A role for the Rcs phosphorelay in regulating expression of plant cell wall degrading enzymes in *Pectobacterium carotovorum* subsp. *carotovorum*

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The Rcs phosphorelay is a signal transduction system that influences the virulence phenotype of several pathogenic bacteria. In the plant pathogen *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) the response regulator of the Rcs phosphorelay, RcsB, represses expression of plant cell wall degrading enzymes (PCWDE) and motility. The focus of this study was to identify genes directly regulated by the binding of RcsB that also regulate expression of PCWDE genes in *Pcc*. RcsB-binding sites within the regulatory regions of the *flhDC* operon and the *rprA* and *rsmB* genes were identified using DNase I protection assays, while *in vivo* studies using *flhDC::gusA*, *rsmB::gusA* and *rprA::gusA* gene fusions revealed gene regulation. These experiments demonstrated that the operon *flhDC*, a flagellar master regulator, was repressed by RcsB, and transcription of *rprA* was activated by RcsB. Regulation of the *rsmB* promoter by RcsB is more complicated. Our results show that RcsB represses *rsmB* expression mainly through modulating *flhDC* transcription. Nevertheless, direct binding of RcsB on the *rsmB* promoter region is possible in certain conditions. Using an *rprA*-negative mutant, it was further demonstrated that RprA RNA is not essential for regulating expression of PCWDE under the conditions tested, whereas overexpression of *rprA* increased protease expression in wild-type cells. Stationary-phase sigma factor, RpoS, is the only known target gene for RprA RNA in *Escherichia coli*; however, in *Pcc* the effect of RprA RNA was found to be *rpoS*-independent. Overall, our results show that the Rcs phosphorelay negatively affects expression of PCWDE by inhibiting expression of *flhDC* and *rsmB*.

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INTRODUCTION

The phytopathogenicity of *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) is largely due its capacity to synthesize and secrete plant cell wall degrading enzymes (PCWDE), including pectinases, cellulases and protease (Heikinheimo *et al.*, 1995; Marits *et al.*, 1999; Mäe *et al.*, 1995; Pirhonen *et al.*, 1991). Survival of infecting *Pcc* largely depends on production of PCWDE for the transition from latent infection to disease state. A number of global and specific regulatory genes contribute to expression of PCWDE in *Pcc* (Burr *et al.*, 2006; Chatterjee *et al.*, 1995; Cui *et al.*, 1995, 1999, 2001, 2005; Eriksson *et al.*, 1998; Flego *et al.*, 2000; Harris *et al.*, 1998; Hyttiäinen *et al.*, 2003; Liu

et al., 1998, 1999; Sjöblom *et al.*, 2006). The post-transcriptional Rsm system plays a key role in regulating the expression of PCWDE (Chatterjee *et al.*, 1995; Cui *et al.*, 1995; Liu *et al.*, 1998). The Rsm system consists of two regulators in *Pcc*: a small protein, RsmA, and RsmB RNA. RsmA is a post-transcriptional global regulator that is thought to bind mRNAs of PCWDE genes and affect their stability, whereas RsmB RNA binds multiple molecules of RsmA, thereby neutralizing its effect (Liu *et al.*, 1998). Recently, the Rsm system was shown to be dependent on expression of a global regulator of flagellar genes, FlhDC. FlhDC activates the expression of PCWDE in *Pcc* strain 71 by modulating transcription of RsmB (Cui *et al.*, 2008).

Motility is a key feature of many microbes' physiology, and flagella provide the necessary propulsion to the microbes in a liquid environment. FlhDC, which regulates the expression of flagellar genes (for a review, see Prüß, 2000), also regulates the consumption of glucose (Entner–Doudoroff pathway) and anaerobic respiration in *Escherichia coli*, as well as induction of purine and pyrimidine synthesis and repression of the urea cycle in *Yersinia enterocolitica* (Kapatral *et al.*, 2004; Prüß *et al.*, 2001, 2003). Flagellar

Abbreviations: *Pba*, *Pectobacterium atrosepticum* SCRI 1043; *Pcc*, *Pectobacterium carotovorum* subsp. *carotovorum*; PCWDE, plant cell wall degrading enzymes; PGA, polygalacturonic acid.

The GenBank/EMBL/DDBJ accession numbers for the *rprA*, *flhDC* and *rsmB* genes are GQ339472, GQ344498, and GQ344499, respectively.

A supplementary table listing predicted RcsB-binding sites in *Pectobacterium atrosepticum* is available with the online version of this paper.

motility is also a pathogenicity determinant in *Pectobacterium*, and appears to be necessary for the successful invasion and infection of a plant host (Pirhonen *et al.*, 1991; Pérombelon, 2002). Mutants defective in either flagellar synthesis or rotation exhibited a reduced ability to elicit soft-rot symptoms, despite the normal expression of PCWDE (Hossain *et al.*, 2005).

Previously, we demonstrated that both PCWDE synthesis and motility are regulated by the Rcs phosphorelay in *Pcc* (Andresen *et al.*, 2007). The Rcs phosphorelay has been implicated in the synthesis of virulence factors in *Pcc* and other plant-pathogenic bacteria and also in human pathogens (Hinchliffe *et al.*, 2008; Mouslim *et al.*, 2004; Wang *et al.*, 2009, 2007). RcsB activates the biosynthesis of capsular exopolysaccharides, transcription of the cell division gene, *ftsZ*, transcription of a cell envelope protein, *osmC*, and transcription of RprA RNA to increase expression of the stationary-phase sigma factor, RpoS, in *E. coli* (Carballes *et al.*, 1999; Davalos-Garcia *et al.*, 2001; Gervais *et al.*, 1992; Gottesman *et al.*, 1985; Majdalani *et al.*, 2002). Francez-Charlot *et al.* (2003) demonstrated that the Rcs phosphorelay system represses motility through the direct binding of RcsB to the promoter of *flhDC* in *E. coli*.

To gain further insight into the regulatory network that controls virulence gene expression in *Pcc*, we focused on the contributions of the Rcs phosphorelay to the expression of PCWDE. We purified RcsB from *Pcc* and investigated its mode of action in regulating the virulence phenotype: whether it directly binds to the promoters of PCWDE to repress the expression, indirectly represses transcription of virulence activator genes, or activates transcription of virulence repressor genes.

METHODS

Bacterial strains, vectors, and growth conditions. Strains and plasmids used in this study are listed in Table 1. *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) strains were grown at 30 °C, and *Escherichia coli* was grown at 37 °C. Bacteria were grown on LB medium or M9 minimal salts medium containing 0.4% glycerol (Miller, 1972; Sambrook & Russell, 2001). When required, media were supplemented with 0.2% (w/v) polygalacturonic acid (PGA; Sigma), 150 µg ampicillin (Amp) ml⁻¹, 100 µg kanamycin (Km) ml⁻¹ or 25 µg chloramphenicol (Cm) ml⁻¹.

Predicted RcsB-binding sites in *Pectobacterium*. Prediction of the RcsB regulon in *Pectobacterium* was performed using a web-based prediction program, Virtual Footprint Version 3.0 (Münch *et al.*, 2005) on the genome sequence of *Pectobacterium atrosepticum* strainSCRI 1043 (*Pba*) (http://www.sanger.ac.uk/Projects/E_carotovora/; Bell *et al.*, 2004). A position-specific weight matrix was generated using RcsB-binding sites described by Wehland & Bernhard (2000), as well as RcsB-binding sequences identified in the promoters of *flhDC*, *rprA*, *osmC* and *ftsZ* genes in *E. coli* (Carballes *et al.*, 1999; Davalos-Garcia *et al.*, 2001; Francez-Charlot *et al.*, 2003; Majdalani *et al.*, 2002). The sensitivity threshold was set to 0.8–11.3, only RcsB-binding sites within 350 bp of the start of translation were considered, and the search was restricted to intergenic regions.

PCR cloning and DNA sequencing. Standard DNA techniques described in Sambrook & Russell (2001) were used. Oligonucleotide primers used in this study for PCR amplification are listed in Table 2. The genomic regions of the *rprA*, *flhDC* and *rsmB* genes of *Pcc* strain SCC3193 were cloned by using primers obtained from the genome sequence of *Pba*. The amplified fragments were cloned into plasmid pTZ57R/T, and sequenced. The GenBank accession numbers for the *rprA*, *flhDC* and *rsmB* genes are GQ339472, GQ344498, and GQ344499, respectively.

Overexpression and purification of histidine-tagged RcsB and RcsBD56E. The *rscB* gene was amplified using primers RcsBNcoI and RcsBHindIII and was cloned into the *Sma*I site of Bluescript SK+ to yield pLA1. The *rscB* gene was subsequently subcloned using *Nco*I and *Hind*III into plasmid pET24d having a C-terminal His₆ tag (pET24d-*rscB*). RcsB was overexpressed from this plasmid as a fusion protein with a C-terminal His₆ tag and purified as described by Hörak *et al.* (2004). The protocol was modified to include buffers A/B [100 mM Tris/HCl (pH 7.5), 1 M NaCl, 25 mM imidazole, 10 mM DTT, 0.1 mM EDTA, 0.1% Triton X-100, 5% glycerol] and C [100 mM Tris/HCl (pH 7.5), 500 mM NaCl, 500 mM imidazole, 10 mM DTT, 0.1 mM EDTA, 0.1% Triton X-100, and 50% glycerol]. Purified protein was dialysed against storage buffer [10 mM Tris/HCl (pH 7.5), 200 mM KCl, 1 mM DTT, 0.5 mM EDTA, 50% glycerol] and stored at –20 °C.

Site-directed mutagenesis and purification of modified protein. The sequence of the *rscB* in which the conserved aspartate codon (D56) was substituted with a codon for glutamic acid was generated as follows. Partial sequence of *rscB* was amplified with primers rcsBalg and RcsBD56E from *Pcc* chromosomal DNA, purified with the UltraClean 15 DNA Purification kit (MO BIO), and subsequently used as a primer in a PCR paired with primer rcsCend, and chromosome of *Pcc* as a template. Amplified PCR product was cloned into the cloning vector pJET1 to yield pJET1-*rscBD56E*, according to the supplied protocol (Fermentas). The *rscBD56E* gene was amplified using primers RcsBNcoI and RcsBHindIII with plasmid pJET1-*rscBD56E* as a template. The subsequent cloning, protein expression and purification procedures were the same as for histidine-tagged RcsB.

DNase I footprinting. DNA fragments for DNase I footprinting assays were amplified by PCR from genomic DNA of *Pcc* SCC3193 and one of the primers end-labelled with [γ -³²P]ATP by polynucleotide kinase (Fermentas). Amplified DNA fragments included: (i) *flhDC* (203 bp) having upstream sequences –374 bp to –171 bp using primers flhD1 and flhD2; (ii) *rprA* (159 bp) including upstream sequences –124 bp to +35 bp using primers rprAFP1 and rprAFP2; (iii) *rsmB* (325 bp) including upstream sequences –287 bp to +38 bp using primers rsmBpA and rsmBpY; and (iv) *prtW* (269 bp) including upstream sequences –55 bp to +214 bp using primers PROS and PRIMEX2. DNase I footprinting reactions were performed as described by Ilves *et al.* (2004), except that 0.01U DNase I (Fermentas) was used and the concentration of RcsB-6His or RcsBD56E-6His ranged from 0.5 to 20 µM. DNA sequencing reactions were performed using a Sequenase Version 2.0 kit (US Biochemicals). Footprinting and sequencing reactions were resolved and dried in 6.5% denaturing polyacrylamide gels and scanned using a Typhoon phosphorimager screen (Amersham Biosciences).

Construction of mutant strains. The *rscB::Km* fragment was amplified using primers rcsBP1 and rcsBP2, with plasmid, pKD4 as template. Replacement of chromosomal *rscB* with *rscB::Km* was performed using the λ Red system (Datsenko & Wanner, 2000) to yield mutant SCC6030. The *flhDC*-negative mutant (SCC6028) was generated as described by Datsenko & Wanner (2000) using primers flhDP1 and flhDP2, which amplified the *cat* (Cm^R) gene from pKD3 with 49 bp of the *flhD* sequence on either side to position the insert +1 with respect to the start codon of *flhD*. The *flhD/rscB* double mutant

Table 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
<i>Pcc</i>		
SCC3193	Wild-type	Pirhonen <i>et al.</i> (1988)
SCC6030	SCC3193 <i>rcsB</i> ::Km ^R	This study
SCC6028	SCC3193 <i>flhD</i> ::Cm ^R	This study
SCC6029	SCC3193 <i>rprA</i> ::Cm ^R	This study
SCC8002	SCC3193 <i>rpoS</i> ::Km ^R	Andersson <i>et al.</i> (1999)
SCC6031	SCC3193 <i>flhD</i> ::Cm ^R <i>rcsB</i> ::Km ^R	This study
<i>E. coli</i>		
DH5 α	<i>supE4</i> <i>AlacU169</i> (<i>lacZAM15</i>) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	BRL
BL21(DE3)	<i>hsdS</i> <i>gal</i> (λ clts857 <i>ind1</i> <i>Sam7</i> <i>nin5</i> <i>lacUV5</i> -T7 gene 1)	Studier & Moffatt (1986)
Plasmids		
pET24d	Protein expression vector (Km ^R)	Novagen
pGP704L	Delivery plasmid for homologous recombination (Amp ^R)	Pavel <i>et al.</i> (1994)
pTZ57R/T	High-copy cloning vector (Amp ^R)	Fermentas
pJET1	High-copy cloning vector (Amp ^R)	Fermentas
pMW119	Low-copy cloning vector (Amp ^R)	Eurogentec
pMW119:: <i>gusA</i>	Plasmid containing <i>gusA</i> gene in the <i>Hind</i> III site in the opposite direction to the <i>lacZ</i> gene	Marits <i>et al.</i> (2002)
pUT mini-Tn5 Cm	Delivery plasmid for mini-Tn5 Cm (Cm ^R Amp ^R)	de Lorenzo <i>et al.</i> (1990)
pET24d- <i>rcsB</i>	Protein expression vector under control of the T7 promoter containing <i>rcsB</i> fused to 6 C-terminal His residues	This study
pJET1- <i>rcsBD56E</i>	High-copy cloning vector containing gene for constitutively active form of RcsB (RcsBD56E)	This study
pET24d- <i>rcsBD56E</i>	Protein expression vector under control of the T7 promoter containing <i>rcsBD56E</i> fused to 6 C-terminal His residues	This study
pLA1	pBluescript SK containing the <i>rcsB</i> gene	This study
pLA2	Vector pTZ57R/T containing the <i>rprA</i> gene	This study
pMW119- <i>flhDC</i>	Vector containing the <i>flhDC</i> operon of <i>Pcc</i>	This study
pMW119- <i>rprA</i>	Vector containing the <i>rprA</i> gene of <i>Pcc</i>	This study
pMW119- <i>flhDC</i> :: <i>gusA</i>	Vector containing <i>gusA</i> and the promoter region of <i>flhDC</i>	This study
pMW119- <i>rprA</i> :: <i>gusA</i>	pMW119 containing <i>gusA</i> and the promoter region of <i>rprA</i>	This study
pMW119- <i>rsmB</i> :: <i>gusA</i>	pMW119 containing <i>gusA</i> and the promoter region of <i>rsmB</i>	This study
pLA9	pTZ57R/T containing the <i>rprA</i> gene inactivated by Cm ^R insertion	This study
pLA10	pGP704L containing the <i>rprA</i> gene inactivated by Cm ^R insertion	This study
pKD46	Red recombinase expression plasmid	Datsenko & Wanner (2000)
pKD3	Derivative of pANTS γ that contains an FRT (FLP recognition target)-flanked Cm ^R (<i>cat</i>) gene from pSC140	Datsenko & Wanner (2000)
pKD4	Derivative of pANTS γ that contains an FRT-flanked Km ^R gene from pCP15	Datsenko & Wanner (2000)

(SCC6031) was generated using the *rcsB*::Km mutant (SCC6030) background and the same methods and primers as described above.

To obtain a knockout mutant of *rprA*, plasmid pLA2 (pTZ57R/T containing a PCR fragment amplified with primers *rprAY* and *rprAA2*) was digested with restriction enzyme *Bpl*I (Fermentas) to disrupt the *rprA* gene sequence at position +25 relative to the transcription start site. The *cat* gene, obtained from plasmid pUT mini-Tn5 Cm as a *Bam*HI fragment, was cloned into *Bpl*I-digested pLA2 to create pLA9. The disrupted *rprA* fragment was amplified from pLA9 using primers *rprAY* and *rprAA2* and cloned into the *Eco*RV-digested vector, pGP704L, to yield pLA10. The *rprA*-negative mutant (SCC6029) was created using the λ Red system (Datsenko & Wanner, 2000), with primers *rprAY* and *rprAA2* and pLA10 as a template.

All of the mutants generated were verified by PCR amplification.

Enzyme assays. For β -glucuronidase (GusA) assays, cells were grown in M9 minimal medium supplemented with 0.4% glycerol with or

without the addition of PGA. GusA activity was measured at 6, 8 and 10 h after inoculation. In the case of pMW119-*flhDC*::*gusA* and pMW119-*rprA*::*gusA* constructs, GusA activity was measured with *p*-nitrophenyl β -D-glucuronide as a substrate (Novel *et al.*, 1974). The degradation product, *p*-nitrophenol (pNP), was detected at 405 nm, and the specific activity of GusA was expressed as nmol pNP liberated min⁻¹ per OD₆₀₀ unit. In the case of the pMW119-*rsmB*::*gusA* construct, GusA activity was measured with 4-methylumbelliferyl β -D-glucuronide (MUG; DUCHEFA Biochemie) as substrate. Five micro-litres of sample was taken at each time point and permeabilized in 90 μ l CTAB buffer (0.05% hexadecyltrimethylammonium bromide, 0.27% β -mercaptoethanol and 1 mM EDTA in 50 mM sodium phosphate buffer pH 7) for 10 min. After addition of MUG (15 μ mol l⁻¹) to the permeabilized cells, accumulation of the fluorescent product 4-methylumbelliferone (MU) was measured with a fluorescence spectrophotometer (GENios Plus; Tecan) using an excitation filter of 360 nm and an emission filter of 465 nm. The slope of relative fluorescence units per minute was calculated. GusA activity of the

Table 2. Oligonucleotide primers used in this study

Name	Sequence (5'–3')*
rcsBP1	CCTAAACGTAATTATTGCCGACGACCATCCCATTGTCCTTTGTGTAGGCTGGAGCTGCTTC
rcsBP2	TTCGCCAACACATTGACCCATTCAATTTGCTCAAGTGATTTTTTATGATGCCAACATATGAATATCCTCCTTA
RcsB <i>Hind</i> III	<u>AAGCTT</u> GTCGACTTGTGTTGCGCCGCC
RcsB <i>Nco</i> I	<u>CCATGGG</u> TAAACCTAAACGTAATTATTGC
flhD1	CATCACGCGGAGAATTTCG
RcsBD56E	CCCGGCATGGATAACTCGGTGAT
rcsBalg	CCTAAAGGGCAGCAAGGTAG
rcsCend	TGATTCTGGTGGTTGACGA
flhD2	CCAGATACAGGTTGGTTCAT
rprAFP1	TCATTGACAAACAGTAGGTTAA
rprAFP2	ATCATTTCTCTAATGCATCAATA
rsmBpA	TTCCTAAAACAATCCGAGTG
rsmBpY	TTTCTGGCCTTAACCCGTTA
PROS	AATAAAAATTAGTCATCATTACC
PRIMEX2	ACTGAGGTATCGTCATCTC
flhDup	AAAGATGTGATCAGGAAATG
flhCA	TTACAGGCTCAGACTGCGTG
flhDP1	CGAACTGGGCGATATGGTCTATCACCTAACTTATGTTTTAAACATAGCCGTGTAGGCTGGAGCTGCTTC
flhDP2	AGATTAATGTCATAAATGTGTTTGAGTAATTCAGAGGTACCCATATTTCCATATGAATATCCTCCTTAG
rprAY	AACCGCAGAACCTCCTCTCTG
rprAA2	GTCCCCATTGTTTTGCCTTC
flhDFw	GCGCAACGCTTAATTAATGATGA
flhDRev	AGTTGCATCAAGATATCTGCCATCTC
ffhFw	CGTATCGCATCACGCATTCT
ffhRev	TCCAGGAAATCGGTCAAATC

*Sites designed for restriction enzymes are underlined.

sample was expressed as nmol substrate hydrolysed h⁻¹ ml⁻¹ per OD₆₀₀ unit, based on a standard curve of MU concentrations. All GusA assays were performed using three technical replications.

To perform semiquantitative agarose plate assays of extracellular protease (Prt) production, cells were grown overnight in M9 medium and washed and suspended in the same medium. Drops of cell suspensions containing ~10⁶ cells were dotted onto milk-containing agar plates (Chatterjee *et al.*, 1995), and the plates were photographed after 3 days of growth at 30 °C. The diameter of the halo produced was considered to be proportional to the amount of enzyme secreted. All assays were performed at least five times.

To quantify the activity of pectate lyase and cellulase, cells were grown on M9 minimal medium supplemented with 0.2% PGA. Three replications were used to measure enzyme activities as described previously (Pirhonen *et al.*, 1991).

Real-time RT-PCR. Wild-type cells harbouring the control plasmid, pMW119, or a plasmid overexpressing *rprA*, pMW119-*rprA*, were grown overnight on milk-containing agar plates at 30 °C. Cells were harvested and total RNA was isolated using the NucleoSpin RNA II kit (Macherey-Nagel). An additional treatment with DNase I (Fermentas) was performed according to the manufacturer's protocol. Real-time RT-PCR was performed using the Verso SYBR Green 1-Step QRT-PCR ROX kit (Thermo Scientific) using the Applied Biosystems 7900 HT real-time thermal cycler. Each reaction contained 2 ng total RNA and 100 nM forward and reverse primers. The sequences of primers used are given in Table 2.

The PCRs were run using the following programme: 50 °C for 15 min, 95 °C for 15 min to inactivate reverse transcriptase, and 40

cycles of 95 °C for 15 s and 60 °C for 30 s. Following PCR amplification, the reactions were subjected to temperature ramping to create the dissociation curve, measured as changes in fluorescence measurements as a function of temperature, by which the non-specific products can be detected. The dissociation programme was 95 °C for 1 min, 60 °C for 15 s, followed by a 2% ramp from 60 °C to 95 °C. Results are presented as mean expression ratios calculated using the 2^{-ΔΔC_t} method as described by Livak & Schmittgen (2001). The *ffh* gene (encoding a signal recognition particle involved in targeting and integration of inner-membrane proteins) was used for normalization of reverse transcriptase real-time PCR data according to Takle *et al.* (2007). All RNA isolations and reverse transcriptase real-time PCRs were performed using three replications.

RESULTS

The response regulator, RcsB, binds to the promoters of *flhDC*, *rprA* and *rsmB*

We previously demonstrated that an *rcsB*-negative mutant expressed higher levels of PCWDE than wild-type *Pcc* (Andresen *et al.*, 2007). To further investigate the regulatory effect of RcsB on PCWDE expression in *Pcc*, the promoter regions of the *prtW*, *pehA*, *pelB*, *pelC* and *celVI* genes were analysed for RcsB-binding sites. Using the Virtual Footprint program (see Methods; Münch *et al.*, 2005), regions upstream of these genes were not found to

contain any putative RcsB-binding sites. Subsequently, the genome of *Pba* was analysed, and 16 putative RcsB-binding sites were found associated with promoter regions of 16 genes (see Supplementary Table S1, available with the online version of this paper). Of these genes, RcsB-binding sites in *flhDC* and *rprA* were previously characterized in *E. coli*, and *rsmB* codes for a global regulator of PCWDE in *Pcc* (Francez-Charlot *et al.*, 2003; Liu *et al.*, 1998; Majdalani *et al.*, 2002). Therefore, *flhDC*, *rprA* and *rsmB* were selected for further analysis.

To identify the DNA-binding sites for RcsB in the promoter regions of *flhDC*, *rprA* and *rsmB*, DNase I footprinting assays were performed using purified, histidine-tagged RcsB. The DNA footprinting assay of *flhD* upstream sequences (−374 to −171) revealed an RcsB-binding site that protected a region spanning 26 bp from −262 to −236 (Fig. 1a). In the *rprA* promoter, bp −124 to +35 and bp −51 to −27 were protected by binding of RcsB (Fig. 1b). In contrast, neither the *rsmB* promoter, nor the promoter of an extracellular protease, *PrtW*, used as a negative control, were protected from DNase I cleavage in the presence of histidine-tagged RcsB (data not shown). The putative RcsB-binding site in the *rsmB* promoter was identified using purified histidine-tagged RcsBD56E. Replacement of conserved aspartate residue in position 56 with glutamic acid is known to make the RcsB protein constitutively active in *E. coli*, without needing RcsC, RcsF or RcsA for its activity (Gupte *et al.*, 1997). DNase I footprinting revealed that the protected regions extended from position −192 to position −211 and from position −213 to position −230 upstream of the start codon of *rsmB* (Fig. 1c). The *prtW* promoter fragment, used as a negative control, was not protected from DNase I cleavage in the presence of histidine-tagged RcsBD56E (data not shown). The nucleotide sequences of the protected regions identified in this study are similar to known RcsB target sites (Fig. 1d).

RcsB modulates expression of *flhDC*, *rprA* and *rsmB* in *Pcc*

To further characterize the influence of RcsB binding on *flhDC*, *rprA* and *rsmB* expression, fusions of *gusA* with *flhDC*, *rprA* and *rsmB* were created, and gene expression for the three constructs was compared between the wild-type strain and an *rcsB*-negative mutant. In the *rcsB*-negative mutant, a fourfold increase in expression of the *flhDC::gusA* fusion was detected, while only low levels of expression of the *rprA::gusA* fusion were detected compared to the wild-type strain. These results indicate that *flhDC* and *rprA* are directly regulated by RcsB in *Pcc*, with RcsB positively regulating *rprA* expression, and negatively affecting *flhDC* expression.

The expression of the *rsmB::gusA* fusion was increased 15–18-fold in an *rcsB*-negative mutant as compared to the wild-type strain (Fig. 2a). We also found that the effect of the *rcsB* mutation on *rsmB::gusA* expression is mediated through *flhDC* in these conditions. We compared the

expression of *rsmB::gusA* fusion in an *flhDC*-negative mutant and in an *flhDC/rcsB* double-negative mutant. No significant differences were found in *rsmB::gusA* expression between these strains in M9 minimal salts medium supplemented with glycerol throughout the entire growth curve (data not shown). When the same mutants were tested for *rsmB::gusA* expression in M9 minimal salts medium supplemented with PGA, *GusA* activity in *flhDC/rcsB* double-negative mutant was similar to that in *flhDC*-negative strain, except at one time point (6 h after inoculation) in the early exponential growth phase, where the *flhDC/rcsB* double mutant expressed 3.4 times higher *GusA* activity than the *flhDC*-negative strain (Fig. 2b).

RcsB modulates expression of PCWDE through *flhDC*

To determine whether the Rcs phosphorelay modulates expression of PCWDE by regulating *flhDC*, *flhDC* mutants were created in *Pcc* strain SCC3193. As shown in Fig. 3, inactivation of *flhDC* resulted in a dramatic decrease in levels of pectate lyases, cellulase and protease compared to the wild-type strain. When expression of *flhDC* was restored using a low-copy plasmid (pMW119-*flhDC*), expression of PCWDE increased in the wild-type strain, and was restored in the *flhDC*-negative strain. An *rscB* null mutation in a wild-type background was also previously shown to increase expression of PCWDE (Andresen *et al.*, 2007). To investigate whether *rscB* inactivation could restore expression of PCWDE in an *flhDC* mutant, enzyme production in an *flhDC/rscB* double mutant was analysed. The double mutant did not regain enzyme production when compared to the *rscB* mutant strain, indicating that the effect of *rscB* inactivation on expression of PCWDE is dependent on *FlhDC*.

Overexpression of *RprA* RNA modulates *rpoS*-independent expression of an extracellular protease and has only a minor effect on expression of pectate lyases and cellulase

As shown in Fig. 2(a), transcription of the *rprA* gene is positively regulated by RcsB. To assess the capacity of *RprA* RNA to regulate expression of PCWDE, an *rprA*-negative mutant was constructed and enzyme assays were performed. The activity of pectate lyases, cellulase and protease in both mutant and wild-type strains was detected. Inactivation of *rprA* did not affect expression of these enzymes (Fig. 3). When *rprA* was overexpressed in the wild-type strain, protease activity increased (Fig. 3). Cellulase activity of the strain overexpressing *rprA* was 1.7 times higher than that of the wild-type strain, while activity of pectate lyase was only slightly elevated (Fig. 3).

RprA RNA was initially discovered and characterized in *E. coli* as a positive regulator of *rpoS* translation (Majdalani *et al.*, 2001). The effect of *RpoS* on *RprA* RNA modulation of PCWDE in *Pcc* was therefore analysed using plate assays. As shown in Fig. 4, overexpression of *rprA* in an *rpoS*-negative

Fig. 1. (a–c) DNase I footprinting assays of histidine-tagged RcsB, histidine-tagged RcsBD56E, and promoter regions of *flhDC* (a), *rprA* (b) and *rsmB* (c). DNA probes labelled on the coding strand were incubated with varying concentrations (0–20 μM) of histidine-tagged RcsB (RcsB-6H) or histidine-tagged RcsBD56E (RcsB_{D56E}-6His) prior to treatment with DNase I. Sequencing gels are shown and the letters A, T, G, and C indicate the DNA sequences of fragments digested by DNase I. (d) The RcsB-binding sites of the *flhDC*, *rprA* and *rsmB* promoters of *Pcc* are aligned with known RcsB-binding sequences in *E. coli*, predicted RcsB-binding sequences in *Pba*, as well as the RcsAB box described by Wehland & Bernhard (2000). Lower-case letters in the RcsAB box indicate nucleotides that have a conservation rate of 50–70% relative to the consensus sequence, and upper-case letters indicate a conservation rate of >70%.

mutant stimulated protease expression, indicating that the effect of *rprA* on protease synthesis is independent of *rpoS*.

Regulation of PCWDE by FlhDC and RprA RNA does not overlap

The results shown in Fig. 3 demonstrate that both *flhDC* and *rprA* are able to enhance protease expression in *Pcc*. To

determine whether *flhDC* and *rprA* can functionally replace each other, an *flhDC* overexpression construct was introduced into an *rprA*-negative mutant and an *rprA* overexpression construct was introduced into a *flhDC*-negative mutant. When *flhDC* was exogenously expressed from pMW119-*flhDC* in a *rprA*-negative background, protease expression was increased. However, when *rprA* was exogenously expressed from pMW119-*rprA* in a

Fig. 2. Expression of promoters fused with reporter gene *gusA* coding for β -glucuronidase (GusA). GusA activity was measured at different times after inoculation and experiments were performed in triplicate. Error bars represent SD. (a) Expression of *flhDC::gusA*, *rprA::gusA* and *rsmB::gusA* fusions was measured in wild-type (wt; SCC3193) and *rcsB*-negative (*rcsB::Km*; SCC6030) strains grown in M9 minimal medium supplemented with glycerol. In the case of the *flhDC* and *rprA* promoter constructs (pMW119-*flhDC::gusA* and pMW119-*rprA::gusA*) GusA activity was measured with *p*-nitrophenyl β -D-glucuronide as a substrate (left axis), whereas in the case of the *rsmB* promoter fusion (pMW119-*rsmB::gusA*) GusA activity was measured with 4-methylumbelliferyl β -D-glucuronide as a substrate (right axis); (b) Expression of *rsmB::gusA* fusion in wild-type (■), *flhDC*-negative mutant (●) and *flhDC/rcsB* double mutant (▲) in minimal medium supplemented with 0.2% PGA.

flhDC-negative background, protease expression did not increase (Fig. 5). To determine whether the positive effect of *rprA* overexpression on protease expression is dependent on an increase in *flhDC* expression, reverse transcription real-time PCR was used to evaluate *flhDC* mRNA levels in a wild-type strain, an *rcsB*-negative mutant, and a strain overexpressing *rprA*. In the *rcsB*-negative mutant, *flhDC* mRNA levels were 1.36 ± 0.15 times higher than in the wild-type strain. In contrast, overexpression of *rprA* decreased *flhDC* mRNA levels compared with wild-type (*rprA* overexpression to wild-type ratio 0.84 ± 0.22). These results demonstrate that activation of protease transcription associated with overexpression of *rprA* is not caused by increased *flhDC* transcription.

DISCUSSION

Genes directly regulated by the Rcs phosphorelay response regulator, RcsB, have been shown to have RcsB-binding sites in their promoters (Wehland & Bernhard, 2000). However, no RcsB-binding sites were identified in any of the PCWDE promoters analysed, although sequences similar to RcsB sites were found in the regulatory regions of the *rsmB*, *flhDC* and *rprA* genes (Supplementary Table S1). *In vitro*, RcsB was shown to directly bind the promoter regions of *flhDC*, *rprA* and *rsmB*. The regulatory regions of *flhDC* and *rprA* were bound by the unphosphorylated form of RcsB, whereas the *rsmB* promoter was bound only by RcsBD56E, a constitutively active form of RcsB (Gupte *et al.*, 1997). These results indicate that the binding sites of RcsB in the *rsmB* promoter region may have lower affinity for unphosphorylated RcsB when compared to the binding sites in the *rprA* and *flhDC*

promoters. This would suggest that *rsmB* is directly regulated by RcsB in response to a signal activating the Rcs phosphorelay, whereas *flhDC* and *rprA* are regulated by RcsB even in the absence of the signal.

In vivo, RcsB positively affected *rprA* transcription and negatively affected *flhDC* and *rsmB* transcription (Fig. 2a), demonstrating that direct transcriptional regulation of *rprA* and *flhDC* expression by the Rcs phosphorelay is not exclusive to *E. coli* and *Salmonella* (Francez-Charlot *et al.*, 2003; Majdalani *et al.*, 2002; Wang *et al.*, 2007). The flagella master regulator, FlhDC, has been previously shown to positively regulate *RsmB* RNA levels in *Pcc* (Cui *et al.*, 2008; Fig. 2b); thus RcsB may regulate transcription of *rsmB* both directly and indirectly through modulating the expression of *flhDC*. Our experiments with an *flhDC*-negative mutant and an *flhDC/rcsB* double-negative mutant indicate that in minimal medium the effect of RcsB on *rsmB* transcription is mediated by FlhDC. When these mutants were grown on minimal medium supplemented with PGA the expression of the *rsmB::gusA* fusion was 3.4 times higher in the *flhDC/rcsB* double-negative mutant than in the *flhDC*-negative mutant at one time point in the early exponential growth phase (Fig. 2b). These results indicate that *rsmB* expression is mainly regulated by RcsB via *flhDC* also in the presence of PGA, but can be directly regulated by RcsB at certain stages of growth. Why the difference in *rsmB::gusA* expression between *flhDC*-negative and *flhDC/rcsB* double-negative mutant appears only in the presence of PGA and at a certain time is currently under investigation.

Our results are consistent with previous studies that have shown that the flagella master regulator, FlhDC, contri-

Fig. 3. Influence of FlhDC and RprA RNA on the expression of PCWDE. (a, b) For assays of pectate lyases (a) and cellulase (b), enzyme activities were measured 10 h after inoculation and the means $\pm$ SD of three independent experiments are shown. (c) Production of protease was observed after 3 days and the diameter of the halo (in mm) was measured from at least five independent experiments. ND, not detectable; wt, wild-type strain SCC3193; *flhDC::Cm*, *flhDC*-negative strain SCC6028; *rcsB::Km*, *rcsB*-negative strain SCC6030; *flhDC::Cm rcsB::Km*, *flhDC*- and *rcsB*-negative double mutant; *rprA::Cm*, *rprA*-negative strain SCC6029; *flhDCpMW119*, *flhDC* overexpression plasmid; *rprApMW119*, *rprA* overexpression plasmid; *pMW119*, control vector.

Fig. 4. Effect of *rprA* overexpression on protease expression in wild-type (wt; strain SCC3193) and RpoS-mutant (*rpoS::Km*; strain SCC8002) strains. Wild-type containing vector pMW119 was used as a control (wt+pMW119). Assay plates were photographed after 3 days and the diameter of the halo (in mm) was measured from at least five independent experiments.

butes to the virulence of *Pcc* (Cui *et al.*, 2008). The results of this study demonstrate that inactivation of *rscB* in an *flhDC*-negative mutant does not restore expression of PCWDE. Since the *flhDC* mutant and the *flhDC/rscB* double mutant exhibited the same phenotype, a model where the Rcs phosphorelay targets PCWDE genes indirectly via FlhDC is supported. Cui *et al.* (2008) reported that FlhDC activates expression of PCWDE indirectly by reducing the activity of the Rsm regulatory system. Based on these data and our results, we hypothesize that the Rcs phosphorelay regulates the expression of PCWDE by modulating the activity of the Rsm system directly (only in certain conditions) by regulating *rsmB* expression and indirectly through *flhDC* (Fig. 6). Furthermore, our results suggest that the effect of the Rcs phosphorelay on motility of *Pcc* presented in our previous paper (Andresen *et al.*, 2007) was most likely due to elevated expression of *flhDC* in the *rsc*-negative mutants.

We were also interested in dissecting the contribution of *rprA* to expression of PCWDE in *Pcc*. In contrast to *flhDC*,

Fig. 5. Effects of overexpressing *flhDC* and *rprA* on protease expression in *rprA*-negative (*rprA::Cm*; strain SCC6029) and *flhDC*-negative (*flhDC::Cm*; strain SCC6028) mutants, respectively. Assay plates were photographed after 3 days of incubation and the diameter of the halo (in mm) was measured from at least five independent experiments. ND, not detectable.

Fig. 6. A proposed model for regulated expression of PCWDE by the Rcs phosphorelay in *Pcc*. The steps of the model indicated on the shaded background are based on data from this study. RcsB affects the expression of PCWDE by directly binding the promoters of *rprA*, *rsmB* and *flhDC*. RcsB positively controls the transcription of small regulatory RprA RNA and negatively affects the transcription of *rsmB* and *flhDC*. A question mark indicates that the exact conditions in which these interactions become important in PCWDE regulation are not yet known. Expression of FlhDC activates *gacA* expression and represses *hexA* expression, which in turn increases the level of RsmB RNA in *Pcc* (Cui *et al.*, 2008) and decreases the pool of free RsmA, resulting in enhanced expression of PCWDE (Liu *et al.*, 1998). Not all of the molecular mechanisms encompassed by this model are fully characterized (indicated with dashed lines). Furthermore, RsmC acts as an anti-FlhDC factor by binding to FlhDC and interfering with FlhDC action (Chatterjee *et al.*, 2009).

which has a significant effect on expression of PCWDE, only increased expression of an extracellular protease was detected in response to exogenous expression of *rprA* in the wild-type strain. Our data further exclude the possibility that RprA RNA is dependent on *flhDC* for regulation of protease expression. Moreover, although exogenous expression of *rprA* did not enhance *flhDC* mRNA levels as detected by reverse transcription real-time PCR assays, it is intriguing to hypothesize that RprA RNA may affect the translation or proteolysis of FlhDC.

In *E. coli* and *Salmonella*, RprA RNA positively affects translation of RpoS by direct interaction with *rpoS* mRNA, which is currently the only known target for RprA RNA (Jones *et al.*, 2006; Majdalani *et al.*, 2001, 2002). In both organisms RprA RNA has an effect on *rpoS* translation in the absence of DsrA RNA (another RNA regulator of *rpoS* translation) under conditions of osmotic shock (Jones *et al.*, 2006; Majdalani *et al.*, 2001). The results of these studies indicate that RprA RNA needs specific conditions for its activity on *rpoS* translation. In this study, *rprA* overexpression was shown to affect protease expression, suggesting that transcription of *rprA* could stimulate

protease expression in the wild-type strain under physiological conditions where the expression level of *rprA* is elevated. Correspondingly, in *E. coli*, *rprA* expression has been shown to increase when the Rcs phosphorelay is artificially activated (Majdalani *et al.*, 2005). Further studies are needed to address the role of RprA RNA as a regulator of protease synthesis in *Pcc* under conditions that activate the Rcs phosphorelay.

In the current study, *rpoS* was not necessary for protease expression induced by RprA RNA (Fig. 4), and this is the first evidence for a role for RprA RNA that is independent of RpoS. It remains unclear whether RprA RNA directly binds the target protease mRNA, or whether RprA RNA indirectly induces protease expression via another regulatory gene.

In summary, we have demonstrated that the Rcs phosphorelay mediates expression of PCWDE mainly through the direct binding of RcsB to the *flhDC* promoter, which in turn causes a subsequent decrease in *rsmB* transcription. Another way to repress *rsmB* transcription is by direct binding of RcsB on the promoter region of *rsmB*, but the conditions in which that becomes important in PCWDE regulation are not yet clear. Lower RsmB RNA levels increase the levels of active RsmA in the cell, which effectuates the degradation of PCWDE gene mRNAs (Liu *et al.*, 1998; Fig. 6). Further study is needed to identify the direct target genes for FlhDC in the regulatory pathway controlling expression of PCWDE genes, the physiological conditions that stimulate the regulatory role of RprA, and the complex relationship between these regulatory factors.

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