügalakturonaasid, pektaatlüaasid, tsellulaasid), mis on võimelised lagundama ja modifitseerima kõrgemate taimede primaarse ja sekundaarse rakukesta ning vahelamellide polüsahhariide — tselluloosi, hemitselluloosi ja pektiini. Rakukesta komponentide lagundamine põhjustab taimerakkude eraldumise ning koe pehmenemise (matseratsiooni), mille tagajärjeks on taimeraku surm vee lekke tõttu protoplastist.

Märgmädanikku põhjustavate erwiniate virulentsuse edukus sõltub kasvust ja levikust peremeestaimes ning massilisest ekstratsellulaarsete ensüümide sünteesist ja sekretsioonist õigel ajal ning õiges kohas, samal ajal vältides taime kaitsereaktsioone. Üks peamisi uurimissuundi patogeneesis on selgitada, milliseid taimseid signaale ja mis süsteemidega tunneb ära patogeen ning kuidas ta väldib taime kaitsereaktsioone või milliste mehhanismidega ta neid maha surub.

Endopolügalakturonaas on üks peamistest ekstratsellulaarsetest ensüümidest, mida *Erwinia carotovora* alamliik *carotovora* sünteesib ja sekreteerib. Endopolügalakturonaasi roll virulentsuses arvatakse olevat infektsiooni alustamine ning teiste ekstratsellulaarste ensüümide sünteesi indutseerimine. Endopolügalakturonaasi süntees on kaltsiumi juuresolekul maha surutud ja ekspressiooniks on vajalik regulaatorlookus *pehR*, mis vahendab ka kaltsiumi mõju. Käesolevas töös me näitasime, et regulaatorlookus *pehR* sisaldab kahest avatud lugemisraamist koosnevat operoni. Vastavad geenid tähistati *pehR* ja *pehS*. Mutatsioonid mõlemas geenis põhjustasid endopolügalakturonaasi negatiivse fenotüübi ja vähenenud infitseerimisvõime *in vitro* kasvatatud tubakaseemikutel. Samas on pektaatlüaaside ja tsellulaaside aktiivsuste tase mõlemas mutandis võrreldav vastavate aktiivsustega metsikul tüvel. Komplementatsiooni katsed näitasid, et mõlemad geenid on vajalikud endopolügalakturonaasi geeni *pehA* ekspressiooniks ja virulentsusvõime taastamiseks. Geenide *pehR* ja *pehS* kodeeritud polüpeptiidide aminohappelised järjestused on väga sarnased *Escherichia coli*'s ja *Salmonella enterica* serotüüp *Typhimurium*'is kirjeldatud kahekomponendilise regulaatorsüsteemi PhoP-PhoQ komponentidele. Geneetilise komplementatsiooni katsetega näitasime, et lisaks struktuurilisele sarnasusele on PehR-PehS ja PhoP-PhoQ süsteemid ka funktsionaalselt sarnased. Veelgi enam, *E. coli* PhoP vahendatud *pehA* ekspressioon on kaltsiumi juuresolekul repres-

seeritud. *Salmonella* PhoP-PhoQ süsteem osaleb 1% geenide regulatsioonil ning seda peetakse peamiseks virulentsuse regulaatoriks. Hüpoteesi kohaselt tunnetab sensorkinaas PhoQ kahevalentsete (Mg^{2+}, Ca^{2+}) ioonide kontsentratsiooni ning selle abil määratleb, millist tüüpi peremeesrakus või -koes bakter asub ning vastavalt sellele modelleerib geenide ekspressiooni.

Kasutades transposoon-mutageneesi kombineeritult promootorita reportergeeniga β-glükouronidaas (*gusA*), isoleerisime *Erwinia carotovora* alamliik *carotovora*`st geeni *prtW*, mille ekspressioon on indutseeritud taimsete ekstraktide juuresolekul. Nimetatud mutandil puudus ekstratsellulaarne proteaasne aktiivsus ning virulentsus tubakaseemikutel ja matseratsioonivõime kartulimugulatel oli vähenenud. Muteeritud lookuse aminohappelise järjestuse analüüs näitas, et *prtW* kodeerib proteaasi, mis on homoloogne *Erwinia chrysanthemi*`s kirjeldatud metalloproteaasidega. Teiste *Erwinia carotovora* tüvedes kirjeldatud proteaaside ja PrtW vahel homoloogia puudus. Peale *prtW* geeni sisaldas kloneeritud genoomi fragment proteaasi inhibiitorit kodeerivat geeni *inh* ja 5` otsa sekretsioonivalku kodeerivast geenist *prtD*. Mõlema geeni kodeeritud valgud on homoloogsed *E. chrysanthemi* vastavate valkudega. Metsiktüve *prtW* alleel introdutseerituna *prtW* mutanti taastas proteaasi aktiivsuse.

prtW ekspressioon sõltub kasvufaasist ning on indutseeritav kartuli- ja selleriekstrakti juuresolekul. Erinevalt teistest ekstratsellulaarsetest ensüümidest, mis saavutavad maksimaalse ekspressiooni varases statsionaarses kasvufaasis, oli *prtW* ekspressioon maksimaalne juba varases eksponentsiaalses kasvufaasis. Nagu teiste ekstratsellulaarsete ensüümide puhul, osalevad ka proteaasi PrtW ekspressiooni regulatsioonil N-atsüül-homoseriin laktoon (kvoorumi tunnetamine), kahekomponendiline süsteem ExpA-ExpS ja repressor KdgR. Peale taimsete ekstraktide indutseerib *prtW* ekspressiooni ka polügalakturoonhappe (PGA) lisamine. Kuid erinevalt kartuliekstraktist, mis indutseeris *prtW* ekspressiooni juba logaritmilise kasvufaasi alguses, oli PGA induktsioon märgatav logaritmilise kasvufaasi keskel. Proteaasi erinevat ekspressiooni PGA ja kartuliekstrakti juuresolekul vahendavad regulaatorid ExpA ja KdgR. Kuna proteaasi ekspresseeritakse vastusena taimsetele signaalidele juba varases kasvufaasis ning proteaasi mutandi virulentsus on vähenenud, siis on alust oletada, et PrtW võib osaleda taime kaitsereaktsioonide mahasurumisel. Proteaas PrtW võib degradeerida valke, mis osalevad taimede kaitsereaktsioonides või signaaliradades, mis on vajalikud resistentsuses osalevate geenide ekspressiooni alustamiseks.

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PUBLICATIONS

I

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A Two-Component Regulatory System, *pehR-pehS*, Controls Endopolygalacturonase Production and Virulence in the Plant Pathogen *Erwinia carotovora* subsp. *carotovora*

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Genes coding for the main virulence determinants of the plant pathogen *Erwinia carotovora* subsp. *carotovora*, the plant cell wall-degrading enzymes, are under the coordinate control of global regulator systems including both positive and negative factors. In addition to this global control, some virulence determinants are subject to specific regulation. We have previously shown that mutations in the *pehR* locus result in reduced virulence and impaired production of one of these enzymes, an endopolygalacturonase (PehA). In contrast, these *pehR* strains produce essentially wild-type levels of other extracellular enzymes including pectate lyases and cellulases. In this work, we characterized the *pehR* locus and showed that the DNA sequence is composed of two genes, designated *pehR* and *pehS*, present in an operon. Mutations in either *pehR* or *pehS* caused a Peh-negative phenotype and resulted in reduced virulence on tobacco seedlings. Complementation experiments indicated that both genes are required for transcriptional activation of the endopolygalacturonase gene, *pehA*, as well as restoration of virulence. Structural characterization of the *pehR-pehS* operon demonstrated that the corresponding polypeptides are highly similar to the two-component transcriptional regulators PhoP-PhoQ of both *Escherichia coli* and *Salmonella typhimurium*. Functional similarity of PehR-PehS with PhoP-PhoQ of *E. coli* and *S. typhimurium* was demonstrated by genetic complementation.

The plant-pathogenic enterobacterium *Erwinia carotovora* subsp. *carotovora* belongs to the soft-rot group of erwinias and has the ability to infect a number of plant species, including several economically important crops. These bacteria secrete a large arsenal of plant cell wall-degrading enzymes, causing maceration of different plant organs and tissues (Pérombelon and Kelman 1980; Collmer and Keen 1986; Barras et al. 1994). The crucial role of these extracellular enzymes in virulence is demonstrated by isolation of avirulent mutants that are defective either in enzyme production (*exp* mutants; Pirhonen et al. 1991, 1993) (*aep* mutants; Murata et al. 1991, 1994; Liu et al. 1993) (*rex* mutants; Jones et al. 1993) or in secretion of the enzymes (*out* mutants) (Andro et al. 1984; Ji et al. 1987; Pirhonen et al. 1991; Thurn and Chatterjee 1985).

The production of these extracellular enzymes is coordinately controlled by a number of global regulators. A central control in this regulatory network is exerted by the cell density-dependent accumulation of a diffusible signal molecule, N-(3-oxohexanoyl)-L-homoserine lactone (OHHL), the *E. carotovora* autoinducer (Jones et al. 1993; Pirhonen et al. 1993). In addition to the *expI* gene directing the synthesis of OHHL, other global regulatory genes required for extracellular enzyme production and virulence have been characterized. These include *expA* and *expS* encoding proteins of the two-component regulator family (Pirhonen et al. 1993; Eriksson et al. 1998), as well as *aepA* and *aepH* (*rsmB*) (Liu et al. 1993; Murata et al. 1994; Liu et al. 1998). However, not only is the synthesis of virulence determinants controlled by the positive factors discussed above, but also a negative global regulator (RsmA) has been described (Chatterjee et al. 1995; Cui et al. 1995; Mukherjee et al. 1996; Liu et al. 1998).

In addition to global regulators, specific regulators, which can modify the expression of one gene or a subset of genes, are also involved in controlling the production of extracellular enzymes. In a related pathogen, *Erwinia chrysanthemi*, differential expression of genes for pectate lyases (Pels) has been observed in different hosts (Beaulieu et al. 1993) and even in different tissues of the same host (Lojkowska et al. 1993). Some Pel isoforms are produced only during infection in planta (Kelemu and Collmer 1993).

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Diana Flego and Reet Marits contributed equally to this paper.

Nucleotide and/or amino acid sequence data have been submitted to the GenBank data base as accession number AF022772.

We have previously characterized mutants of *E. carotovora* subsp. *carotovora* that are impaired in the production of an endopolygalacturonase (PehA) but that produced and secreted Pels and cellulases (Cels) at the wild-type level (Saarilahti et al. 1992). The mutation was shown to affect transcriptional activation of the endopolygalacturonase gene, *pehA*. This suggested that the mutated locus, designated *pehR*, could encode a specific regulator of PehA. Mutations in *pehR*, as well as in the structural gene, *pehA*, exhibited a similar reduction in virulence on in vitro grown tobacco seedlings (Saarilahti et al. 1992). In this work we show that the *pehR* locus contains an operon of two genes, designated *pehR* and *pehS*, both of which are required for *pehA* expression and virulence. We also show that PehR-PehS is both structurally and functionally related to the two-component regulatory system PhoP-PhoQ that controls several pathogenicity properties of *Salmonella typhimurium* and *Escherichia coli*.

RESULTS

The *pehR* locus contains an operon of two genes.

For molecular characterization of the *pehR* locus we first cloned the transposon mutant allele present in strain SCC1935 of *E. carotovora* subsp. *carotovora*. The relevant clone was obtained by selecting for the kanamycin resistance provided by the transposon. Partial sequencing of the clone, carrying a 3.2-kb *SalI-Eco*RI fragment of chromosomal origin, was established and used to design oligonucleotide primers for polymerase chain reaction (PCR) amplification of the wild-type allele. Structural characterization of the *pehR* locus revealed two complete open reading frames (ORFs), apparently present as an operon (Fig. 1; GenBank accession number AF022772). The first 684-bp ORF, identified as *pehR*, is located between nucleotides 253 and 936. This ORF contained the original transposon insertion at 88 nucleotides (nt) downstream from the start codon (Fig. 1). The second 1,455-bp ORF, designated *pehS*, is located between nucleotides 933 and 2387. Both *pehR* and *pehS* coding regions begin with an ATG codon and end with a TGA codon. The start codon of *pehS* overlaps the stop codon of *pehR* by 2 nt, suggesting translational coupling commonly seen with two-component regulators (Govantes et al. 1998). A Shine-Dalgarno sequence, GAGGA, precedes the ATG codon of *pehR* by 7 nt; the *pehS* gene has a sequence AGG 6 nt upstream of the start codon. A putative –10 sequence, GATAAT, is found at nucleotides 125 to 130. The –35 region is replaced by a similar direct hexanucleotide repeat sequence GTTTA(T or C) as originally characterized in the phoP promoter (Soncini et al. 1995). Sequences that may form stem-loop structures and function as transcriptional terminators can be found 134 nt upstream of *pehR* and 82 nt downstream of the termination codon of *pehS*.

Both *pehR* and *pehS* mutants are affected in the production of endopolygalacturonase and exhibit reduced virulence.

We first wanted to assess the role of the individual genes at the *pehR* locus, *pehR* and *pehS*, on virulence and production of extracellular enzymes. To characterize the contribution of the *pehS* gene, we constructed a *pehS* insertion mutant (SCC4194) by marker exchange. Comparison of the extracellular enzyme phenotypes of SCC1935 and SCC4194 showed that they were rather similar. Neither of the mutants produced measurable amounts of polygalacturonase (Fig. 2) but secreted both Pels and Cels close to the wild-type levels (not shown). Northern (RNA) blot analysis of *pehA* expression demonstrated that both mutants failed to accumulate detectable amounts of *pehA* mRNA (Fig. 2).

Virulence tests with in vitro grown tobacco seedlings showed that both mutants exhibit reduced virulence (Table 1). The reduced virulence phenotype of the *pehR* mutant SCC1935 was similar to that of a *pehA* mutant (SCC4080), but the *pehS* mutant SCC4194 appeared to be even less virulent.

For complementation analysis, the genomic fragment harboring the wild-type alleles of both *pehR* and *pehS* was first cloned into pBluescript to obtain plasmid pREM100 (Fig. 1). To facilitate the reliability of the complementation, the 2.5-kb fragment carrying *pehR-pehS* was incorporated into the low copy number vector pMW118. The resulting plasmid, pREM200, and its mutant derivatives, pREM201 (*pehR* mutant-*pehS*$^+$) and pREM202 (*pehR*$^+$-*pehS* mutant), were used for the actual complementation experiments. The mutation introduced into *pehR* (in pREM201) was a 2-bp deletion at the *Nde*I cleavage site resulting in a frameshift. The *pehS* mutant allele (in pREM202) harbored an 859-bp deletion (from *Mlu*I to *Sph*I). Introduction of pREM200 to the mutants restored the Peh$^+$ phenotype to both SCC1935 and SCC4194. In contrast, the mutant plasmids pREM201 and pREM202 only comple-

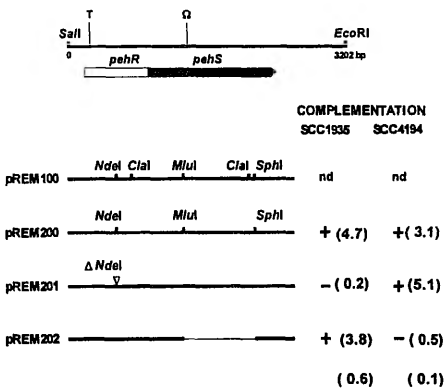


Fig. 1. The organization of the *SalI-Eco*RI chromosomal fragment containing the *pehR-pehS* operon and plasmid constructs derived from this region. Location of transposon insertion in the *pehR* gene of SCC1935 and location of interposon Ω_{Cm} in the *pehS* gene of SCC4194 are indicated by T and Ω, respectively. The *pehR* and *pehS* open reading frames, determined by sequencing, are shown by an open bar and filled bar, respectively. Δ*Nde*I shows position of the 2-bp deletion in the *pehR* gene on pREM201. Restriction enzymes *Mlu*I and *Sph*I were used to delete a 859-bp fragment (thin line) from the *pehS* gene on pREM202. Complementation of the *pehR* mutation in SCC1935 and the *pehS* mutation in SCC4194, determined by Northern (RNA) hybridization and assay of Peh activity, is indicated by + or –, respectively. nd, not determined. The measured Peh activity is shown in parentheses. Nucleotide sequence of the *pehR-pehS* operon of *Erwinia carotovora* subsp. *carotovora* will appear in the GenBank data base under accession number AF022772.

mented the *pehS* and *pehR* mutants, respectively (Fig. 1). The plasmid pREM201, carrying a frameshift-mutated *pehR* gene but an intact *pehS* gene, could not complement the *pehR* mutant SCC1935, but restored the Peh$^+$ phenotype to the *pehS* mutant SCC4194. The plasmid pREM202, carrying an intact *pehR* gene and a deletion of the *pehS* gene, failed to restore the Peh$^+$ phenotype to the *pehS* mutant SCC4194 as expected but complemented the *pehR* mutant SCC1935 (Fig. 1). This was unexpected since the transposon insertion in *pehR* should be polar.

A more extensive complementation analysis by Northern hybridization and enzyme assays showed that pREM200 restores *pehA* expression and Peh activity to both *pehR* (SCC1935) and *pehS* (SCC4194) mutants. The enzyme activity obtained in both backgrounds by the complementing clone was about 35% of the wild-type level (Fig. 2). Furthermore, we assessed the effect of this plasmid on virulence. The results (Table 1) showed that while pREM200 was able to restore the virulence of SCC1935 to wild-type level, the virulence of SCC4194 was only partially restored.

Taken together, these results indicate that both *pehR* and *pehS* are required for *pehA* expression and that the *pehR-pehS* operon controls polygalacturonase production and virulence in *E. carotovora* subsp. *carotovora*.

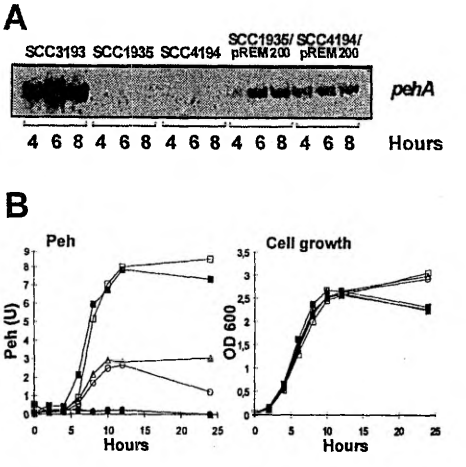


Fig. 2. Effect of the *pehR* and *pehS* mutations on *pehA* expression and production of polygalacturonase (Peh) and complementation of the mutants by a *pehR-pehS* clone. **A**, Northern (RNA) blot analysis of *pehA* expression in *Erwinia carotovora* subsp. *carotovora* wild-type SCC3193, *pehR* mutant SCC1935, *pehS* mutant SCC4194, and mutants carrying the complementing plasmid pREM200 (*pehR*$^+$-*pehS*$^+$). Accumulation of RNA was followed during growth and samples were collected at indicated time points. Each lane contains 5 µg of total RNA. The *pehA* probe was prepared from an 1.2-kb internal *Eco*RV fragment from the *pehA* gene in pHSK24. **B**, Peh activity in the same strains, including the wild-type strain SCC3193 carrying the vector plasmid pMW118. The strains are indicated as follows: SCC3193 (■), SCC3193(pMW118) (□), SCC1935 (▲), SCC1935(pREM200) (△), SCC4194 (●), SCC4194(pREM200) (○). **C**, Corresponding growth curves; strains were grown in L -medium supplemented with appropriate antibiotics at 28°C.

PehR and PehS are structurally similar to the two-component regulators PhoP and PhoQ of *E. coli* and *S. typhimurium*.

The ORFs of *pehR* and *pehS* code for polypeptides of 227 and 484 amino acids with calculated molecular masses of 26 and 55 kDa, respectively. When protein data bases were searched with the deduced amino acid sequences of the PehR and PehS polypeptides, they were found to be similar to the two-component regulatory system PhoP-PhoQ of *E. coli* (Kasahara et al. 1992) and *S. typhimurium* (Miller et al. 1989). The putative response regulators PehR of *E. carotovora* subsp. *carotovora* and PhoP of *E. coli* and *S. typhimurium* share identity throughout the whole amino acid sequence. The amino acid identity of PehR with PhoP of *E. coli* is 76.2% and with PhoP of *S. typhimurium* is 74.6%. The crucial regions for phosphorylation as well as the putative DNA binding region are very well conserved (Fig. 3). The PehS protein is highly similar to the PhoQ proteins of *E. coli* and *S. typhimurium*, which belong to the histidine kinase family. The amino acid identity between PehS and the PhoQ sensor kinase of *E. coli* and *S. typhimurium* is 58.6 and 57.4%, respectively (Fig. 3B). In PehS, the two hydrophobic transmembrane segments are very well conserved (amino acids 16 to 42 and 188 to 216). In contrast, the sequence between them, which by similarity would correspond to the periplasmic loop (Miller et al. 1989) is the part of the polypeptide that differs most. An additional feature that relates PehS to the sensor kinase family is a sequence (amino acids 269 to 277) and a histidine residue (amino acid 275) positioned similarly to those involved in CheA (Hess et al. 1988) and nitrogen regulator I (NR_I) autophosphorylation (Weiss and Magasanik 1988), respectively. Together, these comparisons demonstrate the close structural similarity of the PehR and PehS polypeptides to the two-component regulator PhoP-PhoQ of *E. coli* and *S. typhimurium* and indicate that *pehR-pehS* encodes a two-component regulator system.

In addition to the homology of the *pehR-pehS* and *phoP-phoQ* genes, the flanking regions also appeared similar. The *pehR* gene is preceded by a short ORF encoding the C terminus of adenylosuccinate lyase; the ORF distal to *pehS* encodes the YcfD hypothetical protein. This organization is similar to that of the *phoP-phoQ* flanking regions from *E. coli* K12 as characterized by Kasahara and co-workers (1992).

Table 1. Virulence of *pehR* and *pehS* mutants on axenic tobacco (*Nicotiana tabacum* cv. Samsun) seedlings and complementation of mutants

Strain	Genotype	Virulence: Plants infected[a]	Virulence: %
SCC3193	Wild type	71/72	98
SCC1935	*pehR* mutant	16/72	22
SCC4194	*pehS* mutant	5/72	7
SCC4080	*pehA* mutant	17/72	23
SCC3193 pMW118	Wild type	37/48	77
SCC1935 pMW118	*pehR* mutant	0/24	0
SCC1935 pREM200	*pehR* mutant (*pehR*$^+$-*pehS*$^+$)	18/24	75
SCC4194 pMW118	*pehS* mutant	0/24	0
SCC4194 pREM200	*pehS* mutant (*pehR*$^+$-*pehS*$^+$)	9/24	37

[a] Results show ratio of plants infected to those inoculated.

PehR-PehS is functionally related to PhoP-PhoQ of *E. coli* and *S. typhimurium*.

The structural similarity of PehR-PehS of *E. carotovora* subsp. *carotovora* and PhoP-PhoQ of *E. coli* and *S. typhimurium* prompted us to test whether they were also functionally related. To this end, we transferred a plasmid (pDFL2) harboring a *pehA-lacZ* transcriptional fusion to the *E. coli* wild-type (with respect to PhoP-PhoQ) strain MC4100 and to its *phoP* mutant FS1002 and measured β-galactosidase activity. As a control, the β-galactosidase activity from pDFL2 was measured in the *E. carotovora* subsp. *carotovora* wild-type strain SCC3193, the *pehR* mutant SCC1935, and the *pehS* mutant SCC4194. As expected, β-galactosidase activity was almost abolished in the *pehR* and *pehS* mutants, compared with the activity found in the *E. carotovora* subsp. *carotovora* wild-type SCC3193 (Fig. 4). Interestingly, the β-galactosidase activity in the *E. coli* wild-type strain MC4100 was relatively high, about 42% of the activity in *E. carotovora* subsp. *carotovora* wild type, showing that *E. coli* can support the expression of the *pehA* gene. This activity was abolished in the *phoP* mutant FS1002, indicating that intact *phoP* is required for *pehA* expression in *E. coli*. Our previous work (Flego et al. 1997) has indicated that calcium regulation of *pehA* is mediated by *pehR*. Similarly, the PhoP-PhoQ system is involved in

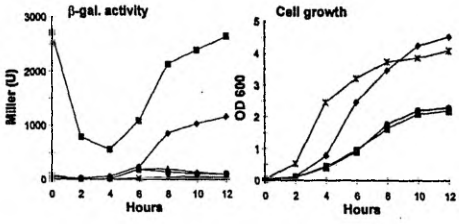
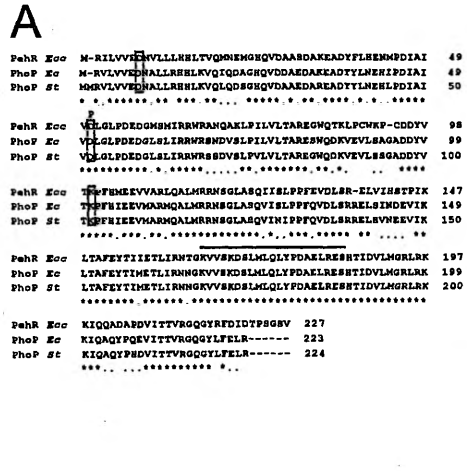


Fig. 4. PhoP-dependent expression of *pehA* in *Escherichia coli*. **A**, β-Galactosidase activity of pDFL2 carrying transcriptional *pehA-lacZ* fusion was determined in *E. coli* strains MC4100 (wild type) and FS1002 (*phoP* mutant), *Erwinia carotovora* subsp. *carotovora* strains SCC3193 (wild type), SCC1935 (*pehR* mutant), and SCC4194 (*pehS* mutant). The strains are indicated as follows: SCC3193(pDFL2) (■), SCC1935(pDFL2) (▲), SCC4194(pDFL2) (●), MC4100(pDFL2) (◆), FS1002(pDFL2) (✱). **B**, Corresponding growth curves; strains were grown in L medium supplemented with ampicillin, *E. coli* strains were grown at 37°C and *E. carotovora* subsp. *carotovora* strains at 28°C.



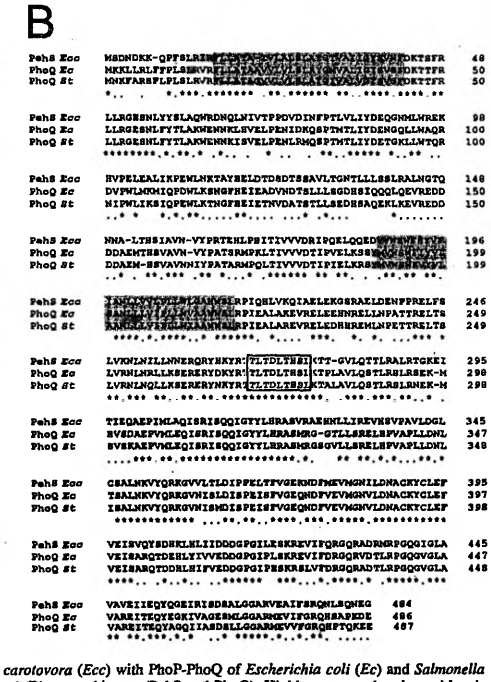


Fig. 3. Amino acid alignment of PehR-PehS of *Erwinia carotovora* subsp. *carotovora* (*Ecc*) with PhoP-PhoQ of *Escherichia coli* (*Ec*) and *Salmonella typhimurium* (*St*). Alignment of (**A**) response regulators (PehR and PhoP) and (**B**) sensor kinases (PehS and PhoQ). Highly conserved amino acid residues in response regulator family are boxed. A potential phosphorylation site is indicated by P; a putative DNA binding domain by overlining. Hydrophobic transmembrane segments in PehS and PhoQ are highlighted by a shaded rectangle; putative autophosporylation site is boxed. * = identical and "." = similar amino acids.

divalent cation (including Mg^{2+} and Ca^{2+}) controlled expression of a number of genes in *E. coli* and *S. typhimurium* (García-Véscovi et al. 1996). To test whether the PhoP-PhoQ system was also involved in Ca^{2+} control of *pehA* expression, we characterized the expression of a *pehA-lacZ* fusion in both wild-type (MC4100) and *phoP* (FS1002) strains of *E. coli*. The results of this analysis (Table 2) demonstrate the presence of Ca^{2+} regulation of *pehA* in *E. coli* and show that this regulation is abolished in the *phoP* mutant, suggesting that PhoP is a functional analogue of PehR also in this respect.

PhoP of *S. typhimurium* was originally discovered as a transcriptional regulator of nonspecific acid phosphatase (NSAP). Both *phoP* and *phoQ* mutants lack NSAP activity. To assess whether *pehR-pehS* is able to complement the NSAP-negative phenotype of the *phoP* and *phoQ* mutants we transferred pREM200 (*pehR*$^+$-*pehS*$^+$), pREM201 (*pehR* mutant-*pehS*$^+$) and pREM202 (*pehR*$^+$-*pehS* mutant) to the *phoP* mutant strain MS7953s and to the *phoQ* mutant strain MS5996s. The *S. typhimurium* wild-type strain ATCC14028s containing the vector pMW118 was used as a control. Strains were grown on phosphorus free M121 medium plates supplemented with 50 mM KH_2PO_4 and stained for NSAP activity (Fig. 5). The *phoP* mutant MS7953s was clearly complemented by plasmids carrying an intact *pehR* gene, pREM200 (*pehR*$^+$-*pehS*$^+$) and pREM202 (*pehR*$^+$-*pehS* mutant). Both plasmids restored the NSAP-positive phenotype to the *phoP* mutant. In contrast, the pREM201 plasmid (*pehR* mutant-*pehS*$^+$) carrying a frameshift mutation in the *pehR* gene could not restore NSAP activity to the *phoP* mutant (Figs. 5 and 1). The *phoQ* sensor kinase mutant MS5996s was complemented by the plasmids carrying the *pehS* wild-type allele, pREM200 and pREM201. Surprisingly, NSAP activity was restored even by pREM202, which contains an intact *pehR* gene but a deletion in the *pehS* gene (Fig. 5). This is in contrast to the results in *E. carotovora* subsp. *carotovora*, where the *pehS* sensor mutant SCC4194 was complemented only by plasmids containing the intact *pehS* gene (Fig. 1).

Table 2. Effect of PhoP on the calcium modulated expression of *pehA* in *Escherichia coli*

	β-Galactosidase activity[a]	
[$CaCl_2$]	MC4100	FS1002 (*phoP*)
0 mM	4,665	76
10 mM	288	73

[a] Both strains harbored a plasmid-borne *pehA-lacZ* transcriptional fusion. The β-galactosidase activity assayed after 8 h of growth is given in Miller units.

It appears that *E. carotovora* subsp. *carotovora* does not produce NSAP activity. Staining of *E. carotovora* subsp. *carotovora* wild type (SCC3193) and both mutants (SCC1935 and SCC4194) for NSAP activity on induced and non-induced conditions did not give any positive reaction. All three strains have regulated phosphatase activity toward p-nitrophenyl phosphate (data not shown).

In conclusion, these data showing PhoP-dependent expression of an *pehR-pehS* controlled target gene *pehA* in *E. coli* and complementation of *phoP* and *phoQ* mutants by *pehR-pehS* in *S. typhimurium* demonstrate that PehR-PehS is functionally similar to the two-component regulator system PhoP-PhoQ in these bacteria.

DISCUSSION

The *E. carotovora* subsp. *carotovora* wild-type strain SCC3193 produces a number of extracellular plant cell wall-

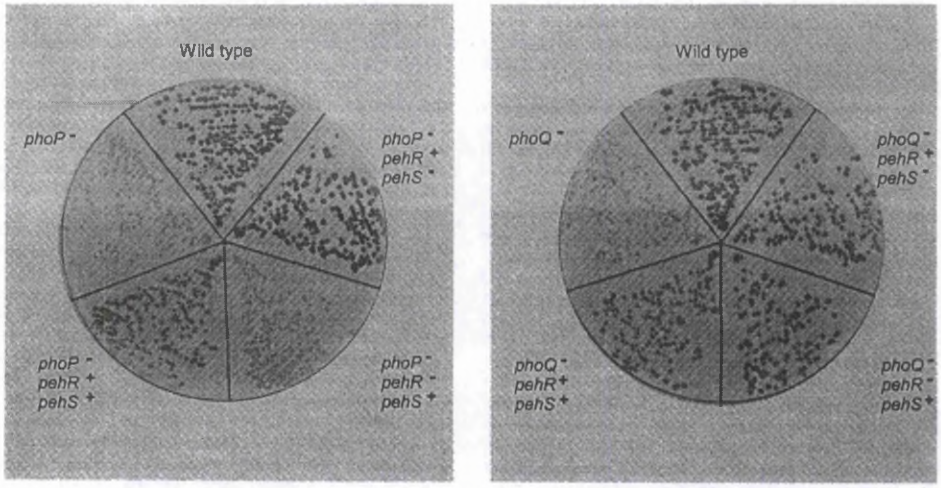


Fig. 5. Complementation of nonspecific acid phosphatase (NSAP) negative phenotype of *phoP* and *phoQ* mutants of *Salmonella typhimurium* by *pehR-pehS* of *Erwinia carotovora* subsp. *carotovora*. The *S. typhimurium* wild-type strain ATCC14028s carrying the vector plasmid pMW118, and its transposon mutant derivatives MS7953s (a *phoP* mutant) and MS5996s (a *phoQ* mutant) carrying pREM200 (*pehR*$^+$-*pehS*$^+$), pREM201 (*pehR* mutant-*pehS*$^+$), pREM202 (*pehR*$^+$-*pehS* mutant), or pMW118 were stained for NSAP activity. Cells producing NSAP are red.

degrading enzymes: an endopolygalacturonase (encoded by *pehA*), at least four Pels (encoded by *pelA* to *pelD*) and two Cels (encoded by *celC, celVI*) (Saarilahti et al. 1990; Heikinheimo et al. 1995; Mäe et al. 1995). All of these virulence genes including *pehA* are controlled by at least two global regulatory systems, quorum sensing (Pirhonen et al. 1993) and the two-component regulator ExpA-ExpS (Eriksson et al. 1998). In addition to these global controls, the *pehA* gene seems to be specifically controlled by the *pehR* locus, in contrast to the other major virulence genes.

In this paper, we describe the molecular characterization of the *pehR* locus. By DNA sequence analysis we show that the locus contains two ORFs. The genes designated *pehR* and *pehS* appear to form an operon with *pehR* being the promoter proximal gene. Analysis of the deduced amino acid sequences suggests that PehR and PehS are members of a two-component regulator system, PehS being the sensor kinase and PehR the response regulator. The evidence for the two proteins belonging to the same regulator system comes partly from the structural organization of corresponding genes as an operon, a usual arrangement for cognate partners of two-component systems, and partly from the phenotypic analysis of *pehR* and *pehS* mutants. Our experiments indicated that both *pehR* and *pehS* are required for *pehA* expression and virulence. Mutations in either of the genes resulted in a Peh-negative phenotype and reduced virulence. The mutations could be complemented by introduction of the respective wild-type alleles on low copy plasmids restoring both *pehA* expression and virulence. Surprisingly, even the plasmid pREM202 (*pehR*$^+$-*pehS* mutant) restored Peh activity to the *pehR* mutant SCC1935, which has a transposon insertion in the *pehR* gene and should be polar on *pehS*. It is possible that the increased amount of PehR is sufficient to activate transcription of *pehA* in the absence of PehS phosphorylation. Alternatively, the increased PehR concentration could stimulate unspecific phosphorylation. A third possibility is that *pehS*, in addition to being co-transcribed with *pehR*, has its own promoter, leading to low level production of PehS in the *pehR* mutant SCC1935.

The observed effect on virulence of *pehR* or *pehS* mutants could be explained by their effect on *PehA* production. Inactivation of *pehA* alone results in reduced virulence (Saarilahti et al. 1992; Flego et al. 1997). The reduced virulence phenotypes of *pehA*, *pehR*, and *pehS* mutants are similar (Table 1) and the phenotypes of *pehA* and *pehR* mutants could not be distinguished. Interestingly, the *pehS* mutant appeared in repeated tests to be even less virulent. Introduction of the *pehR-pehS* clone restored the Peh activity to both mutants. This activity was apparently sufficient for *pehR* mutant to reach the virulence of wild type, while virulence of *pehS* mutant was only partially restored. Currently, we can only speculate about this difference between the phenotypes of *pehR* and *pehS* mutants. Although in many cases a specific sensor kinase-response regulator pair may function as the primary regulator of a particular response, other kinases and regulators could potentially influence each of the components. Evidence for cross-talk in vivo has consistently been seen in mutants that lack a given kinase. It has been also shown that certain low-molecular-weight phosphorylated compounds such as phosphoramidate, carbamyl phosphate, and acetyl phosphate can transfer phosphoryl groups to the response regulator in the absence of a kinase protein. Acetyl phosphate is likely to affect the regulation of many targets, such as cyclic AMP (cAMP), and thus might constitute a global signal (McCleary et al. 1993). The required specificity is obtained by control of the phosphorylation of response regulators and, in some cases, by the highly specific control of the dephosphorylation of response regulators by the regulated phosphatase activities. Based on similarity with OmpR-EnvZ and PhoB-PhoR systems, which belong to the same subfamily as PhoP-PhoQ, we can hypothesize that PehS mediates both phosphorylation of PehR and dephosphorylation of P-PehR (Igo et al. 1989; Parkinson and Kofoid 1992; Gunn et al. 1996). Thus, unspecific phosphorylation of PehR, but not dephosphorylation may occur in the *pehS* mutant, causing the overproduction of phosphorylated PehR. The overproduction of the phosphorylated regulator may modulate expression of genes that are directly or indirectly connected with virulence. It is also possible that PehR is not the only response regulator that is phosphorylated by PehS and that the nonphosphorylated response regulator may also have a functional role. We cannot, however, rule out the possibility that some of the observed effects are due to allele copy number. It has been suggested that low concentration of phosphorylated regulator can cause activation of target gene transcription, while high concentration could cause the opposite reaction—repression of these genes (Stock et al. 1989; Parkinson 1993). This could explain why the *pehR-pehS* wild-type allele cloned into a high copy vector pBluescript could not restore the Peh phenotype of *pehR* and *pehS* mutants (data not shown) and the low copy plasmid pREM200 partly restored Peh activity. However, the results could also suggest that Peh activity is not the only cause for reduced virulence in the mutants.

The regulatory operon *pehR-pehS* encodes proteins that are members of a two-component regulatory family. We here demonstrate both structural and functional similarity of PehR-PehS to the products of the *phoP-phoQ* regulatory operon of *S. typhimurium* and *E. coli*. PhoP was clearly required for expression of *pehA* in *E. coli* and it appeared even to be required for Ca^{2+} controlled repression of *pehA*. Such repression of gene expression was described earlier for *pehA* in *E. carotovora* (Flego et al. 1997) and for a number of PhoP-PhoQ controlled genes in *E. coli* and *S. typhimurium* (García-Véscovi et al. 1996).

It is intriguing that in both *E. coli* and *S. typhimurium* *phoP-phoQ* controls production of more than 40 polypeptides (Miller and Mekalanos 1990; Kasahara et al. 1992). In *S. typhimurium* about 25% of these are involved in virulence. A functional *phoP-phoQ* operon is required for expression of an NSAP, survival within macrophages, resistance to antimicrobial peptides, and acid pH, and is also involved in protein export. The *phoP-phoQ* regulator system can be a repressor for some genes and activator for others. The *phoP-phoQ* modulated genes in *S. typhimurium* are induced under very different conditions and respond in different fashions to the same environmental stimuli such as starvation and rich media, acid pH and neutral pH, and anaerobiosis and aerobiosis. Homologues of *phoP-phoQ* have been found in several bacteria species, including nonpathogens, indicating that the *phoP-phoQ* operon is not ex-

clusively involved in virulence and that it probably plays a central role in the physiology of many Gram-negative bacteria (García-Véscovi et al. 1994; Pegues et al. 1995). Thus, the *phoP-phoQ* regulator system appears to be a central global regulator of many processes in bacteria, including virulence. This is clearly contrasted by the role of *pehR-pehS* in *E. carotovora* subsp. *carotovora*, where we so far have only observed a specific reduction of *pehA* expression in the mutants. In addition, we have observed a slight up-regulation of *pelB* expression in the mutant background (not shown). However, the extensive structural and functional similarity between *pehR-pehS* and *phoP-phoQ* suggests that even *pehR-pehS* could control several genes. Thus, it is feasible to assume that additional genes involved directly or indirectly in virulence could be regulated by *pehR-pehS*.

MATERIALS AND METHODS

Bacterial strains and plasmids.

The *E. coli* strain DH5α (Hanahan 1983) was used for plasmid maintenance and DNA preparations. The *E. carotovora* subsp. *carotovora* wild-type strain SCC3193 (Pirhonen et al. 1988) and its mutant derivatives SCC1935 (*pehR* mutant), SCC4080 (*pehA* mutant) (Saarilahti et al. 1992) have been described. The strain SCC4194 (*pehS* mutant) was isolated in this study (see below). The *E. coli* strain MC4100 (Casadaban 1976) and its *phoP* mutant FS1002 have been described (Groisman et al. 1992). The *S. typhimurium* wild-type strain ATCC 14028s (Fields et al. 1986), its *phoP* mutant MS7953s, and *phoQ* mutant MS5996s have been described (Fields et al. 1989). The pBluescript IISK plasmid was purchased from Stratagene (La Jolla, CA) and the pMW118 from Eurogentec (Seraing, Belgium). The plasmid pHP45Ω-Cm was described by Fellay et al. (1987). The plasmid pHSK24 has been described by Saarilahti et al. (1990) and pHLU102 by Lång and Palva (1993). The plasmids pREM100, pREM200, pREM201, pREM202, and pDFL2 were constructed in this study (see below). Plasmid preparations were done with Qiagen plasmid kits (Qiagen, Hilden, Germany). Transfer of the plasmids was done by standard transformation technique (*E. coli*) or by electroporation (*E. carotovora* subsp. *carotovora*, *S. typhimurium*) with the Bio-Rad gene pulser (Bio-Rad Laboratories, Richmond, CA).

Media, growth conditions, and chemicals.

E. coli and *S. typhimurium* strains were grown in L medium (Miller 1972) at 37°C and *E. carotovora* subsp. *carotovora* strains at 28°C. When necessary, appropriate antibiotics were added to medium at the following concentrations: chloramphenicol (Cm), kanamycin (Km), and tetracycline (Tc) were added to media at 25 μg ml^{-1} and ampicillin (Ap) at 150 μg ml^{-1}. For NSAP assay the *S. typhimurium* strains were grown on phosphorus-free M121 salts medium (Torriani 1966) plates supplemented with KH_2PO_4 (50 mM), thiamin (1 μg ml^{-1}) and glucose (0.4%) as a carbon substrate. *E. carotovora* subsp. *carotovora* were grown on M121 salts medium supplemented with KH_2PO_4 (either 50 mM as excess or 0.1 mM as limiting), casamino acids (0.3%) and glycerol (0.8%) as a carbon source. The restriction enzymes, Klenow fragment, T4 ligase, mung bean nuclease, multiprime DNA labeling kit, radiolabeled dATP, and L-methionine were from Amersham International (Buckinghamshire, UK). The DNA sequencing kit was from United States Biochemicals (Cleveland, OH). Synthetic oligonucleotides were purchased from Kebo Lab (Kebo, Stockholm) and Pharmacia Biotech (Uppsala, Sweden). The p-nitrophenyl phosphate (Sigma 104), α-naphthyl phosphate, tetrazotized o-dianisidine, and polygalacturonic acid (PGA) (P-1879) were from Sigma Chemical (St. Louis, MO). 2-Nitrophenyl-β-D-galactopyranoside was from Merck (Darmstadt, Germany).

Cloning techniques and DNA sequencing.

To clone the mutated *pehR* locus, chromosomal DNA of the mini-kan transposon (Way et al. 1984) mutant SCC1935 was cut by restriction enzymes *Pst*I, *Eco*RI-*Xba*I, or *Sal*I and ligated into pBluescript IISK digested with corresponding enzymes, followed by transformation to the *E. coli* strain DH5α. Mutated allele-containing clones were selected for the kanamycin resistance gene of the transposon. The 3.2-kb *Sal*I-*Eco*RI fragment was further subcloned to smaller fragments suitable for sequencing by the Sanger dideoxy-chain termination method. To sequence both strands, synthetic oligonucleotides designed from the completed sequence were used. For cloning the intact wild-type *pehR-pehS* alleles, the following primers were designed: 5′ GGTACCAACCATTTCCAGCC TG 3′ and 5′ CTCGAGATTGAGCTGATAGAC 3′. PCR was performed with the proofreading Vent DNA polymerase (New England Biolabs, Beverly, MA) with wild-type SCC3193 chromosome as a template. The resulting 2.5-kb fragment was ligated to *Eco*RV-digested pBluescript IIKS vector to create plasmid pREM100. For generation of *pehS* mutant, *Hin*dIII-digested and blunted, 3.5-kb, Cm-specific interposon fragment of the plasmid pHP45-Ω was ligated with *Mlu*I-digested and blunted pREM100. Resulting plasmid pDFL6 was electroporated into the *E. carotovora* subsp. *carotovora* wild-type strain SCC3193. For marker exchange, SCC3193/pDFL6 was grown overnight in L medium with Cm selection and left standing at room temperature over two nights. Recombinants were selected on L-Cm plates and true marker exchange mutants were screened for by loss of the plasmid-encoded ampicillin resistance. The *pehS* mutant, named SCC4194, was purified and controlled by Southern analysis. For complementation the 2.5-kb *Bam*HI and *Hin*dIII fragment carrying *pehR-pehS* in pREM100 was transferred into corresponding sites of the low copy number plasmid pMW118, resulting in pREM200. To generate a mutant of the cloned *pehR*, pREM100 was digested with *Nde*I, the single-strand DNA overhangs removed by mung bean nuclease and the blunt ends ligated. The resulting plasmid pREM101 had lost the *Nde*I cleavage site. Sequencing of both strands showed that two nucleotides T and A (309th and 310th from ATG) were deleted, causing a frameshift in *pehR*. For complementation, the 2.5-kb *Bam*HI-*Hin*dIII fragment of pREM101, carrying the mutant allele of *pehR* but an intact *pehS* gene, was transferred into pMW118, resulting in pREM201. To generate the *pehR*$^+$-*pehS* mutant clone (pREM202), the 859-bp *Mlu*I-*Sph*I fragment was deleted from the *pehS* gene of pREM200. The *pehA-lacZ* transcriptional fusion pDFL2 (Flego et al 1997) was created by first inserting an 1.6-kb *Hin*dIII fragment of pHSK24, carrying the *pehA* promoter region, into the *Hin*dIII site of pBluescript and subsequently moving this promoter as an

*Eco*RI-*Kpn*I fragment to the corresponding sites upstream of the promoterless *lacZ* gene of pHLU102.

Isolation of chromosomal DNA and total RNA, and Southern and Northern hybridization.

Chromosomal DNA of SCC3193 and SCC4194 was isolated as described by Sambrook et al. (1989). Total RNA was isolated by the RNeasy kit (Qiagen) according to the instructions given by the manufacturer. For Northern analysis, 5 μg of total RNA was denatured in formamide and formaldehyde and separated by formaldehyde gel electrophoresis. The blotting onto nylon membranes (Hybond-N; Amersham International) and Southern and Northern hybridization were as described by Sambrook et al. (1989). The gene-specific probes were the 1.2-kb *Eco*RI fragment of pHSK24 for *pehA* and the 1.3-kb *Cla*I fragment of pREM100 for *pehS*. The probe fragments were labeled with ^{32}P-dATP by random priming. The signals were visualized by autoradiography or by phosphorimager and the data were processed with the Image Quant program (Molecular Dynamics, Sunnyvale, CA).

Assay procedures.

The polygalacturonase (Peh) enzyme activity was measured and units defined as described by Pirhonen et al. (1991). The extracellular enzyme indicator plates were according to Pirhonen et al. (1993). The β-galactosidase activity was determined from samples immediately after collection as described by Miller (1972). The NSAP staining procedure was according to Kier et al. (1979). The *S. typhimurium* strains were grown on non-induced conditions to prevent background caused by low activity of acid hexose phosphatase and cyclic phosphodiesterase toward α-naphthyl phosphate as a substrate (Weppelman et al. 1977). Phosphatase activity toward p-nitrophenyl phosphate was determined as described by Brickman and Beckwith (1975).

Assay of bacterial virulence.

The virulence of SCC3193, SCC1935, SCC4194, and SCC4080 was tested on axenic tobacco seedlings (*Nicotiana tabacum* cv. Samsun) grown on MS medium (Murashige and Skoog 1962) supplemented with 2% sucrose on 24-well tissue culture plates. Bacteria were propagated overnight in L medium at 28°C, diluted in 0.9% NaCl, and used to inoculate 3- to 4-week-old axenic tobacco seedlings. Plasmid carrying strains were diluted in 0.9% NaCl supplemented with ampicillin. The leaves were punctured by a needle and 1 μl of bacterial suspension (10^4 CFU) was applied to the damaged site on the leaf. One leaf per well was inoculated. After infection, the plants were incubated at 28°C in high humidity at light. The development of disease symptoms (tissue maceration) was followed up to 72 h. Plants that exhibited clear tissue maceration in 72 h were scored as infected.

ACKNOWLEDGMENTS

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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-Tn*5*Cm^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 *Cla*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.

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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-Tn*5*Cm^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 & Elsden, 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 μg ml^{-1} and with antibiotics at the following concentrations (μg 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 μm 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 $CaCl_2$ 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 *Eco*RI fragment into pUT/mini-Tn*5*Cm^R. The plasmid in which the *gusA* gene was oriented so that it would be transcribed from the outside end of pUT/mini-Tn*5*Cm^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-Tn*5*Cm^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 *Cla*I, ligating this DNA to *Cla*I-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 [^{35}S]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 *Sma*I-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		
Escherichia coli		
DH5α		BRL
S17-1 λ-pir	Km^R Sm^R, *recA thi pro hsdR*$^-$*R*$^+$ RP4:2-Tc^R:Mu:Km^R Tn7 λ-pir	de Lorenzo *et al.* (1990)
Erwinia carotovora subsp. *carotovora*		
SCC3193	Wild-type	Pirhonen *et al.* (1991)
SCC3009	*expS*::Km^R	Pirhonen *et al.* (1991)
SCC3060	*expA*::Km^R	Pirhonen *et al.* (1991)
SCC3065	*expI*::Tn903	Pirhonen *et al.* (1991)
SCC6004	*prtW*::Tn*5-gusA*	This study
Plasmids		
pBluescript SK(+)	Cloning vector (Ap^R)	Stratagene
pUT/mini-Tn5Cm^R		de Lorenzo *et al.* (1990)
pGUS102	Ap^R Tc^R, promoterless 1·8 kb *gusA* gene as an *Eco*RI fragment in pBR322	A. Eriksson, Swedish University of Agricultural Sciences, Uppsala, Sweden
pPRG	Ap^R Cm^R; miniTn5Cm^R::*gusA* (promoterless *gusA* for transcriptional fusions) in pUT/mini-Tn5Cm^R	This study
pROT1	Ap^R, 2·7 kb *Cla*I fragment from SCC6004 containing *prtW* with a miniTn5Cm^R::*gusA* cloned into pBluescript SK(+)	This study
pROT2	Ap^R, 2·7 kb PCR fragment from SCC3193 containing *prtW* 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} $(OD_{600}$ 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 µl ontaining 10^6 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 10^6 c.f.u. bacteria from an overnight culture. The leaves were punctured by needle and 1 µl 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*

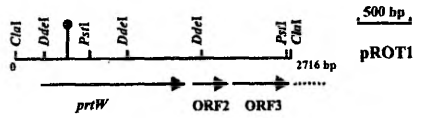

Fig. 1. Localization of the mini-transposon Tn*5*Cm^R::*gusA* in *prtW* mutant SCC6004; the 2·7 kb insert in pBluescript SK(+) is shown. The site of insertion of the transposon leading to the fusion with the protease gene *prtW* is marked with a filled circle. The map also shows the localization and 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 Ca^{2+}- and Zn^{2+}-binding (Baumann *et al.*, 1993). The Zn^{2+}-binding domain was characterized by a well-defined signature HEXXHX-XGXXH (aa 182–192), whereas the Ca^{2+}-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).

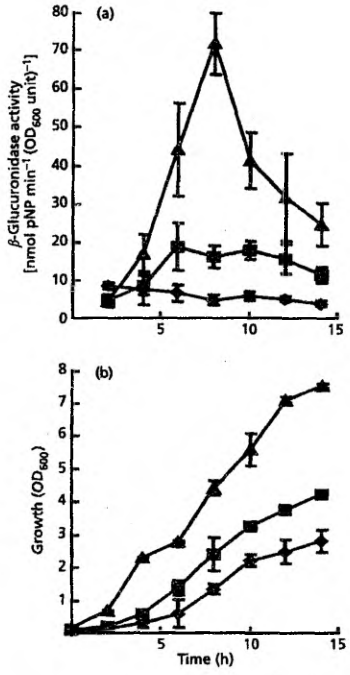

Fig. 2. Expression of the *prtW*::*gusA* fusion in strain SCC6004 under various growth conditions. Cultures were grown in M9 minimal media containing 0·4% glycerol and supplemented as indicated. (a) β-Glucuronidase activity measured as a function of time (h) after inoculation; (b) growth of the bacterial cultures followed by measurement of OD_{600}. ◆, 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 *expI* 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

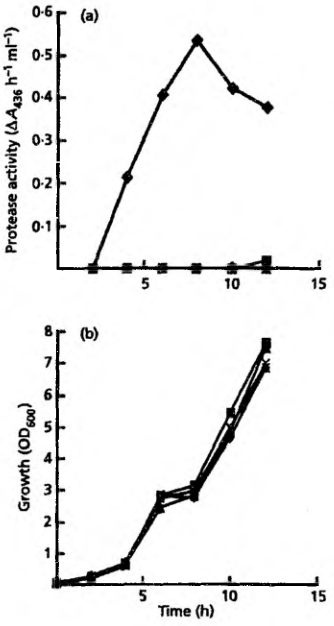

Fig. 3. Growth and protease activity in wild-type and mutant strains. Cultures were grown in M9 minimal media containing 0·4% glycerol and supplemented with 16% potato extract. (a) Protease activity measured as a function of time (h) after inoculation; (b) growth of the bacterial cultures followed by measurement of OD_{600}. ◆, SCC3193 (wild-type); ■, SCC3065 (*expI* 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-Tn*5* insertion into the *prtW* gene (the *prtW* mutant did not show any residual protease activity).

The virulence of the *prtW* mutant and of the *expI* mutant (defective in quorum sensing) was compared with that of the wild-type parent, SCC3193. The results showed that the *expI* 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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III

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

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Erwinia carotovora subsp. *carotovora*, a Gram-negative phytopathogenic bacterium secretes an extracellular metalloprotease PrtW. Our previous results demonstrated that protease activity is necessary for the normal progression of disease symptoms. The present studies revealed that *prtW* gene constitutes an independent transcriptional unit. We demonstrate that introduction of the $prtW^+$ plasmid *in trans* into the $prtW^-$ mutant restored the protease activity in this strain. Gene fusions to the *gusA* reporter were employed to analyze the transcription of *prtW*. The transcription of the *prtW* is dependent on many environmental signals. When the bacteria were grown in the presence of potato extract, the expression of protease gene was markedly higher already in the beginning of the logarithmic growth phase than that apparent when cells were grown in the presence of polygalacturonate. The promoter analysis disclosed that an essential regulatory region reside between 371 and 245 bp 5'of the translational start. As this sequence showed no homology to KdgR box it may be involved in the binding of an unknown negative regulator protein in *Erwinia carotovora* subsp. *carotovora*. The differential responses of *prtW* expression to potato extract and polygalacturonate appeared to be dependent on the KdgR repressor and the response regulator ExpA. According to the presented results it is conceivable that the multiple regulatory network allows flexibility in the expression of *prtW* gene during different stages of infection.

INTRODUCTION

The phytopathogenicity of the plant pathogenic soft-rot enterobacteria of the genus *Erwinia* is correlated with their ability to produce and secrete plant cell wall-degrading enzymes (PCWDE) (Pérombelon & Kelman, 1980; Barras *et al.*, 1994). Many of the plant cell wall-degrading enzymes have been shown also to trigger plant defence responses (probably by releasing cell wall fragments active as elicitors). The mechanism by which extracellular enzymes of *Erwinia carotovora* subspecies *carotovora* (*E. carotovora*) induce defence response 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).

To be able to overcome a defence response and survive in plant tissue, the invading pathogen produce toxins or substances called suppressors that act as pathogenicity factors by suppressing the expression of defence response in the host plant (Basse *et al.*, 1993; Kato *et al.*, 1993, Bender *et al.*, 1999).

As *E. carotovora* is lacking the above mentioned systems for suppression of plant defence, it has to find other measures 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, the *Erwinias* have evolved multiple strategies to sense the environment and to modulate their gene expression both by positive *expA/expS*, *aepA*, *rdgA/rdgB*, *rpfA*, *rpoS*, *hor* and negative *hexA, kdgR* regulators (Liu *et al.*, 1993; Liu *et al.*, 1996; Mukherjee *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 during infection can be achieved also by direct degradation of defence proteins (or proteins involved in signal transduction) in the host plant (Heilbronn *et al.*, 1995). Plant response trigged by *E. carotovora* showed the transcriptional activation of several defence genes already 4–6 hours after infection (Vidal *et al.*, 1997). The expression of the protease *prtW* gene during the early growth phase and the observation that the *prtW* mutant exhibited reduced virulence on potato tubers suggests the possibility that protease might be necessary for the supression of plant defence respons (Marits *et al.*, 1999).

We report here 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 analyzed the expression of each fusion in the wild type strain SCC3193 in response to potato extract and polygalactu-

ronate (PGA) to verify the different effect of these inducers on gene expression. We also tested expression of each fusion in *kdgR*⁻ mutant strain SCC510, *expA*⁻ mutant strain SCC3060 and in the *expA*⁻/*kdgR*⁻ double mutant strain SCC500 to determine if these regulators are modulating *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. 2B. *E. carotovora* was grown at 28°C and *E. coli* was grown at 37°C in L broth (Miller, 1972) or in M9 minimal medium (Sambrook *et al.*, 1982) supplemented with 0.4% glycerol, and trace elements (Bauchop & Elsden, 1960). When required, media were supplemented with 0.4% PGA, 16% potato extract and with antibiotics at the following concentrations (μg ml^{-1}): ampicillin (Ap) 150, kanamycin (Km) 25 and chloramphenicol (Cm) 25. 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 as described in Sambrook *et al.* (1982). Plasmid preparations and isolation of restriction fragments from agarose gels were performed with QIAprep Spin Miniprep Kit and QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). *E. coli* cells were transformed by the $CaCl_2$ method (Sambrook *et al.*, 1982). Plasmids were introduced into *E. carotovora* cells by electroporation (Ausubel *et al.*, 1987; Py *et al.*, 1991).

The DNA was sequenced by the dideoxy method of Sanger *et al.* (1977). Sequencing kit was from USB (United States Biochemicals Corp., Cleveland, OH).

For the PrtW-6His overexpression plasmid, the coding region of *prtW* was amplified by PCR with primers Qia1 (5'-GAGAAGGATCCATGGCTTTACGAGATGACG-3') and Qia2 (5'-TCCTCGTCGACTCACACGATAAAATCGGTT-3'). 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, GmbH, Germany).

RNA isolation and Northern blot analysis. Total RNA was isolated with RNA isolation kit RNeasy (Qiagen, Hilden, Germany). Ten μg of total RNA was denaturated in formamide, separated by electrophoresis through formaldehyde agarose gels and blotted to nylon filters (Sambrook *et al.*, 1982). As a

specific probes the fragment of *prtW* was amplified from the wild type strain SCC3193 by PCR using primers: Qia1 and Qia2. The probe was labelled by using the multiprime DNA labeling kit DecaLabel™ DNA labelling Kit from Fermentas (MBI Fermentas, Lithuania). Prehybridization (1h at 65°C) and hybridization (12h at 65°C) were performed in prehybridization buffer (6x SSC, 2x Denhardt's, 0.1% SDS, 100 μg/ml denatured salmon-sperm DNA). After hybridization, membranes were washed twice for 20 min at 65°C in 2x SSC, 0.5% SDS and then for 30 min at 65°C in 0.5x SSC, 0.5% SDS and were finally examined by autoradiography with X-ray film.

Primer extension. Primer extension assay was performed according to the manufacturer's instructions (MBI Fermentas, Lithuania) with primer PROM1reverse (5´-GTCTCTTGGCGGGATA-3`) and 10 μg RNA. The plasmid pROT4 primed with the same primer 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 (pNP), was detected at 405 nm and β-glucuronidase specific activities were expressed as in nmol p-nitrophenol liberated per minute per OD_{600} unit.

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 carrying pRT1 was grown at 37° C in LB medium containing Km and Amp until the culture reached an OD_{600} value of 0.4. 100 μl cell suspensions were withdrawn 4 h after incubation with 1.0 mM. IPTG (isopropyl-β-D-thiogalactopyranoside) and without IPTG. The cells were collected by centrifugation and solubilized in SDS sample buffer to yield a preparation of total cellular proteins. The samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis according to Laemmli (1970) and visualized by Comassie blue staining.

RESULTS

Identification of PrtW product

The minimal DNA fragment containing *prtW* was amplified from the wild-type strain SCC3193 by PCR (praimers Qia1 and Qia2) and cloned into the *Bam*HI-*Sal*I site of pBluescript(+) vector, yielding pROT3 and pROT3A, respectively in order to produce both orientations with respect to the *lac* promoter. Only the orientation in pROT3, corresponding to the transcription direction of the predicted *prtW* ORF, allowed the complementation of the *prtW* mutant SCC6004 *in trans* (not shown).

By cloning the *Bam*HI-*Sal*I restriction fragment containing the *prtW* gene into the compatible sites of a pQE30 vector, the protease PrtW could be overproduced in *E. coli* M15. SDS-PAGE analysis of crude protein extracts of the *E. coli* M15 (pRT1) strain showed, after IPTG induction, a major over-producing protein with an estimated molecular mass of 50 kDa (Fig. 1). This value is consistent with the 51 kDa molecular mass of the protein PrtW predicted from the 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. Extension terminated sharply at A base located 186 bp upstream from the translational start of *prtW*, no other significant start sites were detected. Inspection of the DNA sequence of this *prtW* region disclosed the presence of a possible sigma 70 promoter with a –10 box 10 bp and a –35 box 33 bp upstream of the transcriptional strat site (Fig. 2A).

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

Deletions in the *prtW* promoter region

To define 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 absence of PGA or potato extract using the transcriptional *prtW::gusA* fusions.

To determine the 5' extent of the *prtW* promoter a 2038 bp *Kpn*I-*Pae*I fragment, which had 3' termini at the 43 bp within the *prtW* coding region and 5' termini 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 cloning vector pMW119::*gusA*, yielding pROT5.

In non-inducing conditions, pROT5 showed low level of β-glucuronidase activity, which remained to the same level throughout the whole growth curve (not shown). The 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 β-glucuronidase activity was markedly higher in the beginning of logarithmic growth phase than that apparent when 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 middle of the logarithmic growth phase. The *gusA* maximum expression 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 β-glucuronidase expression in the presence of potato extract and started to increase only after six hours of growth. The plasmid pROT5 yielded indistinguishable β-glucuronidase activity data from SCC6004 under all conditions tested (Fig. 3). These results indicate that no essential promoter elements reside 3' to the *Pae*I site and 5' to the *Kpn*I site.

As the rate of induction of *prtW* promoter with the potato extract or PGA is maximal after 8 hours of growth the expression of different promoter constructs was observed at this time point. In the first, the enzyme *Hin*dIII, which has a unique site in the *prtW* promoter region located at 683 bp upstream from the first codon (Fig. 2) was employed, and the region upstream of this site was deleted from pROT5. The new construct was designated as pROT6. *E. carotovora* SCC3193 cells carrying the pROT6 showed the same basal level of β-glucuronidase activity when growing under non inducing conditions as pROT5 (Fig. 4A). The induction rates of β-glucuronidase activity in the presence of PGA and 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* gene was cloned from pROT5 by PCR using primers PROM5 (5`-ATGTGAGCTCGTAGCGCGGTTT-3`) and M13/pUC Reverse Primer (–24) into the low copy cloning vector pMW119 and the corresponding plasmid was designated as pROT7. The basal level of β-glucuronidase activity was low, but comparable to the pROT5, however the pROT7 was completely devoid of induction of β-glucuronidase activity even when cells were grown in the presence of potato extract or PGA (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 *Cla*I site located at 245 bp upstream from the first codon. The 2700 bp *Cla*I fragment together with miniTn5Cm^R::*gusA* from pROT1 was cloned into the *Cla*I site of low copy cloning vector pACYC184 to produce plasmid pROT8. The β-glucuronidase activity was measured in the SCC3193 cells under the same conditions as with pROT5 and pROT6. In the case of pROT8, β-glucuronidase expression was increased eightfold relative to the 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* fusions in various regulatory backgrounds

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

To analyze the effect of KdgR on protease expression, we analyzed the expression of the transcriptional *prtW::gusA* fusions (pROT5, pROT6 and pROT8) in *kdgR*$^-$ mutant. In non-inducing conditions *prtW::gusA* transcriptional fusions showed 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, 6 and 8 clearly increased when cells were grown in the presence of potato extract. The addition of PGA to the medium, however, did not result in the 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 uninduced level of β-glucuronidase activity (Fig. 4B). To determine whether the promoter activity in pROT7 was affected by KdgR the pROT7 was introduced into SCC510. The pROT7 was completely devoid of promoter activity on any medium tested (not shown). These results indicate that expression of the *prtW::gusA* fusion in pROT7 appeared to be unaffected by the KdgR.

We also studied the expression of the transcriptional *prtW::gusA* fusions (pROT5, pROT6 and PROT8) in the *expA*$^-$ mutant strain SCC3060. When pROT5 and pROT6 were introduced into SCC3060 the 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 uninduced activity in the wild-type strain (Fig. 4C).

When pROT5 and pROT6 were introduced into *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 SCC3193 (Fig. 4A, D). When pROT8 was introduced into *expA*$^-$ *kdgR*$^-$ double mutant SCC500 the β-glucuronidase activities remained to the same levels in both the presence and the absence of PGA. However, when media with potato extract was used, the relative upregulation of pROT5, pROT6 and pROT8 activity was even greater than in the presence of PGA (Fig. 4D).

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

DISCUSSION

In this study, we have explored some aspects of the transcriptional regulation of the *E. carotovora* protease *prtW* gene. We analyzed both the effect of PGA and 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

Several line of evidence, the Northern analysis and the complementation of protease negative phenotype in the strain SCC6004 by the cloned *prtW* wild-type allele, established that the protease gene is transcribed separately from the inhibitor and secretion genes. In spite of extensive similarity of PrtW with proteases from *Erwinia chrysanthemi* the operon structure of protease, inhibitor and secretions genes in these strains 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 *prtW::gusA* fusions

Genes that encode the PCWDEs are often subject to coordinate regulation, and these regulatory systems are able to respond to various environmental signals that may be encountered during the cycle of infection (Liu *et al.*, 1993; Eriksson *et al.*, 1998; Harris *et al.*, 1998; Liu *et al.*, 1999). The most important signals originate from the infected plant tissues, as this is the main environment for the pathogen during plant microbe interaction. To mimic *in planta* conditions low nutritient media (M9+glycerol) was used.

The expression of protease already during 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 protease could facilitate the establishment of successful infection and makes the protease a possible candidate for the repression of plant defence. The signal to which *prtW* responds is, for the moment, unknown but as the induction occurred already after four hours after infection this factor may be a plant protein(s), phenolic compounds etc., which are released from the damaged plant cells. We also observed the activator effect for PGA on *prtW* expression. The activator function of PGA on *prtW* transcription could result from its degradation products. These degradation products are the result of enzymatic activity of different pectinolytic enzymes which expression is usually activated in the beginning of stationary growth phase (Pirhonen *et al.*, 1991; Eriksson *et al.*, 1998). This explains our results, which showed that expression of *prtW* fusions reached to their maximum in the presence of PGA only in the beginning of stationary phase (Fig. 3).

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

The role of regulators KdgR and ExpA on the expression of *prtW*

The complex regulatory network controlling production of virulence factors has been the subject of intensive studies in many Gram-negative pathogens (Liu *et al.*, 1993; Liu *et al.*, 1996; Frederick *et al.*, 1997; Thompson *et al.*, 1997; Harris *et al.*, 1998). The data presented by Hyytiainen *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 KdgR mutant shows increased *prtW* promoter activity in the absence of PGA, we could still observe the inducing effect of PGA in the *kdgR*$^-$ mutant (Fig. 4B). This may indicate that solely KdgR does not mediate the induction of protease upon addition of PGA, whereas 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 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 to the same levels as in the wild-type strain under non-inducing conditions (Fig. 4A, C). In the *expA*$^-$ *kdgR*$^-$

double mutant the promoter activity of pROT5, pROT6 and pROT8 was similar or slightly increased when compared to those in the *expA*$^-$ mutant (Fig. 4A, D). Similarly, Hyytiäinen *et al.* (2001) showed that under the uninduced 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 uninduced as well as under induced growth conditions.

Deletion analysis identified that an essential regulatory element 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 –371 nt was completely devoid of promoter activity in the wild-type as well as in the *kdgR*$^-$ mutant (not shown). Furthermore, we searched for potential KdgR sites between the 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 it 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 the protease expression to the physiological inducers, such as potato extract and PGA, probably allow flexibility in selectively expressing *prtW* gene. The further studies of different regulatory systems, which must guarantee the coordinate expression of protease, may shed some light on complex regulation of this virulence factor.

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Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Bacterial strains		
Escherichia coli		
DH5α		BRL
M15	Km^R, pREP4	Qiagen
Erwinia carotovora subsp. *carotovora*		
SCC3193	Wild-type	Pirhonen *et al.* (1991)
SCC3060	*expA*::Km^R	Pirhonen *et al.* (1991)
SCC6004	*prtW*::Tn5-*gusA*	Marits *et al.* (1999)
SCC510	*kdgR*::Cm^R	Hyytiäinen *et al.* (2001)
SCC500	*expA*::Km^R; *kdgR*::Cm^R	Hyytiäinen *et al.* (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 *gusA* gene as an *Eco*RI fragment in pBR322	A. Eriksson, Swedish University of Agricultural Sciences, Uppsala, Sweden
pACYC184	Ap^R , Km^R, Cloning vector	New England Biolabs
pMW119::*gusA*	Ap^R, 1.8 kb *Eco*RI fragment containing *gusA* gene from pGUS102 cloned into *Hin*dIII site of pMW119	This study
pROT1	Ap^R, 2.7 kb *Cla*I fragment from SCC6004 containing *prtW* with a miniTn5Cm^R::*gusA* cloned into pBluescript(+)	Marits *et al.* (1999)
pROT3	Ap^R, *placZ::prtW* PCR product of the ORF of *prtW* in pBluescript(+)	This study
pROT3A	Ap^R, Same as pROT3, but opposite insert orientation	This study
pROT4	Ap^R, pBluescript with the 2038 bp *Kpn*I-*Pae*I fragment containing the *prtW* regulatory region and 43 bp of the ORF from SCC3193 inserted into the *Kpn*I-*Pae*I sites	This study

pROT5	Ap^R, Same insert as pROT4, but cloned into the *Sma*I site of pMW118*::gusA*	This study
pROT6	Ap^R, 1312 bp *Kpn*I-*Hin*dIII fragment deleted from pROT5	This study
pROT7	Ap^R, PCR product of pROT5 using primers PROM5 and M13/pUC Reverse Primer (–24) in *Sma*I site of pMW119	This study
pROT8	Ap^R, Cm^R, Same insert as pROT1, but cloned into *Cla*I site of pACYC184	This study
pRT1	Ap^R, *prtW* PCR product of the ORF of *prtW* in expression vector pQE30	This study

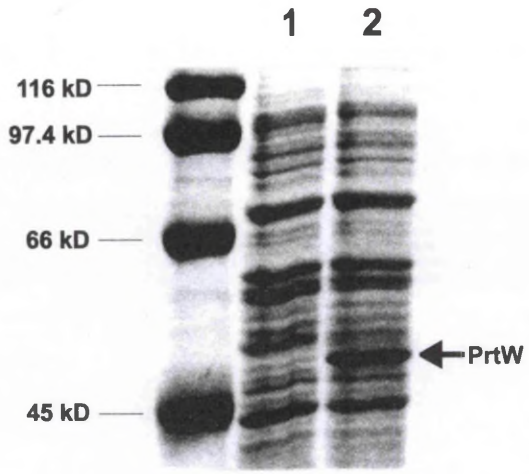

Fig. 1. PrtW protein expression in *E. coli*. Crude extracts were analyzed by SDS-PAGE in a 12% (wt/vol) polyacrylamide gel. Lanes: 1, lysate of M15 carrying pRT1 after 4 h growth without IPTG treatment; 2, lysate of M15 carrying pRT1 after 4 h growth with IPTG treatment. The size of protein molecular weight markers are indicated.

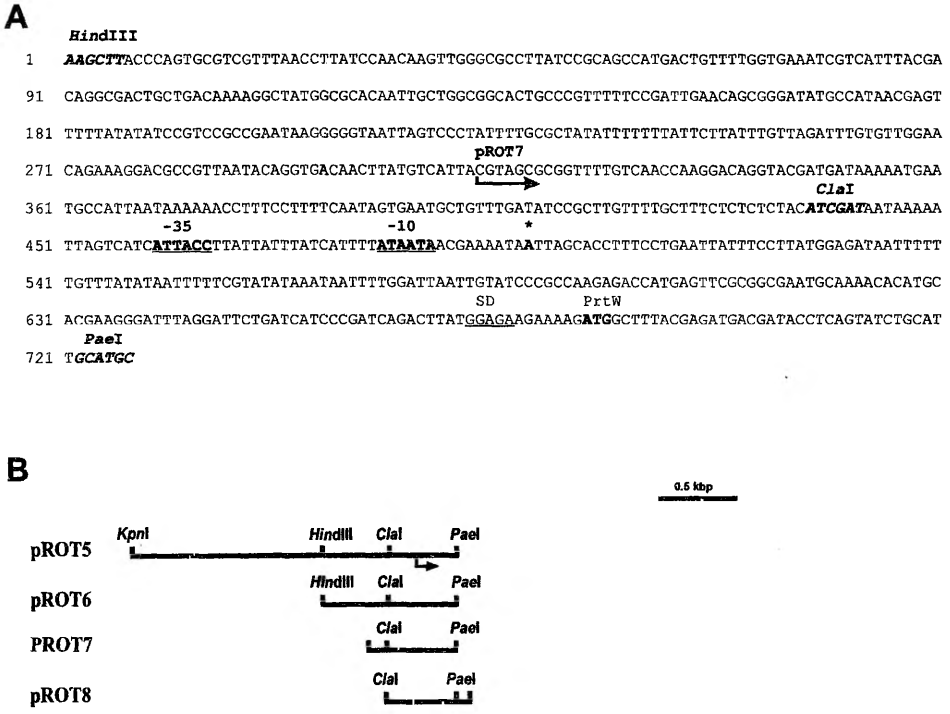

Fig. 2. (A) Summary of the promoter region of the *prtW* gene in *Erwinia carotovora* subsp. *carotovora* strain SCC3193. The translational initiation ATG is shown in bold. The transcriptional start site identified by primer extension analysis is indicated by an asterisk. Potential –35 and –10 promoter elements are in bold and underlined, the potential Shine-Dalgarno (SD) is underlined. *Hin*dIII, *Cla*I and *Pae*I restriction sites utilized for the construction of *gusA* fusions are shown. The beginning of pROT7 is indicated by arrow.

(B) Endonuclease restriction site maps for the inserts of *prtW* in plasmids pROT5, pROT6, pROT7 and pROT8. The transcriptional start of *prtW* is indicated by arrow. The construction of the plasmids is described in the text and Table 1.

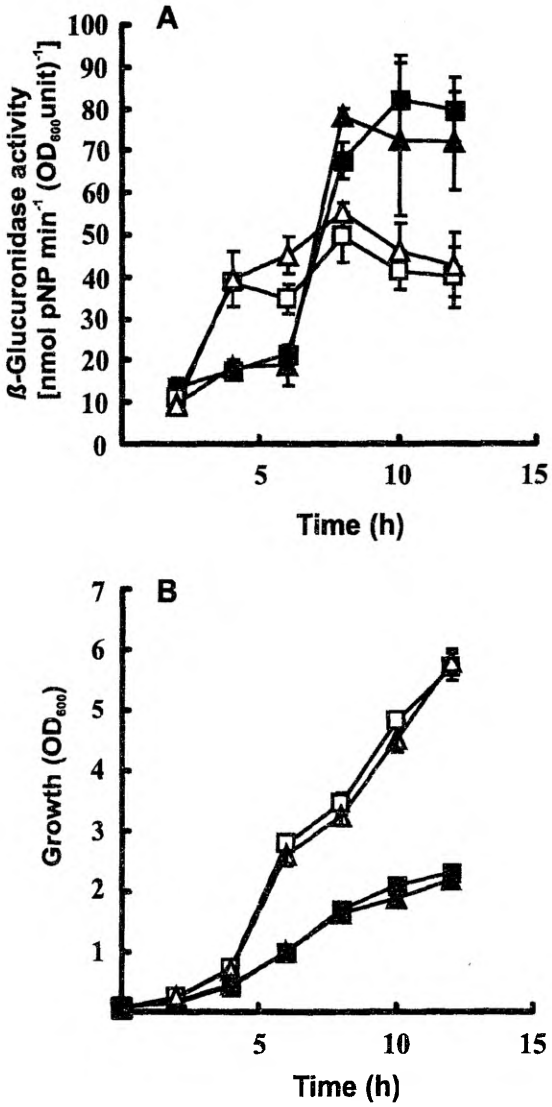

Fig. 3. Expression of the *prtW::gusA* fusion in strain SCC6004 and pROT5 in strain SCC3193 under various growth conditions. Cultures were grown in M9 minimal media containing 0.4% glycerol and supplemented as indicated. (A) β-glucuronidase activity measured as a function of time (h) after inoculation; (B) growth of the bacterial cultures followed by measurement of OD_{600}. ■, SCC6004 in minimal media plus 0.4% PGA; ▲, SCC3193 (pROT5) in minimal media plus 0.4% PGA; □, SCC6004 in minimal media plus 16% potato extract; Δ, SCC3193 (pROT5) in minimal media plus 16% potato extract. Each value represents the mean of three experiments, and the standard deviation is indicated.

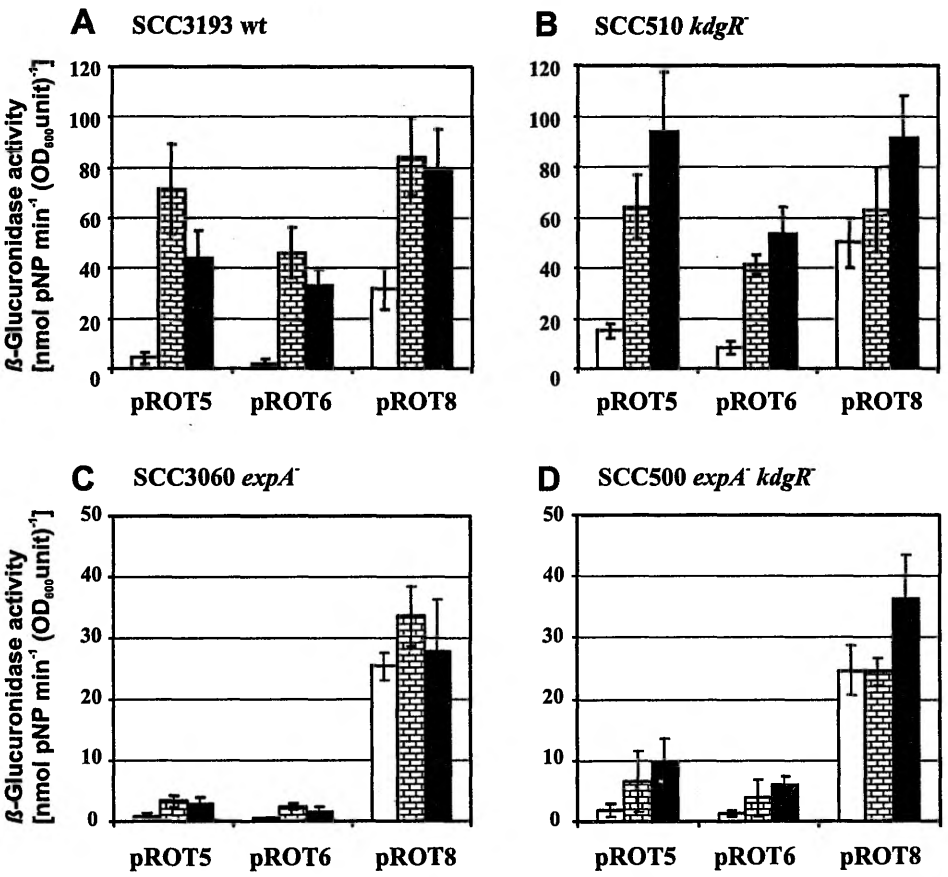

Fig. 4. Expression of the pROT5, pROT6 and pROT8 in different strains of *Erwinia carotovora* subsp. *carotovora.* β-glucuronidase activity measured as a function of time 8h after inoculation. Cultures were grown in M9 minimal media containing 0.4% glycerol and supplemented as indicated. Unsupplemented medium (open bars), medium plus 0.4% PGA (bricks) and medium plus 16% potato extract (black bars). Each value represents the mean of three experiments, and the standard deviation is indicated.

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