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The Rcs phosphorelay system is essential for pathogenicity in *Erwinia amylovora*

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SUMMARY

The Rcs phosphorelay system is a modified two-component signal transduction system found exclusively in *Enterobacteriaceae*. In this study, we characterized the roles of the Rcs system in *Erwinia amylovora*, a highly virulent and necrogenic enterobacterium causing fire blight disease on rosaceous plants. Our results showed that *rcsB*, *rcsC*, *rcsD* and *rcsBD* mutants were non-pathogenic on immature pear fruit. The bacterial growth of these mutants was also greatly reduced compared with that of the wild-type strain in immature pear fruit. In an *in vitro* amylovoran assay, *rcsB* and *rcsD* mutants were deficient in amylovoran production, whereas the *rcsC* mutant exhibited higher amylovoran production than that of the wild-type. Consistent with amylovoran production, expression of the amylovoran biosynthetic gene *amsG*, using green fluorescent protein as a reporter, was not detectable in *rcsB*, *rcsD* and *rcsBD* mutants both *in vitro* and *in vivo*. The expression of *amsG in vitro* was higher in the *rcsC* mutant than in the wild-type, whereas its expression *in vivo* was higher in the wild-type than in the *rcsC* mutant. In addition, *rcs* mutants were more susceptible to polymyxin B treatment than the wild-type, suggesting that the Rcs system conferred some level of resistance to polymyxin B. Furthermore, *rcs* mutants showed irregular and slightly reduced motility on swarming plates. Together, these results indicate that the Rcs system plays a major role in virulence and survival of *E. amylovora* in immature pear fruit.

INTRODUCTION

Fire blight, caused by the Gram-negative bacterium *Erwinia amylovora*, is one of the most devastating bacterial diseases worldwide. *Erwinia amylovora* is capable of infecting blossoms, fruits, vegetative shoots, woody tissues and rootstock crowns, leading to blossom blight, shoot blight and rootstock blight symptoms (Vanneste, 2000). *Erwinia amylovora* is a member of the *Enterobacteriaceae*, which includes many important human and animal pathogens, such as *Escherichia coli*, *Yersinia pestis*, *Salmonella enterica* and *Shigella flexneri*. Like many other plant pathogenic bacteria, *E. amylovora* utilizes the capsular exopolysaccharide (EPS) to cause disease (Bellemann and Geider, 1992). In *E. amylovora*, the EPS amylovoran is an acidic heteropolymer composed of a pentasaccharide repeating unit containing four galactose residues and one glucuronic acid molecule (Nimtz *et al.*, 1996). Although previous studies have established that amylovoran production is required for the pathogen to cause disease, the precise role of amylovoran in the disease process remains unclear, although a number of functions have been suggested. These functions include the plugging of plant vascular tissues, masking cell surface components to elude plant defences, movement through cortical tissue and retention of water and nutrients (Sjulin and Beer, 1978; Vanneste, 2000). Recently, amylovoran production has been shown to be necessary for biofilm formation in *E. amylovora* (Koczan *et al.*, 2008).

In prokaryotes, the two-component signal transduction (TCST) system is a major regulatory system that utilizes phosphorylation as a means for signal transduction (Chang and Stewart, 1998; Parkinson and Kofoid, 1992). Generally, a typical TCST system is composed of a cognate pair of proteins, a membrane-bound histidine kinase (HK) sensor and a cytoplasmic response regulator (RR). TCST systems play critical roles in the sensing of and response to environmental conditions to regulate a wide array of cellular pathways, especially in bacterial pathogenesis (Flamez *et al.*, 2007; Hoch and Silhavy, 1995). The Rcs phosphorelay system, first identified for its role in the transcriptional regulation of genes for capsular polysaccharide biosynthesis in *Es. coli*, is a very complex and well-studied TCST system (Majdalani and Gottesman, 2005, 2007). The gene organization of the Rcs system is conserved among enterobacteria, in which the *rcsBD* operon is transcribed in one direction and the *rcsC* gene is transcribed in the opposite direction (Fig. 1a; Takeda *et al.*, 2001). One unique feature of the Rcs system is that it is composed of three separate proteins containing four signalling domains (Fig. 1b). The RcsC protein is a hybrid sensor kinase that contains a conserved histidine kinase domain (HisKA) and a receiver domain

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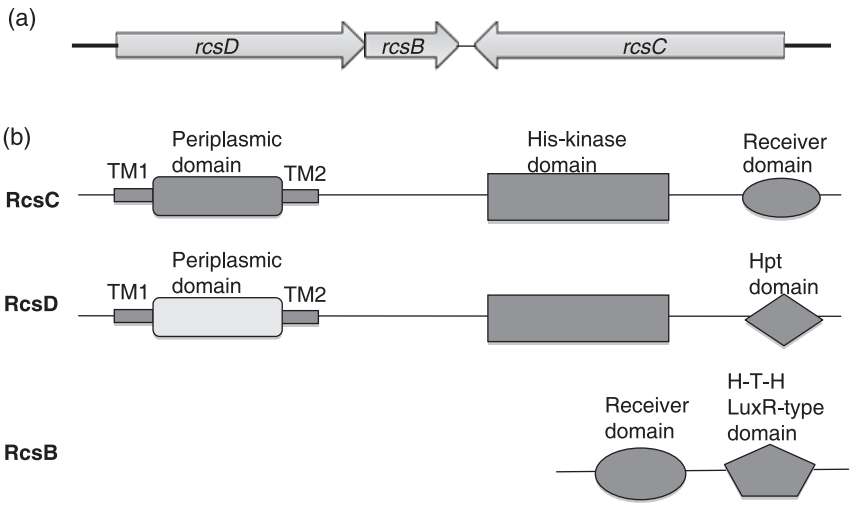

Fig. 1 Schematic maps of genes and proteins of the Rcs phosphorelay system in *Erwinia amylovora*. (a) Organization of the *rcs* genes in *E. amylovora*. (b) Domain organization of Rcs proteins. Domain limits for proteins are derived from the SMART program (www.smart.embl-heidelberg.de). Not drawn to scale. His-kinase, histidine kinase domain; Hpt, histidine phosphotransferase domain; H-T-H, helix–turn–helix motif; TM, transmembrane domain; unlabelled box for RcsD, His-Kinase domain lacking a conserved histidine residue.

(REC) (Rogov *et al.*, 2006). RcsB is the RR and contains a conserved REC (Francez-Charlot *et al.*, 2003). In addition to RcsC and RcsB, a third protein RcsD (also known as YojN), containing a histidine phosphotransfer (HPt) domain, but lacking a conserved histidine residue in the HisKA domain, is also involved in the Rcs phosphorelay process (Huang *et al.*, 2006; Takeda *et al.*, 2001). On stimulation, RcsC is thought to transfer a phosphoryl group from its HisKA domain to its REC domain and then to the HPt domain of RcsD. The phosphoryl group from RcsD travels to the aspartate group of RcsB, and the phosphorylated RcsB further regulates the expression of downstream genes, such as colanic polysaccharide synthesis (*cps*) genes (Huang *et al.*, 2006; Majdalani and Gottesman, 2005; Mizuno, 1998; Parkinson, 1993; Takeda *et al.*, 2001).

In *Es. coli*, the Rcs phosphorelay system positively regulates *cps* gene expression (Jayaratne *et al.*, 1993; Stevenson *et al.*, 1996, Stout and Gottesman, 1990). The colanic acid capsule is produced primarily at low temperature in *Es. coli*, indicating that the colanic acid capsule does not contribute to virulence in *Es. coli* during infection of animals and humans (Allen *et al.*, 1987a,b; Russo *et al.*, 1995). Other studies also report that the Rcs system negatively regulates the *flhDC* operon (Wang *et al.*, 2007), the products of which, in turn, positively act as the master regulator for flagella biosynthesis (Fraser and Hughes, 1999). Mutations in the Rcs system render the pathogen hypermotile (Francez-Charlot *et al.*, 2003). Recently, DNA microarray analysis of the *rcsC* mutant has revealed that more than 150 genes are controlled by *rcsC*; among them, 50% of genes encode proteins either localized to the envelope of *Es. coli* or with activities affecting properties of the bacterial surface (Ferrieres and Clarke, 2003).

Although the Rcs phosphorelay system does not contribute to virulence in *Es. coli*, it plays a role in the regulation of virulence gene expression in other plant and animal pathogens. In the animal pathogen *S. enterica*, the type I capsule, which plays an important role in pathogenesis, is synthesized by genes similar to colanic acid genes of *Es. coli*, and these genes are regulated by the Rcs system (Detweiler *et al.*, 2003; Whitfield and Roberts, 1999). In *Es. coli* and other enterobacteria, type I capsules are involved in protecting the bacteria from desiccation, phages and toxic chemicals, and in biofilm formation. Mutants of *rcsC*, *rcsB* and *rcsD* in *S. enterica* and *Yersinia pseudotuberculosis* poorly colonize host tissues (Hinchliffe *et al.*, 2008; Mouslim *et al.*, 2004). In addition, the Rcs phosphorelay system has been shown to be important for stress resistance *in vitro*, especially for resistance to the cationic peptide polymyxin B in *S. enterica* and resistance to bile in *Y. pseudotuberculosis* (Erickson and Detweiler, 2006; Hinchliffe *et al.*, 2008). In the plant pathogen *Erwinia* (*Pectobacterium*) *carotovora* pv. *carotovora*, the causal agent of soft-rot disease, the Rcs phosphorelay system negatively modulates the expression of plant cell wall-degrading enzymes (Andresen *et al.*, 2007). In *Pantoea stewartii* ssp. *stewartii*, causing Stewart's wilt disease of corn, the synthesis of the EPS stewartan is also dependent on Rcs (Minogue *et al.*, 2005; Wehland *et al.*, 1999). However, Rcs-mediated activation of EPS synthesis is further controlled by a quorum sensing system (EsaI/R) in *P. stewartii* (Koutsoudis *et al.*, 2006; Minogue *et al.*, 2005). A mutation in *esaI* blocks EPS production, even in the presence of a functional Rcs system, thus rendering the mutant avirulent, whereas disruption of the *esaR* gene produces high levels of stewartan constitutively and the mutant is severely attenuated in virulence (Herrera *et al.*, 2008; Koutsoudis *et al.*, 2006).

In *E. amylovora*, the capsule polysaccharide biosynthesis is also regulated by the Rcs system (Bernhard *et al.*, 1993; Wehland *et al.*, 1999). In *E. amylovora*, amylovoran biosynthetic genes are encoded by the *ams* operon from *amsA* to *amsL* (Bugert and Geider, 1995). Previous studies have shown that amylovoran biosynthesis is strongly affected by *rcsA* (Bernhard *et al.*, 1990;

Coleman *et al.*, 1990) and *rcsB* (Bereswill and Geider, 1997; Kelm *et al.*, 1997). RcsB is a typical LuxR family RR with a helix–turn–helix motif in its C-terminal DNA binding domain (Francez-Charlot *et al.*, 2003). However, RcsA is similar to RcsB but without the conserved aspartate residue in its N-terminal REC (Bernhard *et al.*, 1990). Genetic studies have shown that mutation in *rcsB* renders the pathogen non-pathogenic and abolishes amylovoran production, whereas the *rcsA* mutant has reduced virulence but still produces about 10% of amylovoran (Bereswill and Geider, 1997; Bernhard *et al.*, 1990). Further studies have shown that both RcsA and RcsB bind to an 'RcsAB box' (TaA-GaatatTCctA) in the promoter of *amsG* and other genes, as RcsAB heterodimers or RcsB–RcsB homodimers (Wehland *et al.*, 1999). However, the roles of RcsC and RcsD in amylovoran biosynthesis and bacterial virulence have not been studied previously.

The overall goal of this study was to determine the potential roles of the Rcs phosphorelay system in *E. amylovora* virulence and survival. In this study, we first investigated the function of the Rcs phosphorelay system in the virulence of *E. amylovora*. Our results showed that *rcsB*, *rcsC*, *rcsD* and *rcsBD* mutants were unable to grow and cause disease in immature pear tissue. In an *in vitro* assay, both RcsB and RcsD positively regulated the production of amylovoran, whereas RcsC acted as a negative regulator of amylovoran production. Consistent with amylovoran production, *in vitro* gene expression results, using an *amsG* promoter–green fluorescent protein (GFP) fusion as a reporter, demonstrated that the *amsG* gene was not expressed in *rcsB*, *rcsD* and *rcsBD* mutants, but was highly expressed in the *rcsC* mutant. However, expression of the *amsG* gene in the *rcsC* mutant *in planta* was lower than that in the wild-type (WT). Moreover, it was found that the master regulator of flagella biosynthesis, the *flhDC* gene, was up-regulated in all *rcs* mutants, as reported previously in other enterobacteria (Andresen *et al.*, 2007; Francez-Charlot *et al.*, 2003; Hinchliffe *et al.*, 2008). However, *rcs* mutants showed an irregular and partially reduced swarming motility phenotype on swarming plates when compared with WT. Our results also showed that the Rcs phosphorelay system conferred some level of resistance to polymyxin B in *E. amylovora*. Together, these findings indicate that the Rcs phosphorelay system plays a major role in the virulence and survival of *E. amylovora*.

RESULTS

Identification of the Rcs phosphorelay system in *E. amylovora* and generation of the *rcs* mutant

On the basis of the whole genomic sequence of *E. amylovora* (http://www.sanger.ac.uk/Projects/E_amylovora/) and with the aid of a previously reported *rcsB* gene sequence of *E. amylovora* (Bereswill and Geider, 1997; GenBank accession number Y09848), the *rcs* genes within the genome of *E. amylovora* were identified (Fig. 1a). A BLAST search has revealed that RcsB is the most conserved, sharing 92% and 99% amino acid identity and 97% and 100% amino acid similarity with those of *Es. coli* and *E. tasmaniensis*, respectively. RcsC shares 61% and 88% amino acid identity and 76% and 93% amino acid similarity with those of *Es. coli* and *E. tasmaniensis*, respectively. RcsD is the least conserved, sharing 52% and 84% amino acid identity and 70% and 91% amino acid similarity with those of *Es. coli* and *E. tasmaniensis*, respectively. These results indicate that *rcs* genes in *E. amylovora* are more homologous to those of *E. tasmaniensis* than to those of *Es. coli* (Kube *et al.*, 2008).

In order to study the function of the Rcs system in the virulence of *E. amylovora*, we generated four non-polar insertional mutants within the *rcs* genes, including *rcsB*, *rcsC*, *rcsD* and *rcsBD* double mutants, using the λ-Red cloning technique reported previously (Zhao *et al.*, 2006). In these mutants, the endogenous copy of the *rcs* genes was disrupted and replaced with an antibiotic resistance cassette, as described in 'Experimental procedures'.

Mutations in the Rcs phosphorelay system render *E. amylovora* non-pathogenic

To determine the role of RcsB, RcsC and RcsD in *E. amylovora* virulence, we conducted a virulence assay of the *rcs* mutants on immature pear fruits, which have been routinely used to test the pathogenicity of *E. amylovora* (Zhao *et al.*, 2005, 2006). Mutants and WT strains were inoculated on immature pears as described, and disease development was assessed. At 2 days following inoculation, *E. amylovora* WT strain Ea1189 produced water-soaked symptoms in pears with visible bacterial ooze. Four days after inoculation, Ea1189-inoculated immature pears showed necrotic lesions and bacterial ooze formation. After 8 days, the necrosis turned black with more ooze production at the inoculation site. In contrast, no disease symptoms were observed on immature pear fruits inoculated with the *rcs* mutants (Fig. 2a). In another experiment, we inoculated immature pear fruits with an unusually large number [10^{10} colony-forming units (cfu)/mL] of *rcs* mutants, and yet no symptoms were observed (data not shown). These results indicate that the Rcs system is required for *E. amylovora* to induce disease.

To ensure that a lack of disease symptoms of *rcs* mutants was specifically related to mutations in the *rcs* genes, we tested the virulence of *rcs* mutants complemented with cloned original *rcs* genes either in high-copy (for *rcsC* gene) or low-copy (for *rcsBD* genes) vectors. Complemented strains produced similar symptoms and comparable disease progression to the WT strain on immature pear fruits (Fig. 2b). As shown in Fig. 2b, black lesions appeared 4 days following infection and spread systemically in complemented *rcs* mutants by 7 days. These complemented strains also produced ooze, an indicator of amylovoran production.

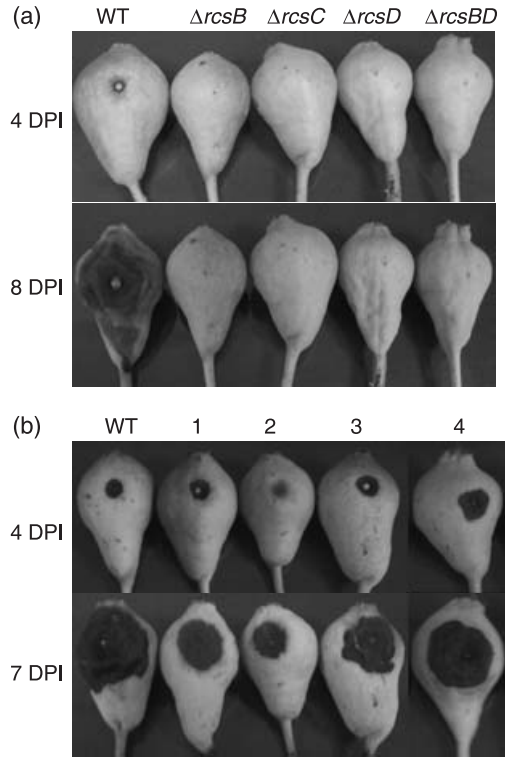

Fig. 2 Pathogenicity tests of *Erwinia amylovora* wild-type (WT) strain, *rcs* mutants and complemented strains of *rcs* mutants on immature pears. (a) Symptoms caused by WT, Δ*rcsB*, Δ*rcsC*, Δ*rcsD* and Δ*rcsBD* mutants in immature pears. (b) Symptoms caused by WT and complementation strains of *rcs* mutants. 1, Δ*rcsB* (pWDP3); 2, Δ*rcsD* (pWDP3); 3, Δ*rcsBD* (pWDP3); 4, Δ*rcsC* (pWDP2). DPI, days post-inoculation.

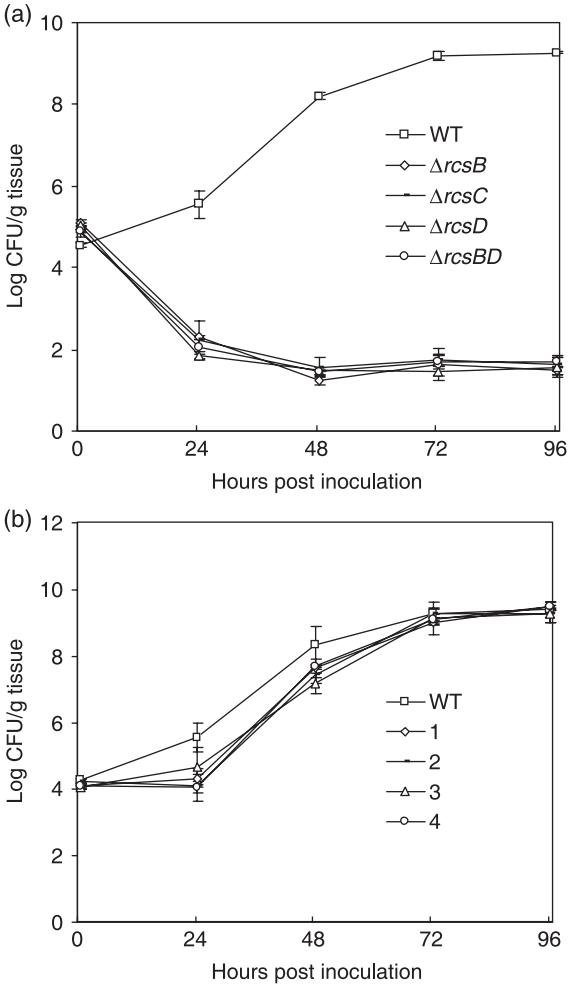

Fig. 3 Bacterial growth of *Erwinia amylovora* wild-type (WT), *rcs* mutants and complemented strains of *rcs* mutants on immature pear fruits. (a) Growth of *E. amylovora* WT, Δ*rcsB*, Δ*rcsC*, Δ*rcsD* and Δ*rcsBD* during infection of immature pears. (b) Growth of WT and complemented strains of *rcs* mutants during infection of immature pears. 1, Δ*rcsB* (pWDP3); 2, Δ*rcsD* (pWDP3); 3, Δ*rcsBD* (pWDP3); 4, Δ*rcsC* (pWDP2). Growth of bacterial strains was monitored at 0, 1, 2, 3 and 4 days after inoculation. Data points represent the means of three replicates ± standard errors. Similar results were obtained in repeated independent experiments. CFU, colony-forming unit.

These results indicate that complementation of *rcs* mutants rescues their virulence. Interestingly, the *rcsB*, *rcsD* and *rcsBD* mutants cannot be complemented by a high-copy-number vector containing the *rcsBD* operon (data not shown). Our results clearly demonstrate that the Rcs phosphorelay system is required for virulence in *E. amylovora*.

The quantification of bacterial growth in infected immature pears was determined for both WT and *rcs* mutants at 4 days post-inoculation. Disease symptoms caused by the WT strain on immature pear fruits were correlated with high levels of bacterial growth in pear tissues (Fig. 3a). In contrast with WT, the bacterial number of *rcs* mutants decreased rapidly following inoculation, representing an approximately 10^6-fold reduction relative to that of WT (Fig. 3a). These results indicate that *rcs* mutants are unable to colonize and survive in immature pears. The growth of complemented strains of *rcs* mutants was also monitored, and these complemented strains were fully restored in growth when compared with the mutant strains by day 3 (Fig. 3b). In summary, these experiments suggest that the Rcs phosphorelay system plays a critical role in the virulence and survival of *E. amylovora* on pears.

The Rcs phosphorelay system regulates amylovoran biosynthesis in *E. amylovora*

To determine whether the *rcs* phosphorelay system of *E. amylovora* affects amylovoran biosynthesis, bacterial cells were grown in amylovoran-inducing medium, and quantitative spectrophotometric assays were performed to determine the levels of amylovoran production *in vitro*. As shown in Fig. 4a, amylovoran production was almost undetectable in *rcsB*, *rcsD* and *rcsBD* mutants, as well as in the *ams* operon deletion

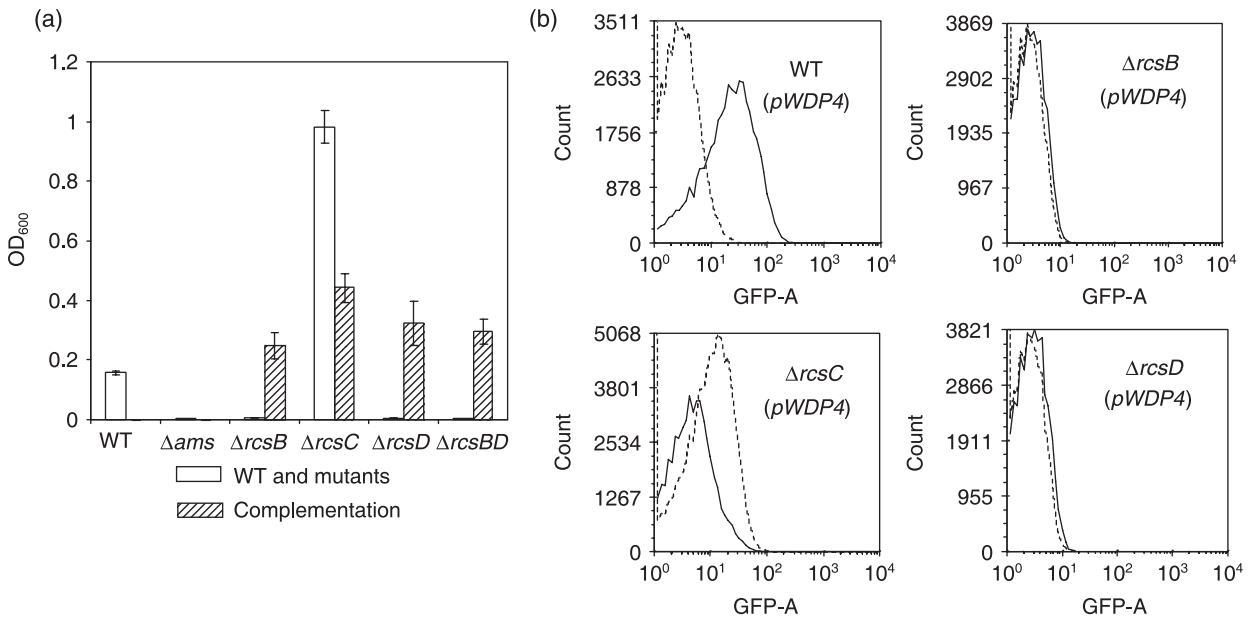

Fig. 4 (a) Amylovoran production of *Erwinia amylovora* wild-type (WT), *rcs* mutants and complemented strains of *rcs* mutants *in vitro*. Bacterial strains were grown in MBMA (3 g KH_2PO_4, 7 g K_2HPO_4, 1 g $(NH_4)_2SO_4$, 2 mL glycerol, 0.5 g citric acid, 0.03 g $MgSO_4$) medium with 1% sorbitol for 72 h at 28 °C with shaking. The amount of amylovoran was measured with the cetylpyrimidinium chloride (CPC) assay and normalized to a cell density of unity. Data points represent the means of three replicates ± standard deviation. Similar results were obtained in three independent experiments. Δ*ams*, an amylovoran biosynthesis operon (*amsA-L*) deletion mutant (Zhao and Sundin, 2008). (b) Expression of the *amsG* gene in WT and *rcs* mutants *in vitro* and *in vivo*. The green fluorescent protein (GFP) intensity in WT strain and *rcs* mutants containing the *amsG* promoter–GFP fusion plasmid was measured by flow cytometry. The experiment was repeated at least three times and similar results were obtained. Full line, *in vivo* on immature pear fruits; broken line, *in vitro* in MBMA plus 1% sorbitol. Count, number of bacterial cells; GFP-A, green fluorescent protein absorbance.

mutant (as a control) (Zhao and Sundin, 2008). In contrast, the *rcsC* mutant produced a 6.2-fold increase in amylovoran production compared with that of WT 3 days after inoculation (Fig. 4a).

To verify whether a lack or overproduction of amylovoran *in vitro* in *rcs* mutants was specifically related to mutations in *rcs* genes, amylovoran biosynthesis was measured by the complementation of *rcs* mutants. We found that amylovoran biosynthesis in the *rcsC* mutant was partially complemented by introducing the original *rcsC* gene in a high-copy-number vector. As shown in Fig. 4a, the complemented strain of the *rcsC* mutant produced less than one-half of the amount of amylovoran relative to that of the *rcsC* mutant, but produced at a level of approximately twofold higher than that of WT. Complemented strains of *rcsB*, *rcsD* and *rcsBD* mutants by a low-copy-number vector containing the *rcsBD* operon also produced relatively higher levels of amylovoran, with optical density at 600 nm (OD_{600}) values of 0.24, 0.32 and 0.30, respectively, compared with 0.16 for WT. These results indicate that the Rcs system regulates amylovoran biosynthesis either as a negative regulator (RcsC) or as a positive regulator (RcsBD) *in vitro*.

In order to correlate amylovoran production with amylovoran biosynthesis gene expression, the promoter of the *amsG* gene, the first gene in the amylovoran operon, was cloned in a promoterless GFP reporter vector. The GFP intensity was measured by flow cytometry in WT and *rcs* mutants containing the *amsG* promoter–GFP fusion plasmid in liquid medium. As shown in Fig. 4b, the *amsG* gene was expressed at a basal level in the WT strain, with a GFP intensity value of 1.70 (geometric mean), relative to the vector control (GFP value of about 1.50). The GFP intensity level of the *amsG* promoter in *rcsB*, *rcsD* and *rcsBD* mutants was measured to be 1.47, 1.48 and 1.46, respectively (Table 1). However, in the *rcsC* mutant, the GFP intensity (geometric mean of 5.64) of the *amsG* promoter was more than three times higher than that of WT (Table 1). The GFP intensity was also measured for both WT and *rcs* mutants containing the *amsG* promoter–GFP fusion following inoculation onto immature pear fruits for up to 2 days. The expression of *amsG* in *rcsB*, *rcsD* and *rcsBD* mutants *in vivo* was similar to that *in vitro*. The GFP intensity of the *amsG* gene in WT increased by more than twofold from *in vitro* (1.70) to *in vivo* (3.95). In contrast, *amsG* expression in the *rcsC* mutant was lower *in vivo* (2.75) than *in vitro* (5.64) (Fig. 4b; Table 1). These results indicate that the expression of *amsG in vitro* and *in vivo* is correlated with amylovoran production in WT and *rcs* mutants. Moreover, the *amsG* gene is induced in WT *in planta*.

Bacterial strain	GFP intensity *in vitro* (geometric mean ± standard deviation)	GFP intensity *in vivo* (geometric mean ± standard deviation)
WT (pWDP4)	1.70 ± 0.04	3.95 ± 0.12
Δ*rcsB* (pWDP4)	1.47 ± 0.02	1.52 ± 0.02
Δ*rcsC* (pWDP4)	5.64 ± 0.05	2.75 ± 0.15
Δ*rcsD* (pWDP4)	1.48 ± 0.04	1.53 ± 0.05
Δ*rcsBD* (pWDP4)	1.46 ± 0.01	1.51 ± 0.03
WT (pSN1)	2.21 ± 0.08	ND
Δ*rcsB* (pSN1)	8.23 ± 0.24	ND
Δ*rcsC* (pSN1)	8.86 ± 0.62	ND
Δ*rcsD* (pSN1)	9.24 ± 0.15	ND
Δ*rcsBD* (pSN1)	9.66 ± 0.36	ND

in vitro, liquid broth; *in vivo*, pear slices; ND, not determined.

Table 1 Green fluorescent protein (GFP) fluorescence intensity in wild-type (WT) and *rcs* mutants containing *amsG* and *flhDC* promoter–GFP fusion plasmid.

The Rcs phosphorelay system negatively regulates *flhDC* gene expression, but swarming motility is only partially affected

In *Es. coli*, mutations in the Rcs system render *Es. coli* hypermotile (Francez-Charlot *et al.*, 2003; Takeda *et al.*, 2001; Wang *et al.*, 2007). Swarming motility was assessed for both WT and *rcs* mutants by inoculating bacterial cells on a swarming plate (0.3% agar) and measuring the diameter of the circle covered by bacterial cells 48 h after inoculation. The *rcs* mutants exhibited irregular and partially reduced motility compared with that of the WT strain, which showed a round circle of 3.01 cm in diameter 2 days following inoculation (data not shown). However, the diameters for the *rcsB*, *rcsC*, *rcsD* and *rcsBD* mutants were about 2.51 ± 0.17, 2.45 ± 0.2, 2.48 ± 0.28 and 2.43 ± 0.11 cm (longest part of the irregular movement), respectively. The irregular movement could only be partially restored in *rcs* mutants complemented with their corresponding genes (data not shown). These results indicate that mutation in the Rcs system in *E. amylovora* does not confer hypermotility as in *Es. coli*, but instead renders them less motile.

To determine whether the Rcs phosphorelay system regulates *flhDC* gene expression in *E. amylovora*, we also constructed a *flhDC* promoter–GFP fusion and introduced it into the WT and *rcs* mutants. The GFP intensity values of the *flhDC* promoter in *rcsB*, *rcsC*, *rcsD* and *rcsBD* mutants were about 8.6, 8.8, 9.2 and 9.6, respectively, approximately four times higher than that detected for WT (2.2) (Table 1). Together, these findings indicate that the *flhDC* gene is up-regulated in *rcs* mutants when bacterial cells are grown on swarming plates, and that the Rcs system acts as a negative regulator for *flhDC* gene expression in *E. amylovora*.

The Rcs phosphorelay system in *E. amylovora* regulates resistance to polymyxin B

To determine whether the Rcs phosphorelay system regulates resistance to polymyxin B in *E. amylovora*, WT and *rcs* mutant strains were tested for their sensitivity to polymyxin B exposure (killing effect), and the survival number of each strain was counted by serial dilution plating. After 1 h of polymyxin B treatment, the survival rate of WT was about 3.2%, whereas the survival rates of *rcs* mutants were about 0.52%, 0.45%, 0.48% and 0.36% for *rcsB*, *rcsC*, *rcsD* and *rcsBD* mutants, respectively (Fig. 5). We also observed slower recovery [smaller colony forming on Luria–Bertani (LB) plates] of *rcs* mutants. The colony of the WT strain was visible after 16 h on LB plates, whereas it took about 25 h for *rcs* mutants to become visible. Complementation of *rcsB*, *rcsC*, *rcsD* and *rcsBD* mutants rescued the survival rates to 3.3%, 2.7%, 2.9% and 2.8%, respectively (Fig. 5). On polymyxin B treatment, complemented strains were visible 18 h after plating on LB plates. These results indicate that the Rcs system confers some level of resistance to polymyxin B in *E. amylovora* and plays a role in the survival of the pathogen.

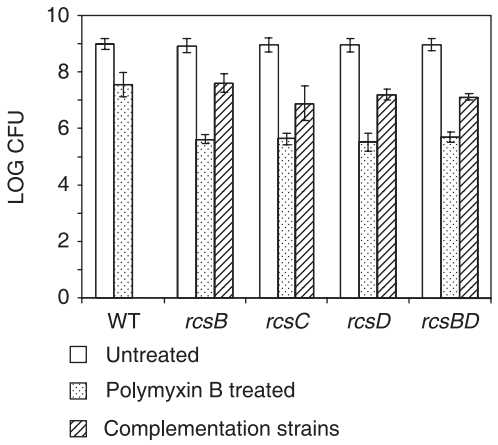

Fig. 5 Polymyxin B killing assay. Bacterial strains were grown overnight in Luria–Bertani (LB) broth and treated with an equal volume of polymyxin B (3 μg/mL) for 1 h. Surviving bacterial cells were counted by plating on LB plates. Data points represent the means of three replicates ± standard deviations. The experiment was repeated at least three times. CFU, colony-forming unit.

DISCUSSION

Fire blight studies have contributed greatly to the elucidation of the genetic, molecular and physiological basis of *E. amylovora* pathogenesis. Early studies revealed that *E. amylovora* utilizes an essential virulence system—the *hrp* type III secretion system (T3SS)—to deliver effector proteins into host plants and to cause disease (Oh and Beer, 2005; Wei *et al*., 1992). In addition to T3SS and its associated effector proteins, other major virulence determinants identified in *E. amylovora*, contributing to pathogenesis and plant colonization, include the acidic extracellular polysaccharide amylovoran (Bernhard *et al*., 1993; Steinberger and Beer, 1988).

In this study, our results have demonstrated that the Rcs phosphorelay system not only regulates amylovoran biosynthesis, but also renders the pathogen more resistant to the antimicrobial peptide (AMP) polymyxin B. This suggests that the Rcs phosphorelay system controls one of the virulence factors in *E. amylovora*, and may also play a major role in the survival of *E. amylovora*.

TCST systems are complicated regulatory systems used by microorganisms to sense and respond to environmental signals which result in gene expression (Stock *et al*., 1990). As one of the most studied TCST systems, the Rcs phosphorelay system has been shown to contribute to the virulence of many pathogenic bacteria, including *Salmonella* and *P. stewartii* (Dominguez-Bernal *et al*., 2004; Mouslim *et al*., 2004; Wehland *et al*., 1999). Previous studies have shown that RcsB contributes to virulence in *E. amylovora* (Kelm *et al*., 1997). Our studies confirm this and, for the first time, further prove that RcsC and RcsD are also essential for virulence. In another experiment, *rcs* mutants remained capable of inducing a hypersensitive response in tobacco (Y. Zhao, unpublished data), thus indicating that T3SS remains functional in *rcs* mutants. These findings suggest that the Rcs system contributes to virulence in *E. amylovora* in part by specifically regulating amylovoran biosynthesis. Interestingly, when the complementation of *rcs* mutants was attempted, we failed to complement *rcsB*, *rcsD* and *rcsBD* mutants when *rcsBD* genes were cloned in a high-copy-number vector (pGEM T-easy), but succeeded when *rcsBD* genes were cloned in a low-copy-number vector (pWSK29). It is likely that an overabundance of unphosphorylated RcsB may provide dominant negative regulation by competing for binding to the *amsG* promoter or by acting as a repressor.

It seems that amylovoran biosynthesis in the *E. amylovora* WT strain is tightly controlled by the Rcs system. In this study, the *rcsC* mutant produced more amylovoran than did WT, and the *amsG* gene was highly expressed *in vitro* in this mutant. At first, it was difficult to explain these findings, as the mutant did not cause disease on immature pear fruits and no ooze was observed. However, similar observations have been reported in *S. enterica*, wherein mutations in *rcsC* resulted in constitutive capsule synthesis, and *rcsC* mutants were severely attenuated in virulence (Garcia-Calderon *et al*., 2005). Furthermore, the *P. stewartii* strain that mutated in *esaR*, having a dominant effect on Rcs-mediated activation of stewartan synthesis, constitutively synthesized stewartan, and was significantly reduced in virulence (Koutsoudis *et al*., 2006; Minogue *et al*., 2005).

The above observations have raised questions as to why mutations in *rcsC* result in the overproduction of EPS, and why the overproduction of EPS renders the pathogen less virulent, in spite of the fact that EPS is a virulence factor. One possibility is that RcsC may be needed to sense an unknown signal *in vivo*, and thus specifically phosphorylates RcsBD *in vivo*; yet RcsBD could be cross-phosphorylated by cross-talking kinases or small phospho-donors *in vitro*. Recent studies have demonstrated that HKs have significant kinetic preferences for their cognate RRs to prevent unwanted cross-talk *in vivo*. In other words, HKs have intrinsic abilities to recognize their cognate substrates *in vivo* (Skerker *et al*. 2005). Further studies have shown that a subset of co-evolving residues around H-boxes is sufficient to control the specificity of HKs (Skerker *et al*., 2008). Nevertheless, given the complex nature of the Rcs phosphorelay system, it has also been proposed that additional input signals may be sensed by RcsD in liquid medium (Majdalani and Gottesman, 2005). Therefore, another possible explanation is that these additional input signals may trigger the phosphorylation of RcsD or RcsB by cross-phosphorylation, thus activating amylovoran biosynthesis *in vitro* in the *rcsC* mutant background, but not *in vivo*. Furthermore, it has been shown that the RcsC sensor kinase in *Es. coli* controls the expression of a regulon in response to growth on a solid surface (Ferrieres and Clarke, 2003). The expression of *cpsB* increases in an RcsC-dependent manner when cells are grown on a solid surface (Ferrieres and Clarke, 2003). This suggests that a solid surface can serve as an environmental signal to activate the RcsC kinase. Our *in vivo* gene expression data support this, thus indicating that a solid surface can be an environmental factor in inducing the expression of the *amsG* gene, and this may depend on RcsC through sensing of this solid surface signal.

It has been reported that RcsC has a dual function in transferring a phosphoryl group to RcsB (as a kinase) or by removing a phosphate from RcsB (as a phosphatase) (Majdalani and Gottesman, 2005). The first evidence for a dual role of RcsC is provided by the expression of the promoter for another target gene of RcsB, the small regulatory RNA *RprA* (Majdalani *et al*., 2002, 2005). In *Es. coli*, the expression of *rprA* is off in the WT strain, but a null mutation of *rcsC* leads to higher levels of *rprA* expression. This suggests that the loss of RcsC (phosphatase) prevents the removal of the phosphate from RcsB, which may be cross-phosphorylated from unknown sources, resulting in the activation of RcsB activity. Further evidence that RcsC acts as a phosphatase is provided by the observation that cells carrying a point mutation in *rcsC137* in *Es. coli* constitutively express *cps*

genes at high levels (Clarke *et al.*, 2002). Recessive *Salmonella rcsC* mutants with constitutive activation of the Rcs system provide further evidence that RcsC has both positive and negative regulatory effects on EPS biosynthesis (Garcia-Calderon *et al.*, 2005). In this study, the *amsG* gene was slightly expressed in the WT strain *in vitro*, but expressed at higher levels in a liquid medium in the *rcsC* mutant *in vitro*, suggesting that the activation of RcsB from an unknown source in liquid medium is not dephosphorylated by RcsC.

In *P. stewartii*, the hypermucoid *esaR* mutant is highly attenuated in virulence (Minogue *et al.*, 2005). It has been proposed that this bacterium may predominantly require an EPS-free state to perform functions such as surface adhesion at low cell density (Herrera *et al.*, 2008; Koutsoudis *et al.*, 2006). Consequently, premature synthesis of EPS interferes with the normal disease process. Further evidence indicates that both spatiotemporal gene expression and EPS production greatly influence the degree of bacterial adhesion during *in vitro* biofilm formation and dissemination within a host plant (Herrera *et al.*, 2008; Koutsoudis *et al.*, 2006). In addition, reduced virulence of *Salmonella rcsC* mutants with constitutive activation of the Rcs system could be partially restored by a double mutation in either the *cps* or *rcsA* gene (Garcia-Calderon *et al.*, 2005). These results suggest that the overproduction of type I EPS is one of the reasons for virulence attenuation in *Salmonella rcsC* mutants. Thus, a similar scenario may be involved in the findings observed in this study, and, indeed, biofilm formation has recently been implied in the pathogenicity of *E. amylovora* (Koczan *et al.*, 2008). However, one of the differences pertains to the fact that the *rcsC* mutant in *E. amylovora* has shown the overproduction of amylovoran *in vitro*, although this was not constitutive. Further studies are needed to determine the roles of amylovoran production, bacterial adhesion and biofilm formation in the virulence of *E. amylovora*.

Several environmental conditions have been reported to activate the Rcs phosphorelay system, including growth on a solid surface, desiccation and changes in osmolarity (Majdalani and Gottesman, 2005). One of the examples for the observed responses to solid surfaces is swarming motility. In both *Es. coli* and *S. enterica*, the RcsCDB phosphorelay system has been shown to negatively regulate swarming motility (Takeda *et al.*, 2001; Wang *et al.*, 2007). Mutations in *rcsC*, *rcsD* and *rcsB* lead to a hypermotile phenotype and higher expression of the *flhDC* operon, the master regulator of flagella biosynthesis. Products of *flhDC* genes, in turn, positively regulate second (such as *fliA*) or third (such as *fliC*) class genes for flagella biosynthesis (McCarter, 2006). This negative regulation of the *flhDC* operon by the Rcs system is in agreement with other supporting evidence for reduced motility under conditions inducing *cps* synthesis in *Es. coli*. Consistent with previous findings, our results demonstrate that the expression of the *flhDC* operon is higher in all *rcs* mutants than in WT in *E. amylovora*. Surprisingly, *rcs* mutants have shown irregular and reduced swarming motilities on swarming plates when compared with those of WT, exhibiting circular movement. It is worth noting here that we have identified several other TCST mutants that are either hypermotile (circle) or non-motile on swarming plates. However, all of these mutants have shown even higher expression of the *flhDC* operon than that in *rcs* mutants (Y. Zhao, unpublished data). These data further indicate that, in addition to flagella, other factors also control swarming motility in *E. amylovora*. Irregular movements in *rcs* mutants may be attributed to the remodelling of bacterial surfaces during growth on a solid surface, as reported recently for *Es. coli* (Ferrieres and Clarke, 2003). Indeed, DNA microarray analysis of the *rcsC* mutant has revealed that more than 150 genes are controlled by *rcsC*, and 50% of these genes encode proteins that are either localized to the envelope of *Es. coli* or have activities affecting properties of the bacterial surface, e.g. the production of colanic acid. Further studies are needed to elucidate why *Es. coli* and *E. amylovora rcs* mutants show different motility behaviour, even though *flhDC* genes are up-regulated in both cases.

Endogenous AMPs produced by plants are known to play important roles in the host defence system and innate immunity (Oard and Enright, 2006). Most AMPs are cationic and are believed to interact with their primary target, the cell membrane. They are reported to kill bacteria by binding to lipopolysaccharides (LPSs) through ionic bonds with negatively charged phosphoryl groups of the lipid A (Rosenfeld and Shai, 2006). Several TCST systems, including PhoP/Q, PmrA/B and the Rcs system, have been reported to regulate resistance to AMPs, including the peptide antibiotic polymyxin B (Erickson and Detweiler, 2006; Hancock and McPhee, 2005; Lee *et al.*, 2004). These regulatory factors are required for the modification of the LPS structure, leading to a reduction of negative charges along the surface, thus altering interactions with AMPs. Many TCST system-targeted genes, including *ugd* and *pbgP*, are responsible for mediating the modifications of LPS necessary for resistance to polymyxin B (Kato *et al.*, 2007, Mouslim and Groisman, 2003). It has been shown that the *rcsC* gene in *S. typhimurium* regulates the expression of *ugd*, a gene that is responsible for the synthesis and incorporation of L-aminoarabinose into LPS to induce resistance to polymyxin B (Mouslim and Groisman, 2003). Our results clearly indicate that the Rcs system in *E. amylovora* confers some level of resistance to polymyxin B. It is probable that the Rcs system in *E. amylovora* also affects properties of the bacterial surface, which leads to resistance to polymyxin B. Although the mechanism remains unknown, it is tempting to speculate that the Rcs phosphorelay system increases *E. amylovora* resistance to AMPs, thus playing a role in the survival of the pathogen during the infection of plant tissues. It will be interesting to investigate whether there are any cross-talks between different

TCST systems in *E. amylovora* that regulate resistance to AMPs, as reported in other enterobacteria.

In summary, we have confirmed previous reports and further demonstrated that the Rcs system is essential for *E. amylovora* to cause disease. Although the Rcs systems in *Es. coli* and *E. amylovora* both control the biosynthesis of extracellular polysaccharide and negatively regulate the *flhDC* operon, the phenotypes of *rcs* mutants in these two bacteria are quite different. The Rcs system does not contribute to the virulence of *Es. coli*, but mutations of the *rcs* system render it hypermotile. However, the Rcs system does contribute to the virulence of *E. amylovora*, but mutations of the *rcs* system only partially affect motility. It is tempting to speculate that, during evolution, the Rcs system maintains its basic function to regulate gene expression; however, it may have adapted to specific host niches and sense specific host signals to control many important cellular processes, including motility and virulence. Our future work will focus on the identification of genes that are regulated by the Rcs system globally, the study of how the Rcs system interacts with other TCST systems, such as PhoP/Q and PmrA/B, and the determination of the roles of other components of the Rcs system, such as *rcsF* (Majdalani *et al.*, 2005), during the infection of host plants.

EXPERIMENTAL PROCEDURES

Bacterial strains and culture media

The bacterial strains and plasmids used in this study are listed in Table 2. LB medium is used routinely for culturing *E. amylovora*. When necessary, the following antibiotics were added to the medium: 50 μg/mL kanamycin (Km) and 100 μg/mL ampicillin (Ap). Amylovoran production was determined by the growth of bacteria in MBMA medium (3 g KH_2PO_4, 7 g K_2HPO_4, 1 g $(NH_4)_2SO_4$, 2 mL glycerol, 0.5 g citric acid, 0.03 g $MgSO_4$) plus 1% sorbitol (Bellemann *et al.*, 1994).

DNA manipulation and sequence analysis

Plasmid DNA purification, polymerase chain reaction (PCR) amplification of genes, isolation of fragments from agarose gels, cloning, and restriction enzyme digestion and T4 DNA ligation were performed using standard molecular procedures (Sambrook *et al.*, 1989). DNA sequencing was performed at the Keck Center for Functional and Comparative Genomics (University of Illinois at Urbana-Champaign, IL, USA). Sequence management 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 (NCBI) (www.ncbi.nlm.nih.gov/BLAST) (Altschul *et al.*, 1997).

Construction of *rcs* mutants in *E. amylovora* by λ-Red recombinase cloning

Erwinia amylovora stable mutants were generated using λ phage recombinases, as described previously (Datsenko and Wanner, 2000; Zhao *et al.*, 2006). Briefly, *E. amylovora* Ea1189 was transformed with plasmid pKD46 expressing recombinases redα, redβ and redγ. The transformant Ea1189 (pKD46) was grown overnight at 28 °C, reinoculated in LB broth containing 0.1% arabinose and grown to exponential phase $OD_{600} = 0.8$. Cells were collected, made electrocomponent and stored at –80 °C. Recombination fragments consisting of a *kan* gene with its own promoter, flanked by a 50-nucleotide homology arm, were generated by PCR using plasmid pKD13 as a template. For the primers used for the generation of *rcs* mutants, the 5′ end of each primer contained 50-nucleotide homology arms corresponding to the first and last 50 bases of the corresponding gene(s), whereas the remaining 21 or 20 nucleotides of each primer at the 3′ end were designed to amplify the *kan* cassette from pKD13. For the generation of the *rcsB*, *rcsC*, *rcsD* and *rcsBD* mutants, the primer pairs B3205F-R, B3207F-R, B3206F-R and B3205F-B3206R were used to amplify *kan* gene fragments from the pKD13 vector. To confirm *rcsB*, *rcsC*, *rcsD* and *rcsBD* mutants by PCR, primers Z3205F, Z3206R, Z3207F-R and internal primer pairs km1 and km2 of the *kan* gene were used. PCR products were gel purified using a gel purification kit from Promega (Madison, WI, USA). Electroporation was performed according to standard protocols, and transformants were plated on LB with Ap and Km. For the resulting mutants, the majority of the coding region of each gene was replaced by the Km^R marker, except for the first and last 50 nucleotides. The *rcsB*, *rcsC*, *rcsD* and *rcsBD* mutants were designated as Z3205Δ*rcsB*, Z3207Δ*rcsC*, Z3206Δ*rcsD* and Z3205-06Δ*rcsBD*, respectively.

Cloning *rcs* genes for complementation of the *rcs* mutants

For complementation of the *rcs* mutants, the regulatory and gene sequences of the *rcs*BD operon and *rcs*C open reading frame (ORF) were used to design primers to amplify fragments of the gene and/or operon and their promoter sequences. Primer pairs rcsBD1-rcsBD2, without restriction sites, and rcsC1-rcsC2 were used to amplify 4.54-kb and 3.88-kb DNA fragments from the *E. amylovora* WT strain, which contain upstream and downstream sequences of the *rcs*BD operon and the *rcs*C gene, respectively. The two PCR fragments were cloned into pGEM T-easy vector through A–T ligation. The final plasmids were designated pWDP1 and pWDP2, respectively.

The *rcsBD* operon was also cloned into a low-copy-number vector pWSK29. Primer pair rcsBD1-rcsBD2, containing *Eco*RI and *Bam*HI restriction sites, was used to amplify the *rcsBD*

Table 2 Bacterial strains, plasmids and primers used in this study.

Strain, plasmid or primer	Relevant characters or sequences (5′–3′)*	Reference or source
Strain		
E. amylovora		
Ea1189	Wild-type, isolated from apple	Burse *et al.* (2004)
Z3205Δ*rcsB*	*rcsB::Km;* Km^R-insertional mutant of *rcsB* of Ea1189, Km^R	This study
Z3207Δ*rcsC*	*rcsC::Km*; Km^R-insertional mutant of *rcsC* of Ea1189, Km^R	This study
Z3206Δ*rcsD*	*rcsD::Km*; Km^R-insertional mutant of *rcsD* of Ea1189, Km^R	This study
Z3205-06Δ*rcsBD*	*rcsBD::Km*; Km^R-insertional mutant of *rcsDB* of Ea1189, Km^R	This study
Z0118Δ*ams*	Km^R-insertional mutant of *ams* operon (15.8 kb) of Ea1189, Km^R	Zhao and Sundin (2008)
Es. Coli		
DH10B	F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*Δ139 Δ(*ara*, *leu*)7697 *gal*U *gal*K λ—*rps*L (Str^R) *nup*G	Invitrogen (Carlsbad, CA, USA)
Plasmid		
pKD46	Ap^R, P_{BAD} gam bet exo pSC101 oriTS	Datsenko and Wanner (2000)
pKD13	Km^R, FRT cat FRT PS1 PS2 oriR6 K rgbN	Datsenko and Wanner (2000)
pGEM® T-easy	Ap^R, PCR cloning vector	Promega
pFPV25	Ap^R, GFP-based promoter trap vector containing a promoterless *gfpmut3a* gene	Valdivia & Falkow (1997)
pWSK29	Ap^R; cloning vector, low copy number	Wang and Kushner (1991)
pWDP1	4.54-kb PCR fragment containing *rcsBD* gene in pGEM T-easy vector	This study
pWDP2	3.88-kb PCR fragment containing *rcsC* gene in pGEM T-easy vector	This study
pWDP3	4.54-kb *Eco*RI-*Bam*HI fragment containing *rcsBD* gene in pWSK29	This study
pWDP4	721-bp KpnI-XbaI DNA fragment containing promoter sequence of *amsG* gene in pFPV25	This study
pSN1	906-bp *Eco*RI-*Bam*HI DNA fragment containing promoter sequence of *flhD* gene in pFPV25	This study
Primer†		
B3205F	ATGTTGCCATATAAATTTCCGCTAACTTCCGGCAATGTAACACGATTTTTGCGATTGTGTAGGCTGGAGCT	
B3205R	TTATTGGTTACCTTGCTGCAGGAGATCTTTGACATAAGTGTCAACTTCACATTCCGGGGATCCGTCGACC	
B3206F	GTGAAGTTGACACTTATGTCAAAGATCTCCTGCAGCAAGGTAACCAATAAGCGATTGTGTAGGCTGGAGCT	
B3206R	TTATTTATCTACCGGCGTCATGCTTACTGATGACAGGTAGTTAAGCAAAGATTCCGGGGATCCGTCGACC	
B3207F	ATGGCCGGGATAACTGGCTTTTTCTGCCTCCCGGCGCTACTGTATCGGCAGCGATTGTGTAGGCTGGAGCT	
B3207R	TTACCGCAACCTGTTACTTAGCGCTTCCCGGCTCTTTCTTACCCGCCCGGATTCCGGGGATCCGTCGACC	
GFP1	ATGAGTAAAGGAGAAGAACTTTTCAC	
GFP2	TTATTTGTATAGTTCATCCATGC	
AmsG1	CGG*GGTACC*GTATCGCTTAAAGGGGAAACG (*Kpn*I)	
AmsG2	CTAG*TCTAGA*CACCTGGAAAGCCATTAATCA (*Xba*I)	
flhD1	CCG*GAATTC*CGTTGTTGCCGATGCTAATA (*Eco*RI)	
flhD2	CGC*GGATCC*CAATACCCAGGCGAAACATC (*Bam*HI)	
RcsC1	GATCAAGGTAACCGAGCGTAAC	
RcsC2	AGCTTAACCGCAGCATTAAAAC	
RcsBD1	CCG*GAATTC*CTAGCACAATTCACAAGGTTGG (*Eco*RI)	
RcsBD2	CGC*GGATCC*CTCCTAATGAACTGCCGCTACT (*Bam*HI)	
Km1	CAGTCATAGCCGAATAGCCT	
Km2	CGGTGCCCTGAATGAACTGC	
Z3205F	GCCAACGCCCGACTGATTACAAG	
Z3206R	GCCGGGAAGCGCTAAGTAACAGGT	
Z3207F	GTTCGCCGCTAAGCCGTGGTCA	
Z3207R	GAGAAAATCAGCGCGGGTGGTTAC	

*Km^R, Ap^R, kanamycin and ampicillin resistance, respectively.
†Italic nucleotides are restriction sites added and the restriction enzymes are indicated at the end of the primers.

operon and its flanking sequences. Following amplification, DNA fragments and the vector were both digested by *Eco*RI and *Bam*HI, and ligated together. The final plasmid was designated as pWDP3. All plasmids were introduced into *E. amylovora* strain by electroporation. Transformants were selected on LB plates supplemented with Km and Ap. Their genotypes were confirmed by both enzymatic digestion and sequencing.

Construction of promoter–GFP fusions for gene expression assays

For gene expression assays, flanking sequences of the *amsG* and *flhD* ORFs were used to design primers to amplify DNA fragments. Primer pairs amsG1-amsG2 and flhD1-flhD2 with restriction sites were used to amplify 721-bp and 906-bp DNA fragments from the *E. amylovora* WT strain, containing promoter sequences of *amsG* and *flhD* genes, respectively. PCR products and the promoter trapping vector pFPV25 were both digested with *Bam*HI and *Eco*RI for the *amsG* gene and with *Pst*I and *Kpn*I for the *flhDC* gene, respectively. The resulting fragments were gel purified, ligated together and cloned to the upstream of the promoterless *gfp* gene. The final plasmids were designated as pWDP4 and pSN1 for the *amsG* and *flhD* genes, respectively, and were confirmed by restriction enzyme digestion and sequencing.

Immature pear virulence assays

For *E. amylovora* WT and mutant strains, bacterial suspensions were grown overnight in LB broth, harvested by centrifugation and resuspended in 0.5 × sterile phosphate-buffered saline (PBS) with bacterial cells adjusted to concentrations of ~1×10^3 to 1×10^4 cfu/μL ($OD_{600} = 0.1$ and then diluted 100 times) in PBS. Immature fruits of pear (*Pyrus communis* L. cv. 'Bartlett') were surface sterilized and pricked with a sterile needle, as described previously (Zhao *et al.*, 2005, 2006). Wounded fruits were inoculated with 2 μL of cell suspensions, and incubated in a humidified chamber at 26 °C. Symptoms were recorded at 2, 4, 6, 7 and 8 days post-inoculation.

For bacterial population studies, fruit tissues surrounding the inoculation site were excised using a #4 cork borer, as described previously (Zhao *et al.*, 2005, 2006), and homogenized in 0.5 mL of 0.5 × PBS. Bacterial growth was monitored by dilution plating of the ground material on LB medium amended with the appropriate antibiotics. For each strain tested, fruits were assayed in triplicate, and each experiment was repeated at least twice.

Cetylpyrimidinium chloride (CPC) assay for the determination of amylovoran concentration

The amylovoran concentration in supernatants of bacterial cultures was determined quantitatively by a turbidity assay with CPC, as described previously (Bellemann *et al.*, 1994; Hildebrand *et al.*, 2006). Briefly, for *E. amylovora* WT, mutants and complemented strains, bacterial suspensions were grown overnight in LB broth with or without appropriate antibiotics, harvested by centrifugation and washed with PBS three times. After the final wash, the bacterial pellet was resuspended in 200 μL of PBS. A total of 100 μL of bacterial suspension was inoculated into 10 mL of MBMA medium with 1% sorbitol. One millilitre of bacterial cells was pelleted 2–3 days after inoculation at 28 °C with shaking. Following centrifugation, 50 μL of CPC at 50 mg/mL was added to 1 mL of supernatant. After 10 min of incubation at room temperature, the amylovoran concentration was determined by measuring OD_{600} 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.

Swarming motility assay

For *E. amylovora* WT and mutant strains, bacterial suspensions were grown overnight in LB broth with or without appropriate antibiotics, harvested by centrifugation, washed with PBS once and resuspended in 200 μL of PBS. Then, bacterial suspensions were diluted 10 times in water, and 5 μL of the diluted bacterial suspension was plated onto the centre of swarming agar plates (10 g tryptone, 5 g NaCl, 3 g agar per litre), as described previously (Hildebrand *et al.*, 2006; Skerker *et al.*, 2005). Swarming diameters were determined following incubation at 28 °C for up to 3 days. The experiments were repeated at least three times.

Polymyxin B killing assay

Bacterial strains were grown overnight in LB broth without antibiotics, collected by centrifugation at 3220 *g* for 10 min and washed with PBS three times. The pellets were resuspended in 200 μL of PBS, and 10-fold serial dilutions of bacterial suspensions were made on 96-well plates. Equal volumes of polymyxin B (3 μg/mL) were added to 100 μL of bacterial suspensions, and incubated at room temperature for 1 h. Then, the bacterial numbers for each dilution were counted by plating on LB medium with or without appropriate antibiotics. The percentage survival was calculated by dividing polymyxin B-treated versus untreated cfus (Erickson and Detweiler, 2006). Dilution of each strain was carried out in triplicate. The experiment was repeated at least three times.

GFP reporter gene assay by flow cytometry

The FACSCanto flow cytometer (BD Biosciences, San Jose, CA, USA) was used to monitor the GFP intensity of WT and mutant strains containing the corresponding promoter–GFP construct (Jacobi *et al.*, 1998). For *in vitro amsG* gene expression, WT and *rcs* mutants containing the *amsG* promoter–GFP fusion plasmid were grown in LB overnight, harvested and resuspended in water. Bacterial suspensions were re-inoculated in MBMA broth with 1% sorbitol and grown at 28 °C with shaking for up to 3 days. Bacterial cultures were then harvested by centrifugation, washed once with PBS and then resuspended in PBS for flow cytometry assay. For *in vivo* gene expression assay, bacterial strains were inoculated onto immature pear fruits by cutting the fruit in half, and adding the bacterial suspensions directly to the surface. For

rcs mutants, several pear slices were inoculated in order to recover sufficient bacterial cells for flow cytometry assay. After incubation at 26 °C for up to 2 days, bacterial cells were collected by either washing or centrifugation, washed twice with PBS and resuspended in PBS for flow cytometry assay.

For *flhDC* gene expression, WT and *rcs* mutants containing the *flhDC* promoter–GFP fusion plasmid were grown on swarming plates as described above. Following incubation for 2 days at 28 °C, bacterial cells were collected, washed and resuspended in PBS for flow cytometry assay. Flow cytometry was performed on LSRII 10-parameter multilaser analysers (BD Biosciences). For both cases, data were collected for a total of 100 000 events and statistically analysed by gating using the flow cytometry software FCS Express V3 (De Novo Software, Los Angeles, CA, USA). A geometric mean was calculated for each sample. Each treatment was performed in triplicate and each experiment was repeated three times.

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REFERENCES

Allen, P., Hart, C.A. and Saunders, J.R. (1987a) Isolation from *Klebsiella* and characterization of two *rcs* genes that activate colanic acid capsular biosynthesis in *Escherichia coli*. *J. Gen. Microbiol.* **133**, 331–340.

Allen, P., Fisher, D., Saunders, J.R. and Hart, C.A. (1987b) The role of capsular polysaccharide K21b of *Klebsiella* and of the structurally related colanic-acid polysaccharide of *Escherichia coli* in resistance to phagocytosis and serum killing. *J. Med. Microbiol.* **24**, 363–370.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389–3402.

Andresen, L., Koiv, V., Alamae, T. and Mae, A. (2007) The Rcs phosphorelay modulates the expression of plant cell wall degrading enzymes and virulence in *Pectobacterium carotovorum* ssp. *carotovorum*. *FEMS Microbiol. Lett.* **273**, 229–238.

Bellemann, P. and Geider, K. (1992) Localization of transposon insertions in pathogenicity mutants of *Erwinia amylovora* and their biochemical characterization. *J. Gen. Microbiol.* **138**, 931–940.

Bellemann, P., Bereswill, S., Berger, S. and Geider, K. (1994) Visualization of capsule formation by *Erwinia amylovora* and assays to determine amylovoran synthesis. *Int. J. Biol. Macromol.* **16**, 290–296.

Bereswill, S. and Geider, K. (1997) Characterization of the *rcsB* gene from *Erwinia amylovora* and its influence on exopolysaccharide synthesis and virulence of the fire blight pathogen. *J. Bacteriol.* **179**, 1354–1361.

Bernhard, F., Poetter, K., Geider, K. and Coplin, D.L. (1990) The *rcsA* gene from *Erwinia amylovora*: identification, nucleotide sequence, and regulation of exopolysaccharide biosynthesis. *Mol. Plant–Microbe Interact.* **3**, 429–437.

Bernhard, F., Coplin, D.L. and Geider, K. (1993) A gene cluster for amylovoran synthesis in *Erwinia amylovora*: characterization and relationship to *cps* genes in *Erwinia stewartii*. *Mol. Gen. Genet.* **239**, 158–168.

Bugert, P. and Geider, K. (1995) Molecular analysis of the *ams* operon required for exopolysaccharide synthesis of *Erwinia amylovora*. *Mol. Microbiol.* **15**, 917–933.

Burse, A., Weingart, H. and Ullrich, M.S. (2004) NorM, an *Erwinia amylovora* multidrug efflux pump involved in *in vitro* competition with other epiphytic bacteria. *Appl. Environ. Microbiol.* **70**, 693–703.

Chang, C. and Stewart, R.C. (1998) The two-component system: regulation of diverse signaling pathways in prokaryotes and eukaryotes. *Plant Physiol.* **117**, 723–731.

Clarke, D.J., Joyce, S.A., Toutain, C.M., Jacq, A. and Holland, I.B. (2002) Genetic analysis of the RcsC sensor kinase from *Escherichia coli* K-12. *J. Bacteriol.* **184**, 1204–1208.

Coleman, M., Pearce, R., Hitchin, E., Busfield, F., Mansfield, J.W. and Roberts, I.S. (1990) Molecular cloning, expression and nucleotide sequence of the *rcsA* gene of *Erwinia amylovora*, encoding a positive regulator of capsule expression: evidence for a family of related capsule activator proteins. *J. Gen. Microbiol.* **136**, 1799–1806.

Datsenko, K.A. and Wanner, B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA*, **97**, 6640–6645.

Detweiler, C.S., Monack, D.M., Brodsky, I.E., Mathew, H. and Falkow, S. (2003) *virK*, *somA* and *rcsC* are important for systemic *Salmonella enterica* serovar *typhimurium* infection and cationic peptide resistance. *Mol. Microbiol.* **48**, 385–400.

Dominguez-Bernal, G., Pucciarelli, M.G., Ramos-Morales, F., Garcia-Quintanilla, M., Cano, D.A., Casadesus, J. and Garcia-del Portillo, F. (2004) Repression of the RcsC-YojN-RcsB phosphorelay by the IgaA protein is a requisite for *Salmonella* virulence. *Mol. Microbiol.* **53**, 1437–1449.

Erickson, K.D. and Detweiler, C.S. (2006) The Rcs phosphorelay system is specific to enteric pathogens/commensals and activates *ydeI*, a gene important for persistent *Salmonella* infection of mice. *Mol. Microbiol.* **62**, 883–894.

Ferrieres, L. and Clarke, D.J. (2003) The RcsC sensor kinase is required for normal biofilm formation in *Escherichia coli* K-12 and controls the expression of a regulon in response to growth on a solid surface. *Mol. Microbiol.* **50**, 1665–1682.

Flamez, C., Ricard, I., Arafah, S., Simonet, M. and Marceau, M. (2007) Two-component system regulon plasticity in bacteria: a concept emerging from phenotypic analysis of *Yersinia pseudotuberculosis* response regulator mutants. *Adv. Exp. Med. Biol.* **603**, 145–155.

Francez-Charlot, A., Laugel, B., Van Gemert, A., Dubarry, N., Wiorowski, F., Castanie-Cornet, M.P., Gutierrez, C. and Cam, K. (2003) RcsCDB His-Asp phosphorelay system negatively regulates the *flhDC* operon in *Escherichia coli*. *Mol. Microbiol.* **49**, 823–832.

Fraser, G.M. and Hughes, C. (1999) Swarming motility. *Curr. Opin. Microbiol.* **2**, 630–635.

Garcia-Calderon, C.B., Garcia-Quintanilla, M., Casadesus, J. and Ramos-Morales, F. (2005) Virulence attenuation in *Salmonella enterica* rcsC mutants with constitutive activation of the Rcs system. *Microbiology*, **151**, 579–588.

Hancock, R.E. and McPhee, J.B. (2005) *Salmonella's* sensor for host defense molecules. *Cell*, **122**, 320–322.

Herrera, C.M., Koutsoudis, M.D., Wang, X. and von Bodman, S.B. (2008) *Pantoea stewartii* subsp. *stewartii* exhibits surface motility, which is a critical aspect of Stewart's wilt disease development on maize. *Mol. Plant–Microbe Interact.* **21**, 1359–1370.

Hildebrand, M., Aldridge, P. and Geider, K. (2006) Characterization of *hns* genes from *Erwinia amylovora*. *Mol. Genet. Genom.* **275**, 310–319.

Hinchliffe, S.J., Howard, S.L., Huang, Y.H., Clarke, D.J. and Wren, B.W. (2008) The importance of the Rcs phosphorelay in the survival and pathogenesis of the enteropathogenic yersiniae. *Microbiology*, **154**, 1117–1131.

Hoch, J.A. and Silhavy, T.J. (1995) *Two-component Signal Transduction*. Washington, DC: ASM Press.

Huang, Y.H., Ferrieres, L. and Clarke, D.J. (2006) The role of the Rcs phosphorelay in *Enterobacteriaceae*. *Res. Microbiol.* **157**, 206–212.

Jacobi, C.A., Roggenkamp, A., Rakin, R., Zumbihl, L., Leitritz, L. and Heesemann, J. (1998) *In vitro* and *in vivo* expression studies of *yopE* from *Yersinia enterocolitica* using the *gfp* reporter gene. *Mol. Microbiol.* **30**, 865–882.

Jayaratne, P., Keenleyside, W.J., MacLachlan, P.R., Dodgson, C. and Whitfield, C. (1993) Characterization of *rcsB* and *rcsC* from *Escherichia coli* O9:K30:H12 and examination of the role of the *rcs* regulatory system in expression of group I capsular polysaccharides. *J. Bacteriol.* **175**, 5384–5394.

Kato, A., Mitrophanov, A.Y. and Groisman, E.A. (2007) A connector of two-component regulatory systems promotes signal amplification and persistence of expression. *Proc. Natl. Acad. Sci. USA*, **104**, 12 063–12 068.

Kelm, O., Kiecker, C., Geider, K. and Bernhard, F. (1997) Interaction of the regulator proteins RcsA and RcsB with the promoter of the operon for amylovoran biosynthesis in *Erwinia amylovora*. *Mol. Gen. Genet.* **256**, 72–83.

Koczan, J., McGrath, M., Zhao, Y.F. and Sundin, G.W. (2008) Biofilm formation in *Erwinia amylovora*: implications in pathogenicity. *Acta Hort.* **793**, 67–71.

Koutsoudis, M.D., Tsaltas, D., Minogue, T.D. and von Bodman, S.B. (2006) Quorum-sensing regulation governs bacterial adhesion, biofilm development, and host colonization in *Pantoea stewartii* subspecies *stewartii*. *Proc. Natl. Acad. Sci. USA*, **103**, 5983–5988.

Kube, M., Migdoll, A.M., Muller, I., Kuhl, H., Beck, A., Reinhardt, R. and Geider, K. (2008) The genome of *Erwinia tasmaniensis* strain Et1/99, a non-pathogenic bacterium in the genus of *Erwinia*. *Environ. Microbiol.* **10**, 2211–2222.

Lee, H., Hsu, F.F., Turk, J. and Groisman, E.A. (2004) The PmrA-regulated *pmrC* gene mediates phosphoethanolamine modification of lipid A and polymyxin resistance in *Salmonella enterica*. *J. Bacteriol.* **186**, 4124–4133.

Majdalani, N. and Gottesman, S. (2005) The Rcs phosphorelay: a complex signal transduction system. *Annu. Rev. Microbiol.* **59**, 379–405.

Majdalani, N. and Gottesman, S. (2007) Genetic dissection of signaling through the Rcs phosphorelay. *Methods Enzymol.* **423**, 349–362.

Majdalani, N., Hernandez, D. and Gottesman, S. (2002) Regulation and mode of action of the second small RNA activator of RpoS translation, RprA. *Mol. Microbiol.* **46**, 813–826.

Majdalani, N., Heck, M., Stout, V. and Gottesman, S. (2005) Role of RcsF in signaling to the Rcs phosphorelay pathway in *Escherichia coli*. *J. Bacteriol.* **187**, 6770–6778.

McCarter, L.L. (2006) Regulation of flagella. *Curr. Opin. Microbiol.* **9**, 180–186.

Minogue, T.D., Carlier, A.L., Koutsoudis, M.D. and von Bodman, S.B. (2005) The cell density-dependent expression of stewartan exopolysaccharide in *Pantoea stewartii* ssp. *stewartii* is a function of EsaR-mediated repression of the *rcsA* gene. *Mol. Microbiol.* **56**, 189–203.

Mizuno, T. (1998) His-Asp phosphotransfer signal transduction. *J. Biochem.* **123**, 555–563.

Mouslim, C. and Groisman, E.A. (2003) Control of the *Salmonella ugd* gene by three two-component regulatory systems. *Mol. Microbiol.* **47**, 335–344.

Mouslim, C., Delgado, M. and Groisman, E.A. (2004) Activation of the RcsC/YojN/RcsB phosphorelay system attenuates *Salmonella* virulence. *Mol. Microbiol.* **54**, 386–395.

Nimtz, M., Mort, A., Domke, T., Wray, V., Zhang, Y., Qiu, F., Coplin, D. and Geider, K. (1996) Structure of amylovoran, the capsular exopolysaccharide from the fire blight pathogen *Erwinia amylovora*. *Carbohydr. Res.* **287**, 59–76.

Oard, S.V. and Enright, F.M. (2006) Expression of the antimicrobial peptides in plants to control phytopathogenic bacteria and fungi. *Plant Cell Rep.* **25**, 561–572.

Oh, C.S. and Beer, S.V. (2005) Molecular genetics of *Erwinia amylovora* involved in the development of fire blight. *FEMS Microbiol. Lett.* **253**, 185–192.

Parkinson, J.S. (1993) Signal transduction schemes of bacteria. *Cell*, **73**, 857–871.

Parkinson, J.S. and Kofoid, E.C. (1992) Communication modules in bacterial signaling proteins. *Annu. Rev. Genet.* **26**, 71–112.

Rogov, V.V., Rogova, N.Y., Bernhard, F., Koglin, A., Lohr, F. and Dotsch, V. (2006) A new structural domain in the *Escherichia coli* RcsC hybrid sensor kinase connects histidine kinase and phosphoreceiver domains. *J. Mol. Biol.* **364**, 68–79.

Rosenfeld, Y. and Shai, Y. (2006) Lipopolysaccharide (Endotoxin)–host defense antibacterial peptides interactions: role in bacterial resistance and prevention of sepsis. *Biochim. Biophys. Acta*, **1758**, 1513–1522.

Russo, T.A., Sharma, G., Weiss, J. and Brown, C. (1995) The construction and characterization of colanic acid deficient mutants in an extraintestinal isolate of *Escherichia coli* (O4/K54/H5). *Microb. Pathog.* **18**, 269–278.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sjulin, T.M. and Beer, S.V. (1978) Mechanism of wilt induction by amylovoran in *Cotoneaster* shoots and its relation to wilting of shoots infected by *Erwinia amylovora*. *Phytopathology*, **68**, 89–94.

Skerker, J.M., Prasol, M.S., Perchuk, B.S., Biondi, E.G. and Laub, M.T. (2005) Two-component signal transduction pathways regulating growth and cell cycle progression in a bacterium: a system-level analysis. *PLoS Biol.* **3**, e334.

Skerker, J.M., Perchuk, B.S., Siryaporn, A., Lubin, E.A., Ashenberg, O., Goulian, M. and Laub, M.T. (2008) Rewiring the specificity of two-component signal transduction systems. *Cell*, **133**, 1043–1054.

Steinberger, E.M. and Beer, S.V. (1988) Creation and complementation of pathogenicity mutants of *Erwinia amylovora*. *Mol. Plant–Microbe Interact.* **1**, 135–144.

Stevenson, G., Andrianopoulos, K., Hobbs, M. and Reeves, P.R. (1996) Organization of the *Escherichia coli* K-12 gene cluster responsible for production of the extracellular polysaccharide colanic acid. *J. Bacteriol.* **178**, 4885–4893.

Stock, J.B., Stock, A.M. and Mottonen, J.M. (1990) Signal transduction in bacteria. *Nature*, **344**, 395–400.

Stout, V., and Gottesman, S. (1990) RcsB and RcsC: a two-component regulator of capsule synthesis in *Escherichia coli*. *J. Bacteriol.* **172**, 659–669.

Takeda, S., Fujisawa, Y., Matsubara, M., Aiba, H. and Mizuno, T. (2001) A novel feature of the multistep phosphorelay in *Escherichia coli*: a revised model of the RcsC → YojN → RcsB signalling pathway implicated in capsular synthesis and swarming behavior. *Mol. Microbiol.* **40**, 440–450.

Valdivia, R.H. and Falkow, S. (1997) Fluorescence-based isolation of bacterial genes expressed within host cells. *Science*, **277**, 2007–2011.

Vanneste, J.L. (2000) *Fire Blight: the Disease and its Causative Agent, Erwinia amylovora*. New York: CABI Publishing.

Wang, Q., Zhao, Y., McClelland, M. and Harshey, R.M. (2007) The RcsCDB signaling system and swarming motility in *Salmonella enterica* serovar *typhimurium*: dual regulation of flagellar and SPI-2 virulence genes. *J. Bacteriol.* **189**, 8447–8457.

Wang, R.F. and Kushner, S.R. (1991) Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. *Gene*, **100**, 195–199.

Wehland, M., Kiecker, C., Coplin, D.L., Kelm, O., Saenger, W. and Bernhard, F. (1999) Identification of an RcsA/RcsB recognition motif in the promoters of exopolysaccharide biosynthetic operons from *Erwinia amylovora* and *Pantoea stewartii* subspecies *stewartii*. *J. Biol. Chem.* **274**, 3300–3307.

Wei, Z.M., Laby, R.J., Zumoff, C.H., Bauer, D.W., He, S.Y., Collmer, A. and Beer, S.V. (1992) Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. *Science*, **257**, 85–88.

Whitfield, C. and Roberts, I.S. (1999) Structure, assembly and regulation of expression of capsules in *Escherichia coli*. *Mol. Microbiol.* **31**, 1307–1319.

Zhao, Y.F. and Sundin, G.W. (2008) A simple genetic tool for studying gene function and signal transduction in *Erwinia amylovora*. *Acta Hort.* **793**, 171–178.

Zhao, Y.F., Blumer, S.E. and Sundin, G.W. (2005) Identification of *Erwinia amylovora* genes induced during infection of immature pear tissue. *J. Bacteriol.* **187**, 8088–8103.

Zhao, Y.F., He, S.Y. and Sundin, G.W. (2006) The *Erwinia amylovora* *avrRpt2*$_{EA}$ gene contributes to virulence on pear and AvrRpt2$_{EA}$ is recognized by Arabidopsis RPS2 when expressed in *Pseudomonas syringae*. *Mol. Plant–Microbe Interact.* **19**, 644–654.