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The Rcs phosphorelay modulates the expression of plant cell wall degrading enzymes and virulence in *Pectobacterium carotovorum* subsp. *carotovorum*

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Keywords: phytopathogenic bacteria, two-component system, mutants, Rcs phosphorelay

Running title: Rcs phosphorelay in the virulence of *Pcc*

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ABSTRACT

Production of plant cell wall degrading enzymes, the major virulence factors of soft-rot *Pectobacterium* species, is controlled by many regulatory factors. *Pectobacterium carotovorum* subsp. *carotovorum* SCC3193 encodes a Rcs phosphorelay system that involves two sensor kinases, $RcsC_{Pcc}$ and $RcsD_{Pcc}$, and a response regulator $RcsB_{Pcc}$ as key components of this system, and an additional small lipoprotein $RcsF_{Pcc}$. This study indicates that inactivation of $rcsC_{Pcc}$, $rcsD_{Pcc}$ and $rcsB_{Pcc}$ enhances production of virulence factors with the highest effect detected for $rcsB_{Pcc}$. Interestingly, mutation of $rcsF_{Pcc}$ has no effect on virulence factors synthesis. These results suggest that in SCC3193 a parallel phosphorylation mechanism may activate the $RcsB_{Pcc}$ response regulator which acts as a repressor supressing the plant cell wall degrading enzyme production. Enhanced production of virulence factors in Rcs mutants is more pronounced when bacteria are growing in the absence of plant signal components.

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INTRODUCTION

Many pathogenic bacteria use phosphorelay signaling cascades in the form of two-component systems (TCS) to sense environmental stress and transmit the information inside the cell. A prototypical TCS consists of two proteins, a sensor kinase and a response regulator (for a review see Stock *et al.,* 2000). Sensor kinases sense environmental signals and activate/deactivate respective response regulators through modulating their state of phosphorylation.

Pectobacterium carotovorum subsp. *carotovorum* (*Pcc*) is a phytopathogenic member of *Enterobacteriaceae* causing soft-rot disease in a wide range of economically important crops (Pérombelon, 2002). Elicitation of soft-rot disease requires production of plant cell wall degrading enzymes (PCWDE), also called virulence factors (Pirhonen *et al*., 1991; Heikinheimo *et al.,* 1995; Mäe *et al*., 1995; Marits *et al*., 1999; Mattinen *et al*., 2004). Coordinated production of pectinolytic enzymes and other virulence factors at a precise stage of infection process is necessary to escape plant defense, adjust to the environment and obtain nutrients from infected plant tissue. Several studies have documented that production of PCWDE is coregulated by plant signals, quorum sensing signals molecules (N-acyl homoserine lactones), various transcriptional factors as well as posttranscriptional regulators (Chatterjee *et al.,* 1995; Harris *et al.,* 1998; Liu *et al.,* 1998; 1999; Cui *et al.,* 1995; 1999; 2001; 2005; Burr *et al.,* 2006; Sjöblom *et al.,* 2006). According to current knowledge, *Pcc* employs at least two different global TCSs, the ExpS-ExpA (Eriksson *et al*., 1998), and the PmrA-PmrB (Hyytiäinen *et al*., 2003) to modulate the expression of virulence genes in response to environmental signals.

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In human pathogens *Salmonella typhi*, *Salmonella enterica*, *Yersinia enterocolitica* and enterohemorrhagic *Escherichia coli,* rapid adjustment to the environment and regulation of the virulence largely relies on Rcs phosphorelay systems (Virlogeux *et al*., 1996; Garcia-Calderon *et al.,* 2005; Tobe *et al*., 2005). The Rcs phosphorelay was initially identified as a positive regulator of the *cps* genes, involved in biosynthesis of capsular exopolysaccharides (EPS) in *E. coli* (Gottesman *et al*., 1985). This system has certain unique features, compared to a classical TCS. The Rcs phosphorelay is known to consist of three proteins: RcsC (a hybrid sensor which has a histidine kinase and a receiver domains but lacks a phosphotransmitter domain), RcsB (a response regulator), and RcsD (a phosphotransmitter) (Fig. 1(a); Gottesman & Stout, 1991; Takeda *et al*., 2001; Clarke *et al*., 2002). In addition, a lipoprotein RcsF has been shown to contribute to the Rcs phosphorelay (Majdalani *et al*., 2005). The core of this system, RcsB, is a classical cytoplasmic response regulator composed of a receiver and a DNA binding domains (Francez-Charlot *et al*., 2003). At present, physiological signals activating the Rcs phosphorelay are not clear yet.

Although the regulatory role of Rcs phosphorelay in human pathogens has been studied extensively, relatively little work has been done to characterize significance of this system in bacterial soft-rot disease caused by pectobacteria. Previous studies have confirmed that EPS synthesis in *Erwinia amylovora* and *Pantoea stewartii* are Rcs-dependent (Kelm *et al.,* 1997; Wehland *et al.,* 1999).

In search for regulatory mutants of *Pcc*, we isolated a class of mutants that produced a high basal level of PCWDE. Subsequent study revealed that the mutants carried disruptions in $rcsB_{Pcc}$, $rcsC_{Pcc}$, and $rcsD_{Pcc}$ genes that constitute a central signal

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transduction pathway of the Rcs-system in *Pcc*. The results of present study propose a model according to which the *Pcc* Rcs phosphorelay suppresses production of PCWDE mainly during non-infective growth outside the host plant.

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METHODS

Bacterial strains, vectors and growth conditions

Strains and plasmids used in this study are listed in Table 1. *Pectobacterium carotovorum* subsp. *carotovorum* strains were grown at 30˚C and *Escherichia coli* was grown at 37˚C. The composition of LB medium and minimal salts medium have been described in previous publications (Miller, 1972; Laasik *et al.,* 2005). When required, media were supplemented with 0.4% (w/v) polygalacturonic acid (PGA; Sigma) and antibiotics were added as follows: ampicillin (Amp) 150 µg ml^{-1}, kanamycin (Km) 100 µg ml^{-1} and chloramphenicol (Cm) 25 µg ml^{-1}.

DNA manipulations

Standard DNA techniques described in Sambrook *et al*. (1989) were used. To analyze the DNA sequences flanking the transposon, arbitary PCR method was used as described by Caetano-Anolles (1993). The first round of PCR was performed using an arbitrary primer ARB1 5′-GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT-3′ paired with the proximal primer of the miniTn5Cm OE_{ext} 5′-GGGACTCCTCAAAGCCGAATTG-3′. The second round of PCR was performed using a primer ARB2 5′-GGCCACGCGTCGACTAGTAC-3′ paired with the distal primer of the miniTn5Cm OE_{int} 5′-GCCGCACTTGTGTATAAGAGTCAG-3′. DNA and protein homology searches in GenBank, EMBL and SWISS-PROT databases were performed using the BLASTN, BLASTX and FASTEMBL programs (University of Wisconsin Genetic Computer Group). Protein domain limits were predicted using PROSITE database of protein families and domains (release 19.36; http://au.expasy.org/prosite).

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Transmembrane domains of RcsC and RcsD were identified using the TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html).

GenBank accession numbers for nucleotide sequences of the *rcsDBC*$_{Pcc}$ region and *rcsF*$_{Pcc}$ are EF415648 and EF415647, respectively.

Isolation and construction of mutant strains

Strains SCC6605 (*rcsB*$_{Pcc}$::Tn5-*gusA*), SCC6011 (*rcsD*$_{Pcc}$::Tn5-*gusA*) and SCC6024 (*rcsC*$_{Pcc}$::Tn5-*gusA*) were selected from a pool of transposon mutants generated as described by Marits *et al* (1999). The mutant SCC6026 (*rcsF*$_{Pcc}$::Cm) was generated as follows. Primers rcsFalg 5′-TTCAATACTCGCTCTTTGA-3′ and rcsFD 5′-GACTCATTGTGCAGAGAC-3′ for *rcsF* gene were designed using the genome sequence of *Erwinia carotovora* subsp. *atroseptica* (http://www.sanger.ac.uk/Projects/E_carotovora/) and the obtained DNA fragment was cloned into plasmid pTZ57R/T. The cloned DNA fragment was sequenced to obtain the *rcsF* sequence of *Pcc.* The mutant SCC6026 was constructed using λ Red system described by Datsenko & Wanner (2000) using primers rcsFP1 5′-ATAGCAACCGGCTACGCCACTAACGGTTTGGCATTCATGTAGGTGTAGGCTGGAGCTGCTTC-3′ and rcsFP2 5′-TAGCTATGTCGCTGACAGGCTGTTCTTTATTTCAGAAGCCACCATATGAATATCCTCCTTAG-3′, which contained the *cat* (Cm^{R}) gene from pKD3 flanked on either side by 42 nt homologous to the upstream and downstream regions of *rcsF*$_{Pcc}$.

The mutant SCC6027 (*rcsD*$_{Pcc}$52::Cm) was made using λ Red system (Datsenko & Wanner, 2000) using primers yojNP1 5′-

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ACATCCGCTGACGACTATTGCCCAAGGTATACAGAAACGCATCGATACTTGT GTAGGCTGGAGCTGCTTC-3′ and yojNP2 5′-AATTGCGCTAATGTTGGCGTCGCTAGCGGACGGTTCACTGTTCAGACTGCCAT ATGAATATCCTCCTTA-3′ and the pKD3 plasmid as a template.

Construction of pMW119::*rcsB*$_{Pcc}$

The functional *rcsB*$_{Pcc}$ gene was amplified from the chromosome of the wild-type strain SCC3193 using primers rcsBL 5′-GATGCGACGCAGGAGGGAGAACAGA-3′ and rcsC1 5′-CCGTGTGAGTTTGCACCGCATGA-3′. The obtained PCR product was cloned into *Sma*I-digested pMW119 to yield pMW119:: *rcsB*$_{Pcc}$. In this construct, *rcsB*$_{Pcc}$ has 365 bp upstream of its translational start and is oriented in the opposite direction with regard to the *lacZ* in the multicloning site.

Construction of pLACFw-*rcsF*$_{Pcc}$

The *rcsF* gene of *Pcc* was amplified from the chromosome of the wild-type strain SCC3193 using the primers rcsFalg 5′-TTCAATACTCGCTCTTTGA-3′ and Fstop 5′-TAGAGGATCTGATTGAAAAC-3′. The resulting PCR product was cloned into *Sma*I-digested pMW119 under the control of the *lacZ* gene promoter to yield pLACFw-*rcsF*$_{Pcc}$ and opposite to the *lacZ* promoter to yield pLACRev-*rcsF*$_{Pcc}$. In these constructs *rcsF*$_{Pcc}$ has 43 bp upstream of its translational start.

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Motility assay

Motility was evaluated on soft-agar LB plates (0.3 % agar). A sample (1 ml) of overnight culture of each strain was used to make a dilution in M9 medium to an OD_{600}=2.0. Diluted cultures were stabbed into the centre of soft-agar plates using a sterile inoculation needle. Plates were incubated at 30°C for 48 h.

EPS production assay

Production of exopolysaccarides was measured using a phenol-sulphuric method described in Dubois *et al* (1956).

Enzyme assay

Semiquantitative agarose plate assay for extracellular protease (Prt) production was performed as described by Chatterjee *et al* (1995). The protease activity on agarose plates was evaluated according to the halo, the size of which is proportional to the amount of secreted enzyme. The extracellular activities of polygalacturonase (Peh) and pectate lyase (Pel) were assayed as described previously by Pirhonen *et al* (1991). The quantitative determination of protease (Prt) activity is described in Marits *et al* (2002).

Potato tuber assay

The potato tuber assay used in this study has been described in Eriksson *et al* (1998). Results were scored after 36 h by cutting the tubers in halves and weighing the macerated tissue removed from the sites of inoculation.

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RESULTS

Identification of Rcs phosphorelay components in *Pcc*

To identify new regulators of virulence that modulate the expression of PCWDE genes, the wild-type *Pectobacterium carotovorum* subsp. *carotovorum* SCC3193 was subjected to transposon mutagenesis. From the constructed mutant pool of ca 10 000 clones, three mutants, that produced larger halos around the colonies on milk plates compared to the parental strain, were selected for further investigation. The nucleotide sequences of DNA regions flanking the transposon insertion were determined and found to be homologous to *rcsB*, *rcsC* and *rcsD* genes, described as components of the Rcs phosphorelay in *E. coli* (Chen *et al*., 2001). The organization of the $rcsB_{Pcc}$, $rcsC_{Pcc}$ and $rcsD_{Pcc}$ genes and location of the transposon in corresponding mutants is shown on Fig 1(b). The truncation of $RcsD_{Pcc}$ after the residue L814 may not completely impair its function. To eliminate possibility of the synthesis of a truncated protein that may be stable and manifest an unusual phenotype, we constructed a new mutant of $rcsD_{Pcc}$ with the insertion of Cm resistance marker after the residue T52. The resulting strain SCC6027 was used in further study. To explore the presence of a *rcsF* homologue in *Pcc* strain SCC3193, we analyzed the sequence data of *Erwinia carotovora* subsp. *atroseptica (Eca)* strain SCRI1043 (http://www.sanger.ac.uk/Projects/E_carotovora) and designed the primers for PCR identification of a potential homologue of *rcsF*. A DNA fragment amplified from *Pcc* SCC3193 exhibited 94% identity to *rcsF* of *Eca* SCRI1043. To study the role of $rcsF_{Pcc}$ in PCWDE production, a respective null mutant was constructed.

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All truncated genes (*rcsB*, *rcsC*, *rcsD* and *rcsF*) were transduced back into the wild-type strain using generalized transducing phage T4GT7. In semiquantitative plate assay all transductants showed protease production similar to their parental mutant strain.

Characterization of the Rcs phosphorelay components of *Pcc*

Comparison of the sequence of $RcsB_{Pcc}$ from *Pcc* to those from *E. coli* and *E. amylovora* revealed 94% and 91% similarity (Bereswill & Geider, 1997; accession: CAA70978). *In silico* analysis of the $RcsB_{Pcc}$ amino acid sequence predicts the protein to be a response regulator as it contains a receiver domain with conserved aspartate in position D56 and a potential DNA binding domain with helix-turn-helix motif at 144-208 aa. Upstream of the $rcsB_{Pcc}$, the $rcsD_{Pcc}$ was revealed. Its deduced sequence of 897 amino acids shows 61% similarity to *E. coli* RcsD protein (accession: ZP_00717815). Like RcsD of *E. coli,* $RcsD_{Pcc}$ contains a pseudo-His-kinase domain, followed by a typical phosphotransmitter domain (Takeda *et al.,* 2001).

According to the predicted structure, the $RcsC_{Pcc}$ protein was suggested to be a hybrid sensor kinase. The deduced amino acid sequence of $RcsC_{Pcc}$ showed 73% similarity to RcsC of *E. coli* (accession: YP_670158). Sequence comparison between the RcsC of *E. coli* and the $RcsC_{Pcc}$ showed high similarity between the histidine kinase and receiver domains (88% and 85% similarity, respectively), while the input domain was less conserved (61% similarity).

The predicted 136 aa $rcsF_{Pcc}$ gene product showed 72% identity and 84% similarity to the *E. coli* outer membrane lipoprotein RcsF that transfers the signal to the sensor RcsC (accession: P69411; Majdalani *et al.,* 2005). Similarity was the highest

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within the C-terminus constituting a periplasmatic domain (Castanié-Cornet *et al.,* 2006). Outer membrane domain which localizes in the N-terminus of the processed RcsF (after the removal of the signal peptide), shows only 24% similarity with the RcsF of *E. coli* (accession: P69411).

Influence of *rcsB*$_{Pcc}$, *rcsC*$_{Pcc}$, *rcsD*$_{Pcc}$ and *rcsF*$_{Pcc}$ mutations on PCWDE synthesis and cell motility

Quantitative spectrophotometric assays were performed to assess the increase in polygalacturonase (Peh), pectate lyase (Pel) and protease (Prt) synthesis in *rcsB*$_{Pcc}$, *rcsC*$_{Pcc,}$ *rcsD*$_{Pcc}$52, and *rcsF*$_{Pcc}$ mutants of *Pcc* under uninduced and induced (0.4% PGA added) conditions. Differences in enzyme activities of the mutants compared to the wild-type strain were the largest at OD_{600}~1.5 of the culture when bacteria entered the late exponential growth phase. As shown in Fig. 2, expression of PCWDE was clearly elevated in the *rcsB*$_{Pcc}$ mutant: under uninduced growth conditions the Peh level was about 18 times, the Pel level about 9 and the Prt level about 19 times higher compared to the wild-type strain. In the *rcsC*$_{Pcc}$ mutant, the Peh, Pel and Prt levels were considerably less affected than in the *rcsB*$_{Pcc}$ mutant: the Peh level was about 9 times, the Pel and Prt levels about 4 times higher than in the parental strain. In the *rcsD*$_{Pcc}$52 mutant, the Peh level was about 9 times, the Pel and Prt levels about 4 times higher compared to the parental strain.

All three studied enzymes were also expressed at elevated levels upon induction, with the *rcsB*$_{Pcc}$ mutant showing a 1.9-fold increase in Pel production, 2-fold increase in Peh production and 3.6-fold increase in Prt production (Fig. 2). The *rcsC*$_{Pcc}$ mutation

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caused 1.4-fold increase in the induced production of Pel and Peh and 2.3-fold increase in Prt production (Fig. 2). The *rcsD*$_{Pcc}$52 mutation resulted in 1.5-fold enhancement of Pel and Peh, and 2.4-fold enhancement of Prt activity under induced conditions (Fig. 2). When we introduced a plasmid copy of the *rcsB*$_{Pcc}$ (pMW119::*rcsB*$_{Pcc}$) into the *rcsB*$_{Pcc}$ mutant, all three studied enzymes were reduced to the wild-type level under all conditions tested (data not shown).

Inactivation of the *rcsF*$_{Pcc}$ did not affect production of the enzymes either in uninduced or induced conditions (Fig. 2). We overexpressed the *rcsF*$_{Pcc}$ to find out whether it is a component of the Rcs phosphorelay in *Pcc*. Multicopy *rcsF*$_{Pcc}$ (pLACFw-*rcsF*$_{Pcc}$) was able to reduce the protease production in the wild-type strain while had no effect on protease activity of different Rcs phosphorelay mutants (Fig. 3).

The homologous Rcs phosphorelay in *E. coli* is known to control genes that mediate EPS production and flagella synthesis (Gottesman *et al.,* 1985; Francez-Charlot *et al.,* 2003). The *rcsB*$_{Pcc}$ mutant showed moderate increase in motility in a plate assay compared to the wild-type strain. (Fig. 4). When a plasmid copy of the *rcsB*$_{Pcc}$ (pMW119::*rcsB*$_{Pcc}$) was introduced into the wild-type strain, its motility was reduced (Fig. 4). No differences in motility between the *rcsC*$_{Pcc}$, *rcsD*$_{Pcc}$52 and *rcsF*$_{Pcc}$ mutants and corresponding parental strain were found under any conditions tested. Also, inactivation of *rcsB*$_{Pcc}$, *rcsC*$_{Pcc}$, *rcsD*$_{Pcc}$ or *rcsF*$_{Pcc}$ had no effect on EPS production in *Pcc* strain SCC3193 (data not shown).

The Rcs phosphorelay affects the pathogenicity of *Pcc*

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Previous studies have established positive correlation between the level of exoenzymes and the virulence of *Pcc* (Pirhonen *et al*., 1993; Chatterjee *et al*., 1995; Marits *et al*., 1999). To characterize the virulence of $rcsB_{Pcc}$, $rcsC_{Pcc}$, $rcsD_{Pcc}$52 and $rcsF_{Pcc}$ insertion mutants, potato tuber maceration experiments were carried out. Results on the Fig. 5 show the average weight of diseased tissue removed from 10 separately infected potato tubers. As regulation of virulence through Rcs-system essentially relies on $RcsB_{Pcc}$ response regulator, the $rcsB_{Pcc}$ mutant caused more extensive maceration of potato tubers than $rcsC_{Pcc}$ and $rcsD_{Pcc}$52 mutants (Fig. 5). Introduction of pMW119::$rcsB_{Pcc}$ into the $rcsB_{Pcc}$ mutant restored virulence of the mutant to the wild-type level (Fig. 5). The maceration capacity of $rcsC_{Pcc}$ and $rcsD_{Pcc}$52 mutants was moderately increased, whereas the $rcsF_{Pcc}$ mutant did not show any difference in tissue maceraration capacity compared to the wild-type strain SCC3193 (Fig. 5). These results are in accordance with the PCWDE production profile of the mutants (Fig. 2).

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DISCUSSION

The cascades governing gene expression in mammalian and plant pathogens have been shown to act through specialized signal transduction pathways.

In this study we identified the Rcs phosphorelay from a plant pathogen *Pectobacterium carotovorum* subsp. *carotovorum*. Previous work has shown that in *E. coli,* the Rcs phosphorelay represses the genes required for flagella biogenesis (Francez-Charlot *et al*., 2003), while activates those needed for capsular biosynthesis (Gottesman *et al*., 1985) and stress tolerance (Davalos-Garcia *et al*., 2001; Boulanger *et al*., 2005). In *Salmonella typhi,* the Rcs phosphorelay differentially modulates the expression of invasion proteins, flagellin and Vi antigen in response to changes of environmental osmolarity (Virlogeux *et al.,* 1996; Arricau *et al*., 1998; Mouslim *et al.*, 2004; Delgado *et al*., 2006). Occurrence of homologues of Rcs phosphorelay genes also in *Erwinia amylovora* (Bereswill & Geider, 1997), suggests that this phosphorelay may represent a common pathway to regulate environmental signal-dependent gene expression. The data presented here show that the Rcs-system is also an important component of regulatory network that modulates expression of virulence factors in *Pcc*.

Studying on the role Rcs phosphorelay in regulation of PCWDE production, we analysed three mutants of *Pcc* in which $rcsB_{Pcc}$, $rcsC_{Pcc}$, and $rcsD_{Pcc}$ genes were insertionally inactivated. In these mutants lacking functional Rcs-system, expression of Peh, Pel and Prt was increased compared to the parental strain under all conditions tested. As shown in Fig. 2, the $rcsB_{Pcc}$ mutant showed 18-fold increase in Peh production, 9-fold increase in Pel production and 19-fold increase in Prt production in uninduced conditions.

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Differences between individual enzyme activities may reflect different role of $RcsB_{Pcc}$ in regulation of each enzyme tested. These findings are in accordance with available evidence, albeit indirect, that the genes for PCWDE in *Pcc* may be differently regulated by the same global regulator (Chatterjee *et al*., 1995; Heikinheimo *et al*., 1995; Marits *et al*., 1999; 2002). For example Chatterjee *et al*. (1995) demonstrated that Peh, Pel and Prt-producing systems in *Pcc* responded differently to inactivation of a global negative regulator $RsmA_{Pcc}$. Interestingly, the $rcsF_{Pcc}$ disruption had no phenotypic effect with regard to PCWDE production (Fig. 2). RcsF has been described as an outer membrane protein playing a critical role in signal transduction from cell surface to RcsC in *E. coli* (Majdalani *et al*., 2005). The failure to observe a visible effect of $rcsF_{Pcc}$ mutation on PCWDE production in *Pcc* suggests that $RcsF_{Pcc}$ is not a necessary component of the Rcs phosphorelay under applied conditions. In *E. coli*, it has been shown that not all Rcs phosphorelay activating signals pass through RcsF (Castanie-Cornet *et al.,* 2006). However, overproduction of RcsF in the wild-type strain *Pcc* but not in $rcsC_{Pcc}$, $rcsD_{Pcc}$ and $rcsB_{Pcc}$52 mutants was able to repress protease production (Fig. 3). This supports the possibility that RcsF is still a member of the Rcs phosphorelay though the actual signal sensed by RcsF remains unknown. Our data also agree with those by Majdalani *et al.* (2002) showing that overproduction of RcsF affects the expression of RcsB-dependent promoters.

In $rcsC_{Pcc}$ and $rcsD_{Pcc}$52 mutants production of PCWDE responsible for plant tissue maceration was less elevated than in the $rcsB_{Pcc}$ mutant (Fig. 2). There are two possibilities to explain this phenomenon. First, RcsB may be phosphorylated by a pathway other than RcsC/RcsD. Fredericks *et al*. (2006) have observed that RcsB of *E.*

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coli can be activated independently of RcsC by accepting a phosphoryl group from acetylphosphate, and Castanié-Cornet *et al.* (2007) have shown that RcsB can regulate target gene expression independently of the RcsC/RcsD pathway. Alternatively RcsB can have partial activity in unphosphorylated state. Further study is needed to verify these hypothesis.

Interestingly, although the basal level of all tested enzymes was increased in all three mutants, we still observed the inducing effect of PGA (Fig. 2). Therefore PGA may induce PCWDE production via *rcs*-independent mechanism(s). The activator function of PGA depends on its degradation products which interact with different regulators. For example, Liu *et al.,* (1999) demonstrated that pectin breakdown products cause $KdgR_{Pcc}$ to dissociate from its binding site thereby elevating the production of PCWDE.

As enhanced motility and increased production of PCWDE both contribute to the virulence of *Pcc* we expected the $rcsB_{Pcc}$ mutant to be more virulent on potato tubers than the wild-type. The effect on virulence was less pronounced in $rcsC_{Pcc,}$ and $rcsD_{Pcc}52$ mutants compared to the $rcsB_{Pcc}$ mutant (Fig. 5) that agrees with their mutant phenotypes of PCWDE production and motility (Fig. 2 and Fig. 4).

Irrespective of the mechanism of action of the Rcs system in *Pcc*, it is certainly an important global regulatory system affecting multiple PCWDE production in that plant pathogen. Coordination of environmental sensing and expression of virulence genes is crucial for successful infection. Further investigation is required to answer the question whether the response regulator $RcsB_{Pcc}$ of the Rcs-system regulates the expression of PCWDE genes by binding directly to their promoters or it controls the expression of its target genes by regulating the expression of another global regulator.

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496 Table 1. Bacterial strains and plasmids

Strains and plasmids	Relevant genotype	Source or reference
Strains		
P. carotovorum ssp. carotovorum		
SCC3193	Wild-type	Pirhonen *et al.*, (1988)
SCC6605	*rcsB*$_{Pcc}$:: Tn5-*gusA*	This study
SCC6011	*rcsD*$_{Pcc}$:: Tn5-*gusA*	This study
SCC6024	*rcsC*$_{Pcc}$:: Tn5-*gusA*	This study
SCC6026	*rcsF*$_{Pcc}$:: Cm^{R}	This study
SCC6027	*rcsD*$_{Pcc}$52:: Cm^{R}	This study
E. coli		
DH5α	*supE4, ΔlacU169, (lacZΔM15), hasdR17, recA1, endA1, gyrA 96, thi-1, relA1*	BRL (San Diego, CA)
Plasmids		
pTZ57R/T	Amp^{R}; cloning vector	Fermentas
pMW119	Amp^{R}; cloning vector	Eurogentec
pMW119::*rcsB*$_{Pcc}$	Vector pMW119 containing *rcsB*$_{Pcc}$ gene with additional 365 bp upstream from start codon in the *Sma*I site	This study
pLACFw-*rcsF*$_{Pcc}$	Vector pMW119 containing *rcsF*$_{Pcc}$ gene with additional 43 bp upstream from start codon in the *Sma*I site under	This study

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	the control of *lacZ* gene promoter	
pLACRev-$rcsF_{Pcc}$	Vector pMW119 containing $rcsF_{Pcc}$ gene with additional 43 bp upstream from start codon opposite direction to *lacZ* gene promoter in the *Sma*I site	This study
pKD3	Derivative of pANTSγ that contains an FRT(FLP recognition target)-flanked Cm^R (cat) gene from pSC140	Datsenko & Wanner, (2000)
pPRG	miniTn5Cm^R::*gusA*	Marits *et al.,* (1999)

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Fig. 1. Model of Rcs phosphorelay in *Enterobacteriaceae* (a) Schematic representation of the Rcs phosphorelay system in *E. coli*. Ⓢ - unknown environmental signal for Rcs phosphorelay; INPUT – the predicted input domains of RcsD and RcsC; KIN – the histidine kinase domain; Htp – phosphotransmitter domain of RcsD; R – receiver domains; HTH- helix-turn-helix DNA binding domain of RcsB; Ⓗ and Ⓓ indicate the histidine and aspartate residues important for the phosphate transfer. Note that histidine residue important for autophosphorylation is missing in the KIN domain of RcsD. (b) The organization of the genes coding for main components of the Rcs phosphorelay in *Pcc*. ♦ indicates localization of the transposon miniTn5Cm^R::*gusA* in $rcsC_{Pcc}$, $rcsD_{Pcc}$ and $rcsB_{Pcc}$ mutants. ♀ indicates localization of Cm^R insertion in the $rcsD_{Pcc}$52 mutant.

Fig. 2. Expression of plant cell wall degrading enzymes in different *Pcc* mutant strains. Cultures were grown in M9 minimal medium containing 0.4% glycerol (uninduced conditions) and in M9 medium plus 0.4% PGA (induced conditions). The production of polygalacturonase (Peh), pectate lyases (Pel) and protease (Prt) by the *rcs* mutants and wild-type strain in uninduced (a, b, c) and induced (e, f, g) conditions. Growth of bacterial cultures followed by measurement of OD_{600} in uninduced (d) and induced (h) conditions. ■ - $rcsB_{Pcc}$ mutant; □ - $rcsD_{Pcc}$52 mutant; ● - $rcsC_{Pcc}$ mutant; ○ - $rcsF_{Pcc}$ mutant; ▲- wild-type. All values are averages of three independent experiments with standard deviations less than 10% in all cases.

Fig. 3. Multicopy *rcsF* was introduced into the wild-type and different *rcs* mutants, and protease production was observed on milk-containing agar plates.

Fig. 4. Motility of $rcsB_{Pcc}$ mutant was tested on 0.3% agar. (a) wild-type; (b) $rcsB_{Pcc}$ mutant ; (c) wild-type (pMW119::$rcsB_{Pcc}$). The agar plates were photographed 48 h after the inoculation.

Fig. 5. The virulence of *rcs* mutants and wild-type strain on potato tubers. The maceration capacity of mutants was compared with that of the wild-type strain SCC3193. Tubers

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were inoculated with 10^4 CFU and the macerated tissue was weighed after 36 h of incubation at 28˚C and 100% humidity. Average amounts (± standard deviation) of macerated tissue collected from 10 different potato tubers are shown.

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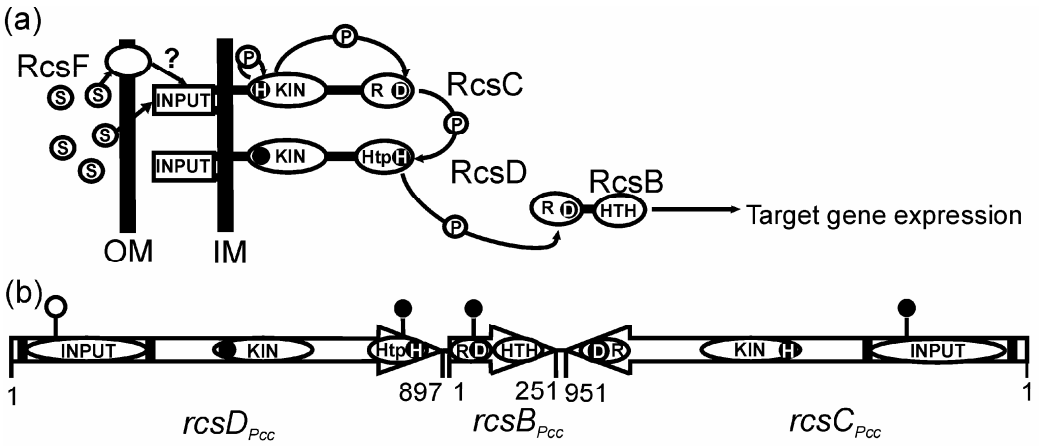

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533 Fig. 1.

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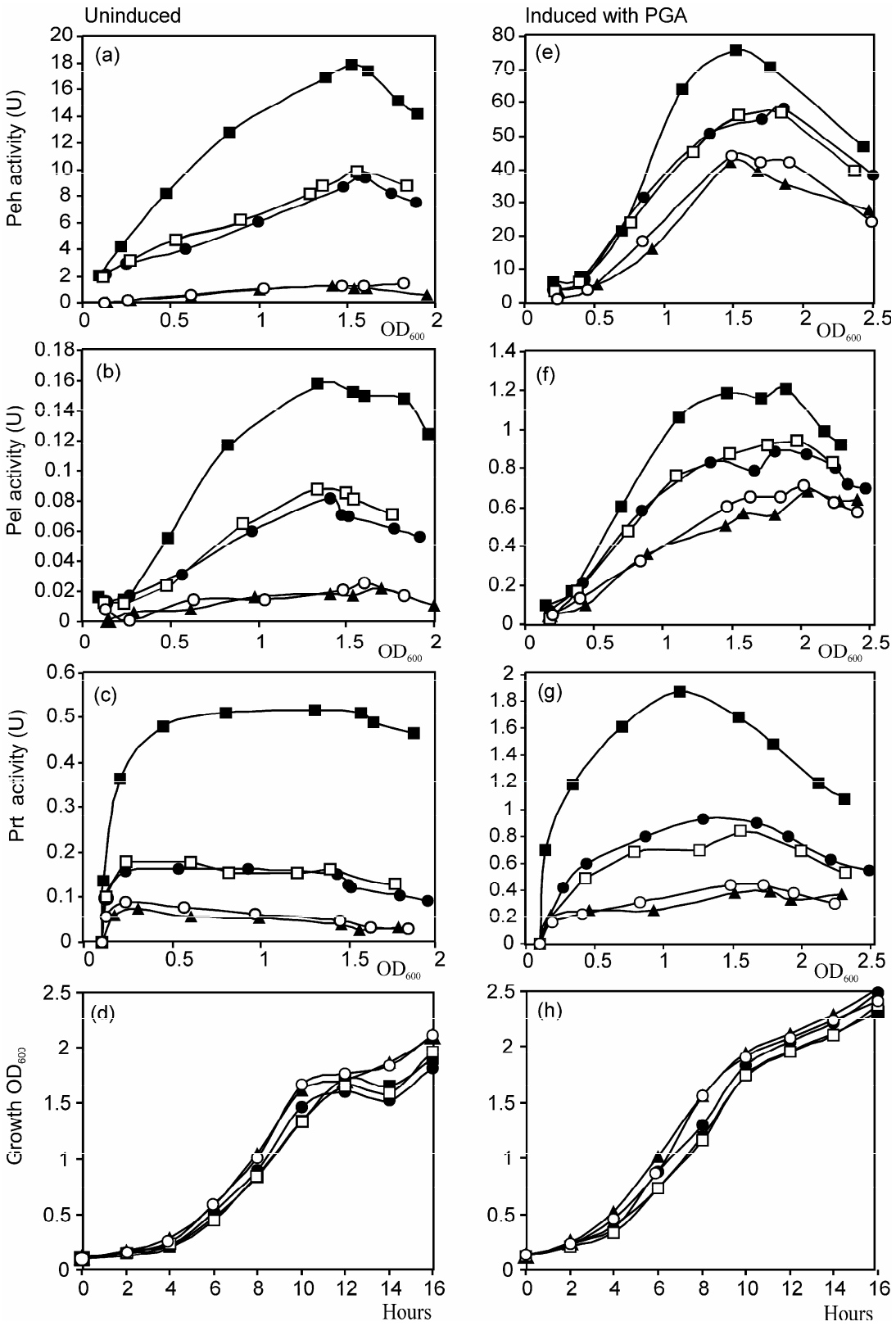
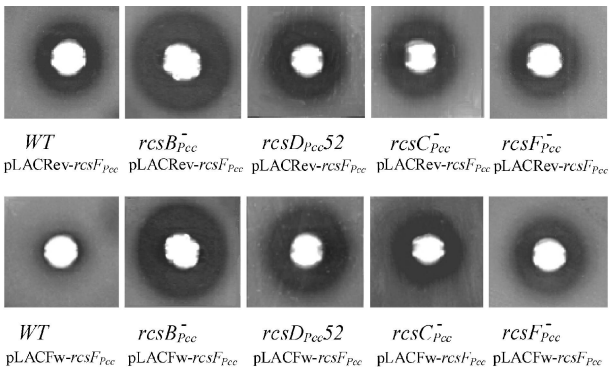

Uninduced
Induced with PGA
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
Peh activity (U)
Pel activity (U)
Prt activity (U)
Growth OD600
OD600
Hours

Fig. 2.



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537 Fig. 3.

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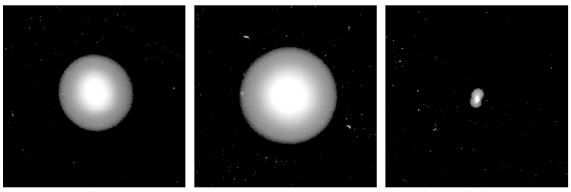

(a) (b) (c)

Fig. 4.

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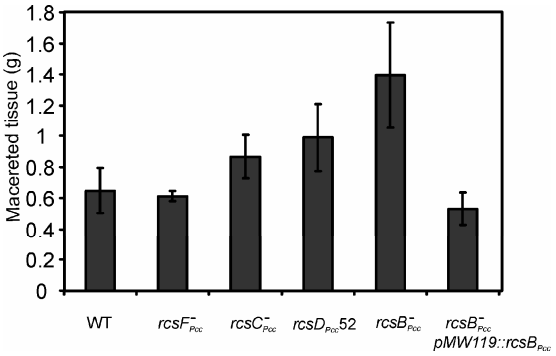

Fig. 5.

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