Characterization of the RcsC Sensor Kinase from *Erwinia amylovora* and Other Enterobacteria

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ABSTRACT

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RcsC is a hybrid sensor kinase which contains a sensor domain, a histidine kinase domain, and a receiver domain. We have previously demonstrated that, although the *Erwinia amylovora* *rscC* mutant produces more amylovoran than the wild-type (WT) strain in vitro, the mutant remains nonpathogenic on both immature pear fruit and apple plants. In this study, we have comparatively characterized the *Erwinia* RcsC and its homologs from various enterobacteria. Results demonstrate that expression of the *Erwinia rscC* gene suppresses amylovoran production in various amylovoran overproducing WT and mutant strains, thus suggesting the presence of a net phosphatase activity of *Erwinia* RcsC. Findings have also demonstrated that *rscC* homologs from other enterobacteria could not rescue amylovoran production of the *Erwinia rscC*

mutant in vitro. However, virulence of the *Erwinia rscC* mutant is partially restored by *rscC* homologs from *Pantoea stewartii*, *Yersinia pestis*, and *Salmonella enterica* but not from *Escherichia coli* on apple shoots. Domain-swapping experiments have indicated that replacement of the *E. coli* RcsC sensor domain by those of *Erwinia* and *Yersinia* spp. partially restores virulence of the *Erwinia rscC* mutant, whereas chimeric constructs containing the sensor domain of *E. coli* RcsC could not rescue virulence of the *Erwinia rscC* mutant on apple. Interestingly, only chimeric constructs containing the histidine kinase and receiver domains of *Erwinia* RcsC are fully capable of rescuing amylovoran production. These results suggest that the sensor domain of RcsC may be important in regulating bacterial virulence, whereas the activity of the histidine kinase and receiver domains of *Erwinia* RcsC may be essential for amylovoran production in vitro.

Additional keywords: fire blight, polysaccharide, regulation.

Erwinia amylovora, the causal agent of fire blight on apple and pear, is a member of the family *Enterobacteriaceae*, which also includes the genera *Escherichia*, *Pantoea*, *Salmonella*, and *Yersinia*. As one of the main virulence factors, *E. amylovora* produces the capsular exopolysaccharide (EPS) amylovoran to cause disease (3,29). Amylovoran is an acidic heteropolymer composed of a branched repeating unit consisting of galactose, glucose, and pyruvate residues (19). Mutant strains deficient in amylovoran production are nonpathogenic (23). It has been reported that amylovoran has multiple functions, including plugging plant vascular tissues, movement through cortical tissue, retention of water and nutrients, and biofilm formation (16,21,22).

In *E. amylovora*, two-component signal transduction systems play critical roles in sensing of and response to environmental conditions to regulate amylovoran production and virulence (31). Among them, the Rcs phosphorelay system, including RcsBCD, is essential for virulence, and it positively regulates amylovoran production (23). The membrane-localized RcsC is a hybrid sensor kinase composed of a sensor (input), a histidine kinase (transmitter), and a receiver (output) domain. The sensor domain contains a periplasmic region flanked by either one or two transmembrane (TM) fragments (Supplemental Figure 1). Although the exact signal for RcsC is unclear, genetic analyses suggest that,

upon stimulation, RcsC autophosphorylates on a conserved histidine residue in the histidine kinase domain. Subsequently, the phosphoryl group is transferred to an aspartate residue in the receiver domain which, in turn, activates the whole Rcs phosphorelay system through RcsD, another membrane-localized protein, and RcsB, the response regulator (15,17,26). Upon phosphorylation, phospho-RcsB binds to DNA and regulates gene expression. Phospho-RcsB can also be dephosphorylated by RcsC and RcsD in reverse reactions (13).

The Rcs phosphorelay system is first identified by its role in transcriptional regulation of genes for capsular polysaccharide in *Escherichia coli* (17). Later studies have demonstrated that the Rcs system is involved in regulation of a wide array of phenotypes such as swarming motility, antibiotic resistance, and biofilm formation (8,12,25). In *Salmonella enterica* and *Yersinia pseudotuberculosis*, the Rcs system positively regulates multiple virulence factors, and *rsc* mutants are highly impaired in their ability to colonize host cells (6,7,11). In *Pantoea stewartii*, the Rcs phosphorelay system also plays a major role in regulating bacterial virulence (18). Previously, we have demonstrated that the Rcs system in *E. amylovora* is essential for regulating virulence (23). The *rscB* and *rscD* mutants are deficient in amylovoran production and cannot cause disease on host plants. Although the *rscC* mutant produces a high level of amylovoran in vitro, it remains nonpathogenic on host plants (23).

It has been reported that RcsC has both kinase and phosphatase activities in *E. coli* (5,17). In *E. coli*, loss of the RcsC function results in phenotypes with increased mucoidy and decreased motility; this is due, in part, to elevated levels of phospho-RcsB, indicating a net phosphatase activity of RcsC (9). The net direction of the flow of phosphoryl groups and strength of the

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output from this signaling pathway is modulated by different environmental signals as well as other periplasmic proteins such as DjlA (5). Recently, comparative functional studies have shown that biofilm formation in the *E. coli* K-12 *rscC* mutant can be rescued by the RcsC homolog from *S. enterica* but not from *Y. pestis* (13). Furthermore, a chimeric protein composed of the sensor domain of *E. coli* K-12 RcsC and the histidine kinase and receiver domains of *Y. pestis* is capable of fully restoring biofilm formation, thus suggesting a different net kinase activity for RcsC of various sources (i.e., different kinase/phosphatase ratio) (13). These results suggest that there is a link between the RcsC sensor domain and biofilm formation in *E. coli*.

In this study, we comparatively characterized *rscC* homologs from five bacterial species within the family *Enterobacteriaceae*. Comparative analyses showed that the nonpathogenic phenotype of *E. amylovora rscC* mutant could not be complemented by *rscC* homologs from *Escherichia coli* but it could be partially restored by those from *P. stewartii*, *Y. pestis*, and *S. enterica*. Further analyses showed that two chimeric constructs containing sensor domains of either *Erwinia* or *Yersinia* RcsC and histidine kinase and receiver domains of *E. coli* RcsC partially restored virulence of the *Erwinia rscC* mutant, whereas chimeric constructs containing the sensor domain of *E. coli* and histidine kinase and receiver domains of *Yersinia* spp. could not rescue bacterial virulence and growth. In addition, amylovoran production in *Erwinia rscC* was not restored by all other RcsC homologs and chimeric proteins, except for the chimeric construct containing the histidine kinase and receiver domains of *Erwinia* RcsC.

MATERIALS AND METHODS

Bacterial strains and culture media. Bacterial strains and plasmids used in this study are listed in Table 1. The Luria-Bertani (LB) medium was routinely used for culturing *E. amylovora*. When necessary, the following antibiotics were added to the medium: kanamycin at 50 µg ml⁻¹ and ampicillin at 100 µg ml⁻¹. Amylovoran production was determined by growing bacterial cells in MBMA medium (3 g of KH₂PO₄, 7 g of K₂HPO₄, 1 g of [NH₄]₂SO₄, 2 ml of glycerol, 0.5 g of citric acid, and 0.03 g of MgSO₄) containing 1% sorbitol (2).

DNA manipulation and sequence analysis. Plasmid DNA purification, polymerase chain reaction (PCR) amplification of genes, isolation of fragments from agarose gels, cloning, restriction enzyme digestion, and T4 DNA ligation were performed using standard molecular procedures (20). DNA sequencing was performed at the W. M. Keck Center for Functional and Comparative Genomics at the University of Illinois at Urbana-Champaign. Sequence alignment and contig assembly were conducted using Sequencher 4.7 software. Database searches were conducted using the BLAST programs at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST) (1).

Cetylpyrimidinium chloride assay for determining amylovoran concentration. The amylovoran concentration in supernatants of bacterial cultures was quantitatively determined by a turbidity assay with cetylpyrimidinium chloride (CPC) as previously described (23,24,31). Briefly, bacterial suspensions were grown overnight in LB broth with or without appropriate anti-

TABLE 1. Bacterial strains, plasmids, and primers used in this study

Strains, plasmids, or primers	Relevant characters or sequences (5'–3') ^a	Reference or source
<i>Erwinia amylovora</i>		
Ea1189	Wild type, isolated from apple	4
Ea273	Wild type, isolated from apple	24
Z3207Δ <i>rscC</i>	Km ^R -insertional mutant of <i>rscC</i> of Ea1189, Km ^R	23,31
Z0271Δ <i>ompR</i>	Km ^R -insertional mutant of <i>ompR</i> of Ea1189, Km ^R	31
Z0270Δ <i>envZ</i>	Km ^R -insertional mutant of <i>envZ</i> of Ea1189, Km ^R	31
Z0270–71		
Δ <i>envZ/ompR</i>	Km ^R -insertional mutant of <i>envZ-ompR</i> operon of Ea1189, Km ^R	31
Z2198Δ <i>grrA</i>	Km ^R -insertional mutant of <i>grrA</i> of Ea1189, Km ^R	31
Z3742Δ <i>grrS</i>	Km ^R -insertional mutant of <i>grrS</i> of Ea1189, Km ^R	31
Z2797Δ <i>hns</i>	Km ^R -insertional mutant of <i>hns</i> of Ea1189, Km ^R	This study
<i>Escherichia coli</i>		
DH10B	F [–] <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) Φ80 <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>recA1</i> <i>endA1</i> <i>ara</i> Δ139 Δ(<i>ara, leu</i>)7697 <i>galU</i> <i>galK</i> λ- <i>rpsL</i> (Str ^R) <i>nupG</i>	Invitrogen
Plasmids		
pGEM T-easy	Ap ^R , polymerase chain reaction (PCR) cloning vector	Promega Corp.
pWDP2	3.88-kb PCR fragment containing <i>rscC</i> _{Ea} gene in pGEM T-easy vector	23
pWDP782	3.41-kb PCR fragment containing <i>rscC</i> _{Ps} gene in pGEM T-easy vector	This study
pBMM101	Ap ^R , pTrc99a-derivative plasmid carrying a <i>c-myc</i> epitope tag	5
pBMM102	<i>rscC</i> _{Ec} in pBMM101	5
pBMM631	<i>rscC</i> _{Yp-Ec} in pBMM101	13
pBMM639	<i>rscC</i> _{Ec-Yp} in pBMM101	13
pWDP777	<i>rscC</i> _{Ea} in pBMM101	This study
pWDP778	<i>rscC</i> _{Yp} in pBMM101	This study
pWDP781	<i>rscC</i> _{Se} in pBMM101	This study
pWDP779	<i>rscC</i> _{Ea-Ec} in pBMM101	This study
pWDP780	<i>rscC</i> _{Ec-Ea} in pBMM101	This study
Primers ^b		
Ea1	TCGAGCCATGGCCGGGATAACTGGCTTTTCT (<i>Nco</i> I)	...
Ea2	ATAGCTCTAGACGCAACCTGTTACTTAGCGCT (<i>Xba</i> I)	...
Ea3	CAGAAACATCGATTTAGACTGGCTTGC (<i>Cl</i> aI)	...
Ea4	AAACATCGATGTTCTGGCGACCGTGA (<i>Cl</i> aI)	...
Yp1	TCGAGCCATGGTTTTTCATGGTATCCGATTG (<i>Nco</i> I)	...
Yp2	ATAGCTCTAGACGTGATCGCCGTACTTGGTC (<i>Xba</i> I)	...
Se1	TCGAGCCATGGAATACCTTGCTTCTTTCGAAC (<i>Nco</i> I)	...
Se2	ATAGCTCTAGAGCCCGCGTTTACGTACCCGC (<i>Xba</i> I)	...
Ps1	TGGTAAGAACCCGTGTCTCC	...
Ps2	TCGACAAAGAGTAAGTTTTCAGG	...

^a Km^R and Ap^R = kanamycin and ampicillin resistance, respectively.

^b Underlined nucleotides are restriction sites added and the restriction enzymes are indicated at the end of primers.

biotics, harvested by centrifugation, and washed three times with phosphate-buffered saline (PBS). After the final wash, pelleted bacterial cells were resuspended in 200 µl of PBS, and 100 µl of bacterial suspension was added to 10 ml of MBMA medium with 1% sorbitol. Following 2 days of incubation at 28°C with shaking, bacterial cells were pelleted by centrifugation, and 50 µl of CPC (50 mg ml⁻¹) was added to 1 ml of supernatant. After 10 min of incubation at room temperature, amylovoran concentration was determined by measuring optical density at 600 nm (OD₆₀₀) turbidity. The final concentration of amylovoran production was normalized for a cell density of 1.0. For each strain tested, the experiment was repeated at least three times.

Cloning *rscC* genes from *E. amylovora*, *S. enterica*, *Y. pestis*, and *P. stewartii*. The *rscC* genes from *E. amylovora* (*rscC_{Ea}*), *S. enterica* serovar Typhimurium LT2 (*rscC_{Se}*), and *Y. pestis* (*rscC_{Yp}*) were cloned into pBMM101 vector under the control of the *ptc* promoter (5). Briefly, genes were amplified from genomic DNA using high-fidelity platinum *Pfu* DNA polymerase (Invitrogen) and the following primer pairs: Ea1 and Ea2 for *rscC_{Ea}*, Se1 and Se2 for *rscC_{Se}*, and Yp1 and Yp2 for *rscC_{Yp}*. PCR products were digested with *Nco*I and *Xba*I and ligated with the *Nco*I/*Xba*I digested vector, resulting in plasmids pWDP777 (*rscC_{Ea}*), pWDP778 (*rscC_{Yp}*), and pWDP781 (*rscC_{Se}*) (Fig. 1). For cloning the *rscC* gene from *P. stewartii*, the primer pair Ps1-Ps2, without restriction sites, was used to amplify 3.41-kb DNA fragments from the *P. stewartii* strain containing upstream and downstream sequences of the *rscC* gene. The PCR fragment was cloned into the pGEM T-easy vector through A-T ligation. The final plasmids were designated as pWDP782. All plasmids were introduced into *E. coli* DH10B strain by electroporation. Transformants were selected on LB plates supplemented with ampicillin. Their genotypes were confirmed by enzymatic digestion and sequencing.

Construction of chimeric *rscC* constructs. A 1.4-kb DNA fragment encoding the N-terminal region of *rscC_{Ea}* was amplified

using primers Ea1 and Ea3. The Ea3 primer introduced a *Clal* site into the *rscC_{Ea}* sequence and the fragment was digested with *Nco*I and *Clal*. This *Clal* site occurs naturally in the *rscC_{Ec}* allele. Plasmid pBMM102, encoding *rscC_{Ec}*, was digested with *Nco*I/*Clal* and ligated with the digested N-terminal region of *rscC_{Ea}* product, resulting in plasmid pWDP779. Similarly, primers Ea2 and Ea4 were used to amplify a DNA fragment coding for the C-terminal end of *rscC_{Ea}*, while also introducing a *Clal* site at the 5' end of the fragment. This DNA fragment and pBMM102 were digested with *Clal* and *Xba*I and ligated, thus resulting in plasmid pWDP780. The protein sequence around the *Clal* site (SKSM) in the histidine kinase domain is identical among *Erwinia* and *Yersinia* spp. and *E. coli*. All plasmids are sequenced to confirm integrity of the inserts (Fig. 1). Chimeric constructs for *rscC_{Yp-Ec}* (pBMM631) and *rscC_{Ec-Yp}* (pBMM639) have been generously provided by Dr. David Clarke (13).

Virulence assays on apple plants and immature pear fruit. Virulence assays were performed using young annual shoots of 'Gala' apple, 22 to 25 cm in length, by pricking the tip with a needle and pipetting 5 µl of pathogen suspension (OD₆₀₀ = 0.1) onto the wounded tissue. For each bacterial strain, six to seven shoots were inoculated. Plants were kept in a greenhouse at 25°C and a 16-h light photoperiod, and periodically evaluated for disease development for up to 8 days following inoculation by measuring length of the necrotic tissue. The experiment was performed at least three times.

Virulence and bacterial growth assays were performed using immature pear fruit as previously described (27,28,30). Wounded fruit was inoculated with 2 µl of cell suspension (OD₆₀₀ = 0.001) and incubated in a controlled humidity chamber at 26°C. Fruit tissues surrounding the inoculation site were excised using a no. 4 cork borer as described previously (27,28), and homogenized in 0.5 ml of 0.5× PBS. Bacterial growth was monitored by dilution plating on LB medium amended with appropriate antibiotics. For

Fig. 1. Schematic map showing the domain architecture of RcsC homologs from *Escherichia coli* K-12 (*RcsC_{Ec}*), *Erwinia amylovora* (*RcsC_{Ea}*), *Yersinia pestis* (*RcsC_{Yp}*), *Salmonella enterica* (*RcsC_{Se}*), *Pantoea stewartii* (*RcsC_{Ps}*), and four chimeric RcsC constructs used in this study. The *Clal* restriction site and the residues involved in the phosphorelay (H = histidine and D = aspartate) are indicated. Numbers indicate the position of individual residues. HK: histidine kinase; pBMM102: *rscC_{Ec}*; pWDP777: *rscC_{Ea}*; pWDP778: *rscC_{Yp}*; pWDP779: *rscC_{Ea-Ec}*; pWDP780: *rscC_{Ec-Ea}*; pBMM631: *rscC_{Yp-Ec}*; pBMM639: *rscC_{Ec-Yp}*; pWDP781: *rscC_{Se}*; and pWDP782: *rscC_{Ps}*.

each strain tested, fruit were assayed in triplicate, and each experiment was performed at least three times.

RESULTS

Comparative in silico analysis of RcsC from enterobacterial species. The predicted amino acid lengths of RcsC are 880 (*P. stewartii*), 933 (*E. coli*), 948 (*S. enterica*), 957 (*Y. pestis*), and 987 (*E. amylovora*). As predicted by the SMART program, RcsC homologs contain two TM domains, except for *P. stewartii* RcsC, which has a single TM domain (Supplemental Figure S1). Multiple sequence alignment analysis of RcsC homologs has revealed that length variations are mainly due to variations in amino acid residues in sensor domains. A BLAST search has revealed that *E. amylovora* RcsC shares 60, 61, 57, and 66% sequence identities and 75, 75, 73, and 80% sequence similarities with those of *E. coli*, *S. enterica*, *Y. pestis*, and *P. stewartii*, respectively. The sensor domain of *E. amylovora* RcsC shares 41, 43, 39, and 43% sequence identities and 63, 64, 64, and 67% sequence similarities with those of *E. coli*, *S. enterica*, *Y. pestis*, and *P. stewartii*, respectively. Moreover, the histidine kinase and receiver domain of *E. amylovora* RcsC share 68, 68, 65, and 73% sequence identities and 80, 79, 75, and 84% sequence similarities with those of *E. coli*, *S. enterica*, *Y. pestis*, and *P. stewartii*, respectively. These analyses suggest that the histidine kinase and receiver domain of the RcsC sensor kinase is more conserved than the sensor domain in terms of sequence similarities among the five enteric bacteria.

RcsC suppresses amylovoran production in vitro. Previously, we reported that RcsC negatively regulated amylovoran production in vitro (23). The *rscC* mutant produced approximately sevenfold more amylovoran compared with that of the wild type (WT) at 48 h. A similar effect was observed for *rscC*_{Ea} cloned in pBMM101 and pGEM T-easy vectors; both partially restored the amylovoran production phenotype of the mutant (Fig. 2B, Ea1 and Ea2). The complemented strains produced less than half of the amylovoran compared with that of the *rscC* mutant (23). When RcsC was overexpressed in other amylovoran overproducing WT or mutants, decreased amylovoran production by ≥50% in both WT or mutant strains was observed (Fig. 3A). These results indicated that *rscC*_{Ea} acted as a negative regulator of amylovoran production in vitro, suggesting that it possessed a net phosphatase activity.

All RcsC but a single chimeric construct could not restore the amylovoran production phenotype in the *rscC* mutant. To determine whether amylovoran biosynthesis could be complemented by different RcsC homologs and chimeric proteins, bacterial strains were grown in MBMA medium for 48 h and the amylovoran level was quantified by a CPC turbidity assay as described above. In this study, all *rscC* homologs were not capable of complementing amylovoran production in the *rscC* mutant; instead, these homolog-complemented strains produced higher levels of amylovoran than those of the *rscC* mutant (Fig. 3B). These results indicated that RcsC homologs from other enterobacteria could not complement the amylovoran production phenotype in vitro, suggesting that these homologs might yield net kinase activities in *E. amylovora*.

Amylovoran production of the *rscC* mutant complemented by chimeric constructs was also determined (Fig. 3C). Complemented strains containing *rscC*_{Yp-Ec}, *rscC*_{Ec-Yp}, and *rscC*_{Ea-Ec} produced a higher level of amylovoran than that of *rscC* mutant. In contrast, *rscC*_{Ec-Ea} was able to rescue amylovoran production, and the complemented strain produced an amount of amylovoran equivalent to that of the strain expressing *rscC*_{Ea}. These results indicated that the histidine kinase and receiver domains of the *Erwinia* RcsC are essential for amylovoran production in vitro.

All but an RcsC_{Ec} homolog could partially rescue virulence of the *rscC* mutant in vivo. Previously, we demonstrated that

RcsC was required for virulence in *E. amylovora* (23). To test whether different RcsC homologs could rescue virulence, the virulence of the *rscC* mutant complemented with different homolog constructs was tested on Gala apple shoots. Disease severity was periodically measured for up to 8 days as the length of symptom development from the original inoculated site. The WT strain caused visible necrosis around the inoculated site 2 days post inoculation (dpi). The disease rapidly progressed along the shoot, with a mean rate of ≈2.75 cm/day and covered 23.0 cm at 8 dpi (Figs. 2 and 4A). As expected, the *rscC* mutant and *rscC* mutant containing the vector plasmid pBMM101 did not cause

Fig. 2. Amylovoran production of bacterial strains in vitro. **A**, Amylovoran production of the wild-type (WT) and mutant strains with or without expressing *rscC*_{Ea} (pWDP2 or pWDP777). **B**, Amylovoran production of WT, *rscC* mutant, and complementation strains. Vector: Δ*rscC* (vector pBMM101); Ea1: Δ*rscC* (*rscC*_{Ea}, pWDP2); Ea2: Δ*rscC* (*rscC*_{Ea}, pWDP777); Ec: Δ*rscC* (*rscC*_{Ec}, pBMM102); Se: Δ*rscC* (*rscC*_{Se}, pWDP781); Yp: Δ*rscC* (*rscC*_{Yp}, pWDP778) and Ps: Δ*rscC* (*rscC*_{Ps}, pWDP782). **C**, Amylovoran production of WT, *rscC* mutant and *rscC* mutant harboring chimeric constructs. Ea-Ec: Δ*rscC* (*rscC*_{Ea-Ec}, pWDP779); Ec-Ea: Δ*rscC* (*rscC*_{Ec-Ea}, pWDP780); Yp-Ec: Δ*rscC* (*rscC*_{Yp-Ec}, pBMM631); and Ec-Yp: Δ*rscC* (*rscC*_{Ec-Yp}, pBMM639). Bacterial strains were grown in MBMA media with 1% sorbitol for 48 h at 28°C with shaking. The amount of amylovoran was measured with the cetylpyrimidinium chloride assay and normalized to a cell density of 1. Data points represent the means of three replicates ± standard deviations. Similar results were obtained in three independent experiments. OD₆₀₀ = optical density at 600 nm.

disease (Fig. 2). Both *rscC_{Ea}* constructs fully complemented virulence of the mutant on Gala shoots as expected, and mean lengths of diseased tissues were 23.5 and 22.0 cm at 8 dpi (Figs. 2 and 4A; data not shown). Other homologs, including *rscC_{Yp}*, *rscC_{Ps}*, and *rscC_{Se}*, also promoted disease development at varying levels but they were not as effective as *rscC_{Ea}*. However, the *rscC_{Ec}* did not restore disease formation in the *rscC* mutant, suggesting that it could not complement the mutant (Figs. 2 and 4A).

The sensor domain of RcsC_{Ec} affects its ability to complement the *rscC* mutant. To better understand why RcsC_{Ec} cannot complement the virulence of the *rscC* mutant, we constructed several chimeric constructs (Fig. 1). To test whether chimeric *rscC* constructs could complement the *rscC* mutant, a virulence test was performed for the *rscC* mutant harboring different chimeric constructs on Gala plants, and disease severity was determined as described above. Replacement of the sensor domain of *rscC_{Ec}* with that of either *rscC_{Ea}* or *rscC_{Yp}* restored disease formation, with lengths of diseased tissues reaching 18.8 and 4.2 cm, respectively (Figs. 2 and 4B). In contrast, when the sensor domain of *rscC_{Ea}* and *rscC_{Yp}* was replaced with that of *rscC_{Ec}*, the ability to complement the *rscC* mutant to cause disease was completely eliminated (Figs. 2 and 4B). These results indicated that the sensor domain of RcsC might play an important role in the virulence of *E. amylovora*.

Fig. 3. Virulence tests of *Erwinia amylovora* wild type (WT), *rscC* mutant, and *rscC* mutant harboring different *rscC* constructs on 'Gala' apple shoots. Vector: *ΔrscC* (vector, pBMM101); *rscC_{Ea}*: *ΔrscC* (*rscC_{Ea}* pDPW777); *rscC_{Ec}*: *ΔrscC* (*rscC_{Ec}* pBMM102); *rscC_{Se}*: *ΔrscC* (*rscC_{Se}* pWDP781); *rscC_{Ps}*: *ΔrscC* (*rscC_{Ps}* pWDP782) and *rscC_{Yp}*: *ΔrscC* (*rscC_{Yp}* pWDP778). *rscC_{Ea-Ec}*: *ΔrscC* (*rscC_{Ea-Ec}* pWDP779), *rscC_{Ec-Ea}*: *ΔrscC* (*rscC_{Ec-Ea}* pWDP780), *rscC_{Yp-Ec}*: *ΔrscC* (*rscC_{Yp-Ec}* pBMM631), and *rscC_{Ec-Yp}*: *ΔrscC* (*rscC_{Ec-Yp}* pBMM639). Symptoms were shown for WT, *rscC* mutant, and complementation strains at 8 days postinoculation.

Impact of RcsC homologs and chimeric constructs on bacterial growth on immature pear fruit. Symptom development and bacterial growth were also monitored on immature pear fruit for the *rscC* mutant complemented by various homologs and chimeric constructs. As expected, the *rscC* mutant complemented by *rscC_{Ea}* fully restored its virulence and bacterial growth (Fig. 5A; data not shown). RcsC homologs from *Yersinia* and *Pantoea* spp. were also capable of fully rescuing bacterial growth on immature pear fruit (Fig. 5A). However, symptom development for *rscC_{Se}* was slightly delayed and bacterial population size was reduced by ≈ 10 -fold at 2 and 3 dpi (Fig. 5A; data not shown). Furthermore, *E. coli* RcsC was not capable of supporting growth of the *rscC* mutant (Fig. 5A). Survival of the *rscC* mutant containing *rscC_{Ec}* was similar to that of the *rscC* mutant or with the empty vector (data not shown).

Growth of the *rscC* mutant complemented with RcsC chimeric constructs was also monitored. The *rscC_{Ea-Ec}* fully restored the ability of the *rscC* mutant to proliferate on pear fruit and cause symptom development (Fig. 5B; data not shown). Though the *rscC_{Yp-Ec}* also restored the ability of the *rscC* mutant to proliferate

Fig. 4. Disease severity of bacterial strains on 'Gala' apple shoots. **A**, Disease severity of wild type (WT), *rscC* mutant, and complementation strains on apple shoots. **B**, Disease severity of WT and *rscC* mutants harboring chimeric constructs. Length of disease progress was measured at 2, 4, 6, and 8 days postinoculation. Similar results were obtained in repeated independent experiments. pBMM101: vector; pBMM102: *rscC_{Ea}*; pDPW777: *rscC_{Ea}*; pWDP778: *rscC_{Yp}*; pWDP781: *rscC_{Se}*; pWDP782: *rscC_{Ps}*; pWDP779: *rscC_{Ea-Ec}*; pWDP780: *rscC_{Ec-Ea}*; pBMM631: *rscC_{Yp-Ec}*; and pBMM639: *rscC_{Ec-Yp}*. Lines for pBMM101 (I) and pBMM102 (X) were coincided on the X-axis.

on pear fruit, symptom development was slightly delayed (data not shown) and bacterial population size was reduced by ≈ 10 -fold at 2 and 3 dpi (Fig. 5B). Furthermore, the population size of the *rscC* mutant complemented with *rscC*_{Ec-Ea} was significantly reduced by 100-fold compared with that of the WT strain (Fig. 5B), and disease development was also greatly delayed. However, *RcsC*_{Ec-Yp} did not support bacterial growth and disease development (Fig. 5B). These results suggested that the *rscC* sensor domain might greatly contribute to virulence and survival of *E. amylovora* on immature pear fruit, and that the histidine kinase and receiver domains were also involved in these activities.

DISCUSSION

Several genetic factors have been reported to be involved in the regulation of amylovoran, a major virulence factor in *Erwinia amylovora* (23,31). One of the major and direct regulators of amylovoran production is the Rcs phosphorelay system. We have previously demonstrated that mutations in a single *rsc* gene results in its inability to cause disease. In particular, the *rscC* mutant yields interesting phenotypes as it produces a high level of amylovoran in vitro but the mutant remains nonpathogenic on host plants. In this study, we have further characterized the RcsC sensor kinase and its homologs from several enteric bacteria. Our results provide strong evidence that the RcsC kinase has both phosphatase and kinase activities, and these activities play a major role in its ability to control amylovoran production in vitro and disease development in vivo. Our results have also demonstrated that only the chimeric protein *RcsC*_{Ec-Ea} could complement in vitro amylovoran production in the *rscC* mutant, whereas several homologs and *RcsC*_{Yp-Ec} and *RcsC*_{Ea-Ec} chimeric constructs could partially complement virulence of the *rscC* mutant on apple shoots. These results indicate that the sensor domain of RcsC may be important in regulating bacterial virulence, whereas the histidine kinase and receiver domains of *Erwinia* RcsC may be essential for suppressing amylovoran production in vitro. Moreover, these findings also suggest that an unknown in planta signal may be involved in this process, and that the level of disease resistance or susceptibility in the plant host may also play a role in this interaction.

It has been shown that amylovoran biosynthesis in the *E. amylovora* WT strain is induced in planta and the RcsC sensor kinase is required for this process (23). Expression of the *amsG* gene is lower in the *rscC* mutant than in the WT strain, thus suggesting that RcsC might possess net kinase activity during plant infection (23). However, under normal growth either in LB or minimum media, expression of the *amsG* gene (and, thus, amylovoran production) is higher in the *rscC* mutant than in the WT strain. This suggests that RcsC with net phosphatase activity acts as a suppressor of both *ams* gene expression and amylovoran production (23). It has also been reported that RcsC in *E. coli* has a dual function in transferring a phosphoryl group to RcsB (as kinase) or by removing a phosphate from RcsB, in which it acts as a phosphatase (17). However, it remains unclear how RcsC has different functions in planta and in vitro. One possibility is that the kinase/phosphatase ratio may change for the RcsC protein during plant infection. Plant infection may activate some unknown mechanism to suppress phosphatase activity of the RcsC protein in planta so that the kinase activity of the RcsC protein becomes dominant. It is also possible that, during plant infection, the phosphoryl group transfer reactions from RcsC to RcsD and then RcsB may be much stronger than the reversible reactions. Host signals that trigger the phosphorelay reactions may play a role in the function change of the RcsC protein by continuously activating this system (pressure), which is lacking in the in vitro condition. Evidence to prove these hypotheses may help elucidate the nature of such in planta signals.

Our results further confirm the net phosphatase activity of RcsC sensor kinase in the liquid medium as it reduces amylovoran production in *rscS*, *rscA*, *envZ*, *ompR*, and *hns* mutants. These results also indicate that RcsC's role in amylovoran biosynthesis is independent of the signaling pathways controlled by GrrS/GrrA, EnvZ/OmpR, and H-NS, and it is more direct. This is consistent with the hypothesis that the Rcs phosphorelay system is the most downstream pathway in the regulation of amylovoran biosynthesis in *E. amylovora*. It has been proposed that the phosphatase activity of RcsC is mainly dependent on the phosphoreceiver domain. Point mutation studies have demonstrated that the RcsC phosphatase activity requires presence of D859 in the phosphoreceiver domain but it is highly independent of the presence of H463 in the histidine kinase domain in *E. coli* (5). Our results partially support this hypothesis by revealing that only the chimeric protein *RcsC*_{Ec-Ea} containing the phospho-

Fig. 5. Bacterial growth of *Erwinia amylovora* wild type, *rscC* mutant, and complementation strains of the *rscC* mutant on immature pear fruit. **A**, *rscC* mutant complemented with *rscC* homologs. **B**, *rscC* mutant complemented with chimeric constructs. Growth of bacterial strains was monitored at 0, 1, 2, 3, and 4 days postinoculation. Similar results were obtained in repeated independent experiments. pBMM102: *rscC*_{Ec}; pDPW777: *rscC*_{Ea}; pWDP778: *rscC*_{Yp}; pWDP781: *rscC*_{Sc}; pWDP782: *rscC*_{P₅}; pWDP779: *rscC*_{Ea-Ec}; pWDP780: *rscC*_{Ec-Ea}; pBMM631: *rscC*_{Yp-Ec}; and pBMM639: *rscC*_{Ec-Yp}.

receiver domain of *E. amylovora* RcsC fully restores amylovoran production in *rscC* mutant. These results have also demonstrated that all RcsC homologs cannot complement amylovoran production in the *rscC* mutant, suggesting low functional conservation in the phosphoreceiver domain between *E. amylovora* and other homologs, and pointing to different net phosphatase activities or even net kinase activities in vitro. These results further confirm previous reports of a different net kinase activity for the RcsC proteins of various sources (i.e., different kinase/phosphatase ratio) (13).

Although RcsC from *Yersinia*, *Pantoea*, and *Salmonella* spp. were not capable of complementing amylovoran production in vitro, they partially restored bacterial virulence in the *Erwinia rscC* mutant in planta. Knowledge of how RcsC homologs influencing amylovoran production in vivo is hindered by our inability to measure amylovoran production under these conditions due to technical difficulties. In the immature pear fruit assay, similar amounts of ooze production were observed in both the WT and those strains complemented by RcsC from *Yersinia*, *Pantoea*, and *Salmonella* spp., thus suggesting that they might synthesize similar levels of amylovoran in vivo (data not shown). Similar results were also observed between two different WT strains. Ea273 produced >10-fold higher levels of amylovoran than those produced by Ea1189 in vitro, whereas the two strains produced similar amounts of amylovoran and caused similar disease severity on immature pear fruit (24).

It is likely that, upon receiving signals from plants, all RcsC homologs may act as kinases to promote expression of *ams* genes and amylovoran production. It is also possible that the phosphoreceiver domains of *E. amylovora* RcsC homologs behave differently under in vitro and in vivo conditions (i.e., different kinase/phosphatase ratio) (13). Interestingly, we have found that homologs from pathogenic strains (plant or human and animals) could rescue the virulence phenotype of the *rscC* mutant on apple shoots. However, the homolog of a nonpathogenic strain (*E. coli* K-12) could not do so, thus suggesting that there may be some unknown mechanism involved in this process.

Several domain-swapping studies have demonstrated the essential role of the sensor domain for sensing signals (10,13,14). In *E. coli*, the KdpD sensor kinase regulates expression of the *kdpFABC* operon that encodes the high-affinity K⁺ transport system. Domain-swapping studies using KdpD homologs from *E. coli*, *S. enterica*, *Agrobacterium tumefaciens*, and *P. aeruginosa* have demonstrated that full responses to salt stress are only achieved with chimerical constructs containing the *E. coli* KdpD sensor domain (10). The functional variability of the sensor domain has also been observed within a single bacterial species (14). In *Streptococcus pneumoniae*, the ComD sensor kinase responds to pheromone competence stimulating peptide and phosphorylates the response regulator ComC (14). In 17 sequenced *S. pneumoniae* strains, ComD had four types of divergent sequences in its first 70 residues. Further studies have shown that these different sensor domains appear to sense two different types of signal peptides (14). Similarly, a chimeric protein composed of the sensor domain of *E. coli* K-12 RcsC and the histidine kinase and receiver domains of *Y. pestis* are capable of fully restoring biofilm formation in *E. coli* (13). Our results have shown that the sensor domain of RcsC_{Ep}/RcsC_{Yp} is required for RcsC_{Ec} to be able to complement the *rscC* mutant of *E. amylovora* to cause disease. Because *E. coli* K-12 is not a pathogen, our results suggest that the sensor domain of RcsC may have evolved to sense specific host signals. Although this host signal remains unknown, it is likely that this signal may be common among animal and plant hosts. Further studies are needed to identify such signals that may provide opportunities to develop effective disease control measures.

Taken together, our results indicate the existence of functional modularity within the RcsC sensor kinase. The sensor domain of

RcsC may be important for regulating bacterial virulence, whereas the histidine kinase and receiver domains of *Erwinia* RcsC may be essential for amylovoran production in vitro by acting as phosphatase. Due to current technical difficulties, future work pursuing gene expression, especially in vivo gene expression on apple, would further elucidate the functionality of these genes or various domains. Future studies will also focus on identifying sequences or residues in the sensor domain that are involved in the regulation of bacterial virulence and those signals that are sensed by RcsC during host infection.

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