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Deciphering the Components That Coordinately Regulate Virulence Factors of the Soft Rot Pathogen *Dickeya dadantii*

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The bacterial soft rot pathogen *Dickeya dadantii* utilizes the type III secretion system (T3SS) to suppress host defense responses, and secretes pectate lyase (Pel) to disintegrate the plant cell wall. A transposon mutagenesis fluorescence-activated cell sorting screen was used to identify mutants with altered promoter activities of the T3SS pilus gene *hrpA*. Several insertion mutations, resulting in changes in *hrpA* expression, were mapped to a new locus, *opgGH,* which encodes the gene cluster responsible for osmoregulated periplasmic glucan (OPG) synthesis proteins. Our data showed that OPG was involved in T3SS and Pel regulation by altering the expression of the regulatory small RNA RsmB. Through genome searching, the mechanism of two novel regulatory components, the RcsCD-RcsB phosphorelay and CsrD on OPG and the *rsmB* gene, was further investigated. The Rcs phosphorelay and OPG inversely regulated *rsmB* at transcriptional and post-transcriptional levels, respectively. CsrD exhibited dual functionality in T3SS and Pel regulation by manipulating levels of RsmB RNA and cyclic diguanylate monophosphate (c-di-GMP). CsrD positively regulated the promoter activity of the *rsmB* gene but negatively controlled RsmB RNA at the post-transcriptional level via OpgGH. In addition, CsrD contains both GGDEF and EAL domains but acted as a c-di-GMP phosphodiesterase. When the expression of the *csrD* gene was induced, CsrD regulated T3SS expression and Pel production through controlling intracellular c-di-GMP levels.

The infection of host plants by phytopathogenic bacteria is a complicated process which involves adherence and entry into host tissues, colonization of the apoplastic spaces between plant cells, suppression of the host defense response, and development of disease symptoms (Alfano and Collmer 1996).

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The gram-negative bacterium *Dickeya dadantii* (formerly *Erwinia chrysanthemi*) is a phytopathogen that causes soft rot disease in a wide range of plant species, including many economically important crops (Hugouvieux-Cotte-Pattat et al. 1996). A number of virulence factors have been identified as required for the successful infection of plant hosts by *D. dadantii*. These include several plant cell wall–degrading enzymes (pectinases, cellulase, and proteases) and the type III secretion system (T3SS) (Bauer et al. 1994; Glasner et al. 2011; Hugouvieux-Cotte-Pattat et al. 1996). Among the cell wall–degrading enzymes, pectate lyase (Pel), which is secreted through the type II secretion system, is a major virulence factor that causes disintegration of plant cell wall structures and maceration of plant tissues. In *D. dadantii*, the T3SS is reported to play a key role in the initial infection in plant hosts (Bauer et al. 1994; Yang et al. 2002). The T3SS of *D. dadantii* 3937 is encoded by two divergent operons, which contain the *hrp*/*hrc*/*dsp* gene clusters. Among them, *hrpA* encodes a structural protein of the T3SS pilus, *hrpN* encodes a harpin protein that is translocated by the T3SS, and *dspE* encodes an effector protein in *D. dadantii* 3937 (Yap et al. 2006).

The expression of T3SS genes is modulated by abiotic and biotic environmental factors, including different carbon sources, divalent cations, and other metabolites released by the host plant. For example, the T3SS genes are expressed at a very low level in nutrient-rich media but can be induced in infected plant tissues or in artificial *hrp*-inducing minimal medium (Alfano et al. 1997). In addition, some natural phenolic compounds in the plant either induce or inhibit the expression of T3SS genes in *D. dadantii* 3937 (Li et al. 2009; Yang et al. 2008). Intensive studies on the regulation of the T3SS in recent years have revealed a number of transcriptional and post-transcriptional regulatory elements (Büttner 2012; Li et al. 2010; Tang et al. 2006; Yang et al. 2007). In *D. dadantii* 3937, two main regulatory pathways are involved in controlling the expression of the T3SS. The two-component system (TCS) HrpX/HrpY activates the expression of *hrpS*, which encodes an NtrC-family transcriptional enhancer-like protein (Fig. 1). HrpS interacts with σ factor RpoN (σ^{54}) and then initiates transcription of *hrpL* (Peng et al. 2006; Yap et al. 2005). HrpL is a member of the extracytoplasmic family of alternative σ factors and positively controls many genes downstream within the T3SS regulatory cascade due to its interaction with a specific binding site in the promoter region of the target genes, called the "*hrp* box" (Nissan et al. 2005; Xiao and Hutcheson 1994). The

TCS GacS/GacA positively controls the T3SS via a regulatory cascade involving the regulatory small RNA RsmB and the RNA-binding protein RsmA (Cui et al. 2001; Yang et al. 2008). In *D. dadantii* 3937, the RNA-binding protein RsmA binds to and promotes the degradation of *hrpL* mRNA (Fig. 1) (Lebeau et al. 2008). The degradation effect of RsmA on *hrpL* mRNA can be alleviated by the antagonistic action of the regulatory small RNA RsmB, whose expression is controlled by the TCS GacS/GacA (Cui et al. 2001; Yang et al. 2008).

In addition to the HrpX/HrpY and GacS/GacA signaling pathways, the expression of theT3SS is regulated by additional factors. For instance, a mutation of *pecT* or *pecS* has been shown to significantly influence the expression of *hrpN* (Nasser et al. 2005). PecS was reported to bind the *hrpN* regulatory region and inhibit *hrpN* transcript elongation in *D. dadantii* 3937. In addition, bis-(3′-5′)-cyclic diguanylate monophosphate (c-di-GMP), a global second messenger which controls the lifestyles of many bacteria, was reported to regulate the T3SS of *D. dadantii* 3937 (Jenal and Malone 2006; Yi et al. 2010). OpgG and OpgH are two membrane-bound proteins responsible for the production of osmoregulated periplasmic glucans (OPG), an intrinsic component of the gram-negative bacterial cell envelope (Cogez et al. 2001; Page et al. 2001). In *D. dadantii*, the production of these periplasmic glucans plays an important role in Pel production and secretion (Page et al. 2001). From a transposon mutagenesis screen, we uncovered that, in addition to Pels, OPG are also intimately involved in T3SS regulation, suggesting that the different virulence factors are coordinately regulated in bacterial pathogens. By using *D. dadantii* 3937 as a model system, we investigated the coordination of virulence programs in gram-negative bacteria. We elucidated a complex but sophisticated regulatory circuit consisting of *opgGH,* the RcsCD-RcsB regulatory system, c-di-GMP, and a central regulatory small RNA, RsmB, that coordinately control expression of the T3SS and Pel in *D. dadantii* 3937.

RESULTS

Identification of *D. dadantii* mutants with altered *hrpA* expression.

We utilized a transposon mutagenesis screen combined with a green fluorescent protein (GFP) reporter fluorescence-activated cell sorting (FACS) technique to screen for novel regulators of *hrpA* in *D. dadantii* 3937. Mutant strains Ech239, Ech240, Ech241, Ech242, and Ech243 exhibited reduced *hrpA* promoter activity, and contained transposon disruptions at the *opgH* or *opgG* gene loci (Table 1). In *Escherichia coli*, the *opgGH* operon encodes two proteins (OpgG and OpgH) required for the synthesis of OPG (Page et al. 2001). In a previous report, an *opg* mutant of *D. dadantii* showed a reduction of protease, cellulase, and Pel production (Page et al. 2001). The five mutants identified from the screen with reduced *hrpA* expression had transposon insertions in the *opgGH* operon, indicating that the production of the virulence factors T3SS and Pel must be coordinated by some key regulators in the bacterium.

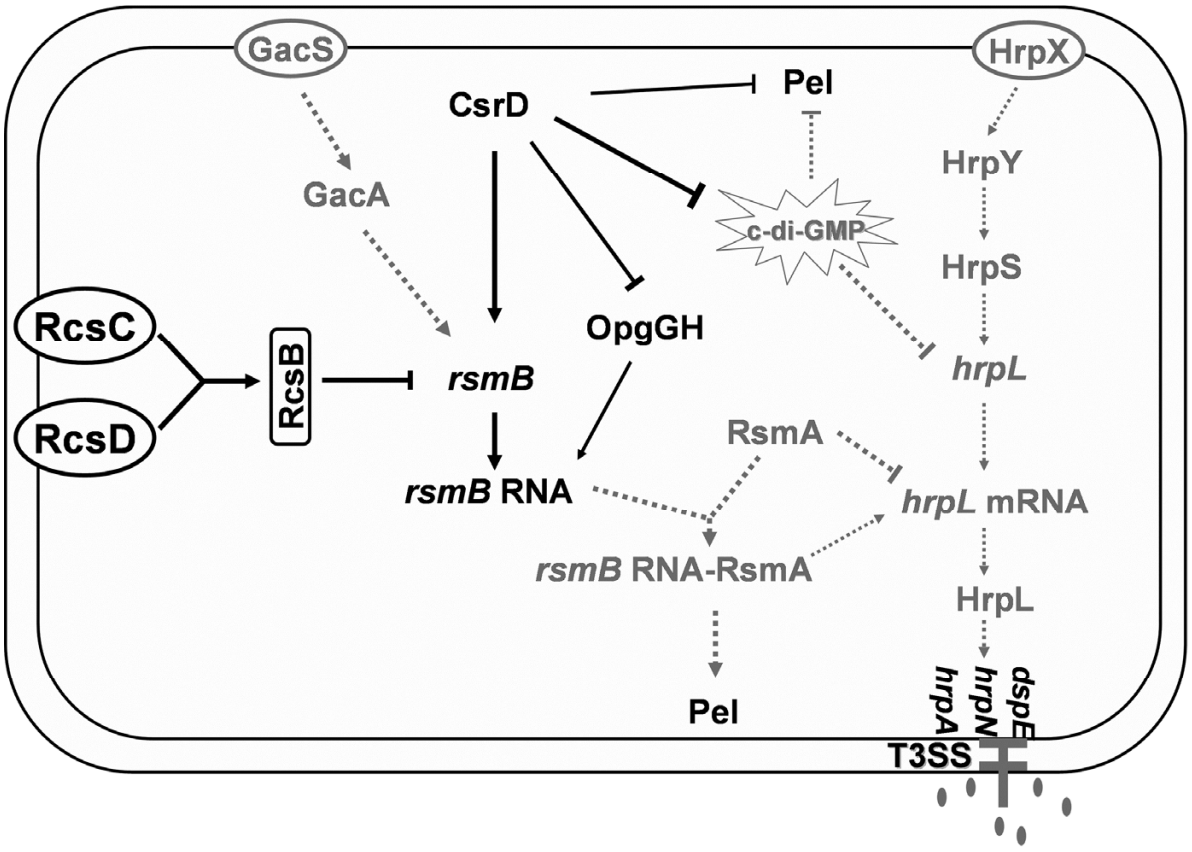

Fig. 1. Model of type III secretion system (T3SS) and plant cell wall–degradation enzyme pectate lyase (Pel) regulatory cascade in *Dickeya dadantii* 3937. The *D. dadantii* 3937 T3SS was previously reported to be regulated by the HrpX/HrpY-HrpS-HrpL pathway at the transcriptional level and the GacS/GacA-RsmB-HrpL pathway at the post-transcriptional level (Li et al. 2010; Yang et al. 2008, 2010; Yap et al. 2005). In this study, the RcsCD-RcsB phosphorelay is observed to repress the regulatory small RNA RsmB at the transcriptional level, which is required for virulence in *D. dadantii* 3937. Although CsrD positively regulates the promoter activity of the *rsmB* gene, CsrD negatively controls the RsmB RNA at the post-transcriptional level via the OpgGH. In addition, CsrD modulates the level of c-di-GMP by its c-di-GMP phosphodiesterase activity. High levels of c-di-GMP suppress synthesis of the virulence factors T3SS and Pel. Thus, under induction, CsrD controls virulence factors by manipulating the intracellular level of c-di-GMP in the bacterial cell. Dotted lines represent regulatory components identified previously while solid lines denote regulators studied in this article.

Expression of T3SS structural and effector genes and HrpN production were reduced in a *D. dadantii* Δ*opgH* mutant.

Early studies showed that mutations in the *opgG* or *opgH* genes abolish OPG synthesis in *D. dadantii* (Page et al. 2001). To verify that transposon insertion mutations in the *opgGH* operon were responsible for the decreased *hrpA* expression in *D. dadantii* mutants Ech239, Ech240, Ech241, Ech242, and Ech243, we generated an *opgH* deletion mutant (Δ*opgH)* of *D. dadantii* 3937. The *opgH* gene encodes the enzyme glucosyltransferase. The promoter activity of *hrpA* was then measured in this mutant and the wild-type strain. Expression of *hrpA* decreased approximately eightfold in the *opgH* mutant compared with that of the wild-type strain *D. dadantii* 3937 (Fig. 2B). Complementation of Δ*opgH* with the *opgGH* operon restored *hrpA* expression to the wild-type level (Fig. 2B). A Northern blot assay further showed that levels of *hrpA* mRNA were significantly decreased in Δ*opgH* compared with its parental strain (Fig. 2D). In addition to the effects seen on the *hrpA* gene, the promoter activities of other HrpL-regulon genes, *hrpN* and *dspE,* were also decreased in the Δ*opgH* mutant using the promoter fusion plasmids pPhrpN and pPdspE, respectively (Fig. 2A and C). Complementation of Δ*opgH* with the *opgGH* operon restored the *hrpN* and *dspE* expression near the wild-type level (Fig. 2A and C). Finally, consistent with the *hrpN* expression level in the Δ*opgH* mutant, this mutant produced lower amount of the T3SS harpin protein HrpN than wild-type strain *D. dadantii* 3937 (Fig. 2E). Together, these results demonstrate that the *opgGH* operon positively controls T3SS expression in *D. dadantii* 3937.

The effect of OPG on T3SS expression is mediated by the regulatory small RNA RsmB.

In plant-pathogenic bacteria such as *Erwinia* and *Dickeya* strains, the transcription of T3SS structural and effector genes such as *hrpA, hrpN*, and *dspE* is controlled by the master regulator HrpL (McNally et al. 2012; Tang et al. 2006; Yang et al. 2010). To determine whether the observed regulation of the T3SS by OPG in *D. dadantii* is mediated by HrpL, the expression of *hrpL* was measured in the Δ*opgH* mutant. The *hrpL-gfp* transcriptional fusion pPhrpL in the Δ*opgH* mutant was expressed at levels similar to those in the wild-type strain (Fig. 3A). However, the quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) assay showed that the amount of *hrpL* mRNA was only 0.24-fold in the Δ*opgH* mutant compared with that of *D. dadantii* 3937 ($P < 0.05$) (Fig. 3B), suggesting that the effect of OPG on *hrpL* expression may occur at the post-transcriptional level.

The regulatory small RNA RsmB plays an essential role in the post-transcriptional regulation of *hrpL* by alleviating the degradation effect of RsmA on *hrpL* mRNA (Cui et al. 2001; Yang et al. 2008). To test whether the effect of OPG on *hrpL* mRNA was dependent on the RsmB/RsmA system, we investigated the levels of *rsmA* and RsmB RNA in the Δ*opgH* mutant by qRT-PCR. Although no statistical difference for *rsmA* mRNA was observed (Fig. 3B), the level of RsmB RNA was significantly lower in Δ*opgH* ($P < 0.05$) (Fig. 3B). To confirm that OPG affect T3SS expression through RsmB, plasmid pMLrsmB, which contains the *rsmB* gene in vector pML123, was transferred into the Δ*opgH* mutant. Compared with the Δ*opgH* mutant with pML123, the promoter activity of *hrpA* was increased when *rsmB* was expressed in the Δ*opgH* mutant carrying plasmid pMLrsmB (Fig. 3C). Thus, OpgH positively regulates the level of regulatory small RNA RsmB which, in turn, controls the expression of *hrpA* through modulating the stability of *hrpL* mRNA.

The Rcs phosphorelay and OPG inversely regulate *rsmB* at transcriptional and post-transcriptional levels.

Previous studies showed that the virulence of the Δ*opg* mutant of *D. dadantii* is restored by inactivation of the RcsCD-RcsB phosphorelay (Bouchart et al. 2010). From the information above, we hypothesized that RcsCD-RcsB may play a role in T3SS regulation. We also hypothesized that OPG may regulate the *rsmB* gene by controlling Rcs phosphorelay. To elucidate the regulatory relationship on the T3SS pathway among the Rcs phosphorelay, OPG, and Rsm systems, the level of RsmB RNA was assessed in wild-type strain *D. dadantii* 3937, Δ*rcsB,* and Δ*opgH* Δ*rcsB* double mutant strains. Northern blot analysis revealed an increase in RsmB RNA in the Δ*rcsB* mutant compared with that of the wild-type strain *D. dadantii* 3937, suggesting that *rcsB* negatively regulates *rsmB* (Fig. 4A). In contrast, the RsmB RNA level was decreased in the Δ*opgH* mutant compared with the wild-type strain *D. dadantii* 3937. Thus, OpgH positively regulates the level of RsmB RNA which, in turn, controls the expression of *hrpA* through modulating the stability of *hrpL* mRNA (Fig. 4A). Interestingly, the level of RsmB RNA in the Δ*rcsB* Δ*opgH* double mutant was lower than in the Δ*rcsB* mutant but higher than the Δ*opgH* mutant, suggesting that the Rcs phosphorelay regulated RsmB RNA in parallel with the OpgGH pathway (Fig. 4A). To further determine whether *rcsB* and *opgH* functioned in parallel signaling pathways, we determined the level of transcription induction of *rsmB* in Δ*opgH,* Δ*rcsB,* and Δ*rcsB* Δ*opgH* double mutants. Our result showed that the Rcs phosphorelay but not OPG regulated the promoter activity of *rsmB* (Fig. 4B). These data demonstrate that the Rcs phosphorelay and OPG inversely regulate *rsmB* via different regulatory pathways. Whereas the Rcs phosphorelay negatively regulates the expression of *rsmB* at the transcriptional level, the effect of OPG on RsmB RNA is post-transcriptional.

IgaA (YrfF in *E. coli* and *D. dadantii*) was reported to repress the Rcs phosphorelay at the post-transcriptional level in *Salmonella enterica* (Cano et al. 2002; Tierrez and Garcia-

Table 1. Expression of *hrpA* in *Dickeya dadantii* 3937 (3937) and its mini-*Himar* RB1 mutants in minimal medium

Strains[a]	MFI[b]	Name of gene mutated	ASAP ID[c]	Products of the gene disrupted by the transposon
3937	151.7 ± 2.7			
Ech239 (*opgH*$^-$)	24.2 ± 3.4	*opgH*	19948	Osmoregulated periplasmic glucan synthesis protein
Ech240 (*opgH*$^-$)	19.8 ± 1.3	*opgH*	19948	Osmoregulated periplasmic glucan synthesis protein
Ech241 (*opgH*$^-$)	18.0 ± 2.1	*opgH*	19948	Osmoregulated periplasmic glucan synthesis protein
Ech242 (*opgH*$^-$)	25.9 ± 4.6	*opgH*	19948	Osmoregulated periplasmic glucan synthesis protein
Ech243 (*opgG*$^-$)	17.1 ± 2.5	*opgG*	19949	Osmoregulated periplasmic glucan synthesis protein

[a] *D. dadantii* 3937 and mini-*Himar* RB1 mutants carrying Green fluorescent protein (GFP) reporter plasmid pPhrpA.

[b] Promoter activities at 12 h of bacterial growth were determined (bacterial cells were at the exponential growth phase). GFP mean fluorescence intensity (MFI) was determined for gated populations of bacterial cells by flow cytometry. Values were representative of two independent experiments and three replicates were used for each experiment. Differences of the GFP MFI intensity between the wild type and all of the mini-*Himar* RB1 mutants are significant ($P < 0.01$, Student's *t* test).

[c] ASAP is a systematic annotation package for community analysis of genomes.

del Portillo 2004). To further investigate whether YrfF plays a role in the RcsB-RsmB regulatory pathway, a Δ*yrfF* mutant was constructed and the promoter activity of *rsmB* was measured. Similar levels of promoter activity were observed between wild-type strain *D. dadantii* 3937 and the Δ*yrfF* mutant (Supplementary Fig. S1). Similar promoter activities of *hrpA* were also observed between the Δ*yrfF* mutant and the wild-type strain3937. Taken together, these results suggest that YrfF does not affect genes in the RcsCD-RcsB regulon in *D. dadantii*.

CsrD regulates *rsmB* at the post-transcriptional level through OpgGH.

Proteomic assays revealed that the level of the OpgG protein in *Serratia* strain American Type Culture Collection number 39006 was significantly elevated in the Δ*pigX* mutant (Fineran et al. 2007). PigX is an ortholog of CsrD of *D. dadantii*. To identify other key regulatory components of the T3SS, we investigated the role of CsrD in the OPG-Rsm pathway. To elucidate the effect of CsrD on the T3SS, the expression of *hrpA* in the wild-type strain *D. dadantii* 3937 and Δ*csrD* mutant was examined. A mutation of *csrD* resulted in increased promoter activity of *hrpA* (Fig. 5A). In addition, Northern analysis showed that the mRNA level of *hrpA* was increased in the *csrD* mutant versus that of wild-type *D. dadantii* (Fig. 5D). These results showed that CsrD negatively regulates *hrpA* expression.

The regulatory small RNA RsmB positively regulates *hrpA* through minimizing the degradation effect of RsmA on *hrpL* mRNA (Yang et al. 2008). To elucidate whether CsrD regulates the T3SS through the Rsm pathway, we compared the promoter activity and RsmB RNA level between the wild-type strain and the Δ*csrD* mutant. We hypothesized that, if CsrD negatively regulates *hrpA* through RsmB RNA, an increased level of *rsmB* promoter activity will be observed in the Δ*csrD* mutant. Unexpectedly, the promoter activity of *rsmB* was reduced in the Δ*csrD* mutant (Fig. 5B). The contribution of CsrD on the regulation of RsmB RNA was further examined in the bacterial strains. In contrast to the promoter activity of *rsmB*, an increased level of RsmB RNA was observed in the Δ*csrD* mutant (Fig. 4A). Together, our data demonstrate that CsrD negatively regulates *rsmB* promoter activity but posi-

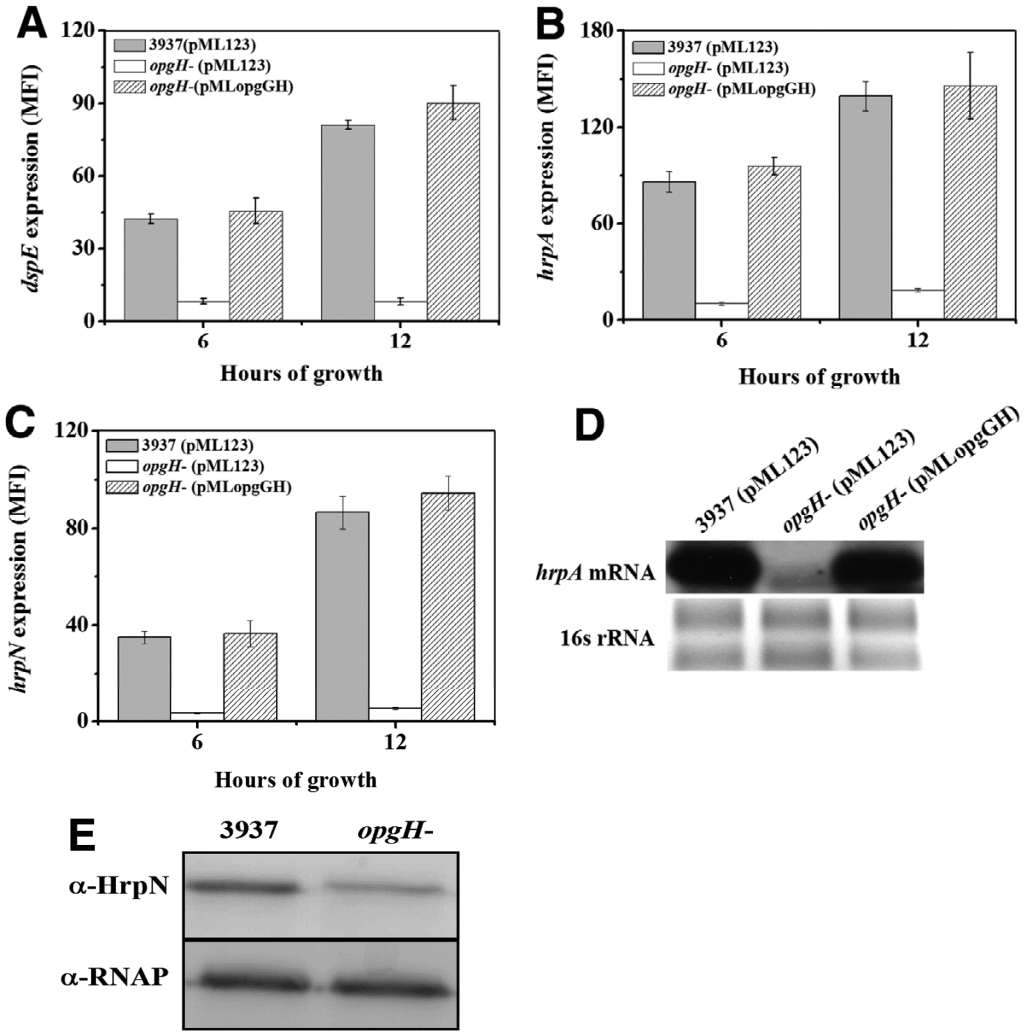

Fig. 2. Osmoregulated periplasmic glucans (OPGs) affect type III secretion system gene expression and harpin production. In the parental strain 3937 with the vector pML123, the Δ*opgH* mutant Ech191 with pML123, and the Δ*opgH* mutant Ech191 with pMLopgGH, the promoter activities of **A,** *dspE*; **B,** *hrpA*; and **C,** *hrpN* were measured in each bacterial strain carrying plasmids pPdspE, pPhrpA, and pPhrpN, respectively. Green fluorescent protein (GFP) intensity was determined on gated populations of bacterial cells by flow cytometry. Values of mean fluorescence intensity (MFI) are an average GFP fluorescence intensity of total bacterial cells with standard deviations (SD). **D,** Northern blot analysis of *hrpA* mRNA (10 μg of total cellular RNA was loaded onto each lane) in wild-type strain *Dickeya dadantii* 3937 and its derivatives. 16 S rRNA is used as an internal control in this and later assays. **E,** Western blot analysis of total crude lysates (containing 5 μg of total protein, as determined by the Bradford assay) prepared from the wild-type strain *D. dadantii* 3937 and the Δ*opgH* mutant Ech191. An antibody directed against RNA polymerase subunit β subunit (α-RNAP) is used as a loading control in this and later blots. Bacterial strains were grown in minimal medium for 12 h during the exponential growth phase in this and later assays.

tively regulates *rsmB* at the RNA level. In addition, these results also suggest that CsrD negatively regulates RsmB RNA at a post-transcriptional level through an unknown factor. The unknown factor was further identified and described below.

Because OPG regulate *rsmB* at the post-transcriptional level (Fig. 4A and B), we suspected that CsrD may regulate *rsmB* post-transcriptionally through OPG. To verify this, we first compared the promoter activity of the *opgGH* operon in the

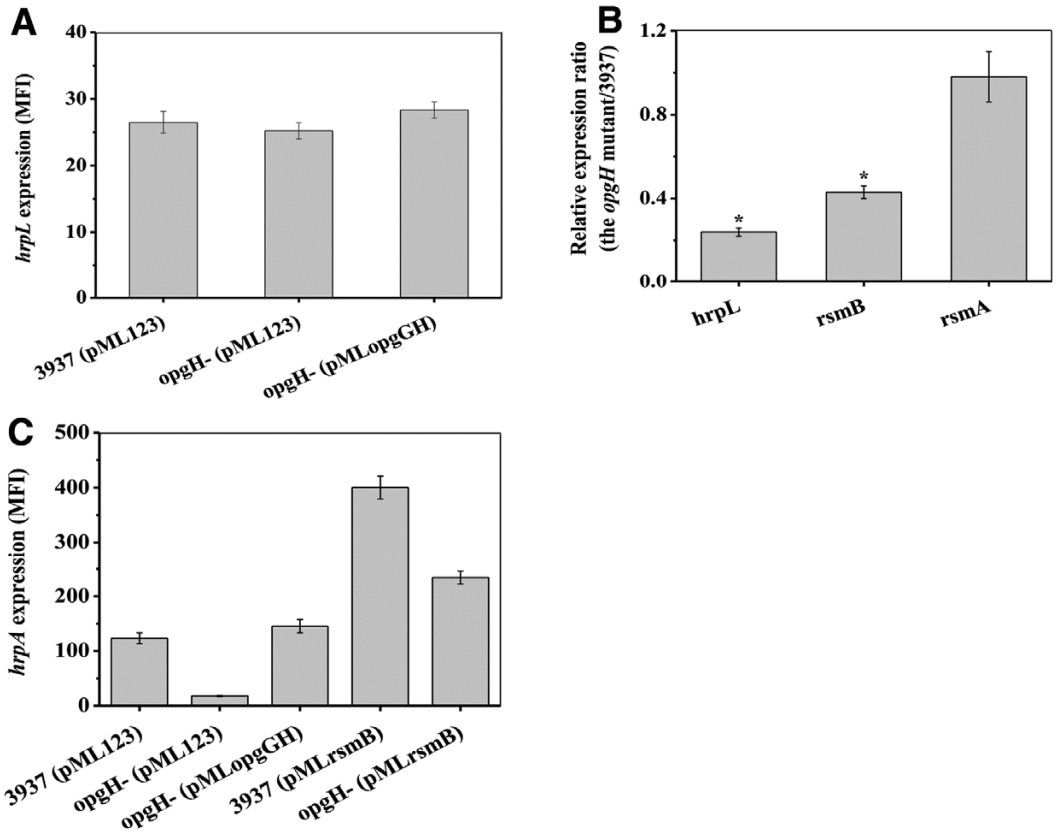

Fig. 3. Osmoregulated periplasmic glucans (OPGs) affect type III secretion system via RsmB in *Dickeya dadantii* 3937. **A,** Promoter activities of *hrpL* were measured using plasmid pPhrpL in strains *D. dadantii* 3937 carrying empty vector pML123, the Δ*opgH* mutant Ech191 carrying empty vector pML123, and the Δ*opgH* mutant Ech191 carrying complement plasmid pMLopgGH. Values of mean fluorescence intensity (MFI) are an average green fluorescent protein (GFP) fluorescence intensity of total bacterial cells with standard deviations (SD). **B,** The relative mRNA/RNA levels of *hrpL*, *rsmA*, and RsmB in Δ*opgH* mutant Ech191compared with that of *D. dadantii* 3937. Total RNA was isolated, and the mRNA of target genes was measured by real-time reverse-transcriptase polymerase chain reaction. Asterisks indicate statistically significant differences in mRNA/RNA levels of the mutants compared with that of the wild type ($P < 0.05$). Similar results were observed in two independent experiments. **C,** Promoter activities of *hrpA* were measured using plasmid pPhrpA in *D. dadantii* 3937 carrying pML123, the Δ*opgH* mutant Ech191 carrying pML123, the Δ*opgH* mutant Ech191 carrying pMLopgGH, *D. dadantii* 3937 carrying pMLrsmB, and the Δ*opgH* mutant Ech191 carrying pMLrsmB. Values of MFI are an average GFP fluorescence intensity of total bacterial cells with SD.

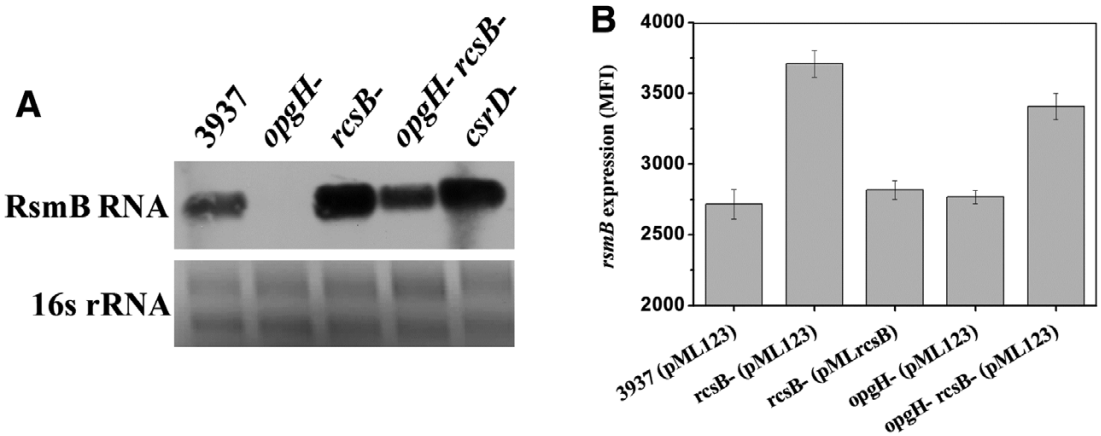

Fig. 4. Rcs phosphorelay and osmoregulated periplasmic glucans (OPGs) inversely regulate *rsmB* at transcriptional and post-transcriptional levels. **A,** Northern blot analysis of RsmB (10 μg of total cellular RNA was loaded onto each lane) in wild-type strain *Dickeya dadantii* 3937, the Δ*opgH* mutant Ech191, the Δ*rcsB* mutant Ech195, the Δ*opgH* Δ*rcsB* double mutant Ech196, and the Δ*csrD* mutant Ech193. **B,** Promoter activities of *rsmB* were measured using plasmid pPrsmB in strains *D. dadantii* 3937 carrying empty vector pML123, the Δ*rcsB* mutant Ech195 carrying empty vector pML123, the Δ*rcsB* mutant Ech195 carrying pMLrcsB, the Δ*opgH* mutant carrying empty vector pML123, and the Δ*opgH* Δ*rcsB* double mutant Ech196 carrying empty vector pML123. Values of mean fluorescence intensity (MFI) are an average green fluorescent protein fluorescence intensity of total bacterial cells with standard deviations (SD).

wild-type strain *D. dadantii* 3937 and the Δ*csrD* mutant. Similar levels of promoter activity were observed between wild-type strain *D. dadantii* 3937 and the Δ*csrD* mutant using the *opgGH-gfp* transcriptional fusion (Fig. 5C). To examine the levels of OpgG protein in these bacterial strains, we constructed a chromosomal *his*-tagged *opgG* allele in strain 3937 and the Δ*csrD* mutant. The result showed that the His_6-OpgG protein was produced at a higher level in the Δ*csrD* mutant (Fig. 5E), indicating that a negative effect of CsrD on *opgG* occurs at the post-transcriptional level. Taken together, these results suggested that CsrD was involved in the OpgGH-dependent regulatory pathway. CsrD positively regulates *rsmB* at the transcriptional level but negatively regulates *rsmB* at post-transcriptional level through OpgGH.

CsrD controls T3SS as a c-di-GMP phosphodiesterase.

Sequence analysis indicated that CsrD has two N-terminal transmembrane domains and a central GGDEF domain (amino acids 231 to 385), as well as a C-terminal EAL domain (amino acids 405 to 637). Instead of a conserved GGDEF motif, which is necessary for the synthesis of c-di-GMP, CsrD contains a GGDEF domain with the noncanonical active-site sequence YHSDF, indicating that CsrD is unlikely to catalyze c-di-GMP synthesis (Christen et al. 2006; Jenal and Malone 2006). Analysis of the EAL domain of CsrD revealed that CsrD contains the conserved amino acids ELL, and biochemical data have demonstrated the importance of the EAL motif in c-di-GMP phosphodiesterase (PDE) activity (Tamayo et al. 2005).

A recent study showed that c-di-GMP negatively regulates T3SS expression in *D. dadantii* 3937 (Yi et al. 2010). We investigated whether CsrD regulated the T3SS through manipulating the c-di-GMP levels in *D. dadantii* 3937. EcpC is an active PDE enzyme, and the expression of *hrpA* in an *ecpC* mutant was dramatically decreased due to increased levels of c-di-GMP (Yi et al. 2010). We expressed the *csrD* gene in an *ecpC* mutant from pPSV35 under the control of the *lacUV5* promoter following induction with isopropyl-β-D-1-thiogalactopyranoside (Rietsch et al. 2005). We predict that, if CsrD is a PDE, then in trans expression of *csrD* will reduce the c-di-GMP level in the *ecpC* mutant and cause an increase of *hrpA* expression in the bacterial strain. Remarkably, our result showed that the expression of *hrpA* in the *ecpC* mutant could be partly restored to wild-type levels by the overexpression of the *csrD* gene (Fig. 6A). Similarly, the expression of the *hrpA-gfp* fusion in the wild-type strain was also increased when the expression of the *csrD* gene was induced (Fig. 6A). In addition, CsrD lost its ability to alter *hrpA* expression when a mutation disrupting the conserved ELL motif (ELL→ALL) was introduced (Fig. 6A). Finally, the expression of *hrpA* was increased when the EAL domain of CsrD alone was overexpressed (Fig. 6A). A Western blot assay further showed that levels of the HrpN protein were increased in strains overexpressing the *csrD* gene (Fig. 6B). Finally, the σ factor RpoN is needed for full induction of the promoter activity of *hrpL*. c-di-GMP negatively regulates *hrpL* expression by reducing the *rpoN* mRNA level (Yi et al. 2010). To confirm that CsrD regu-

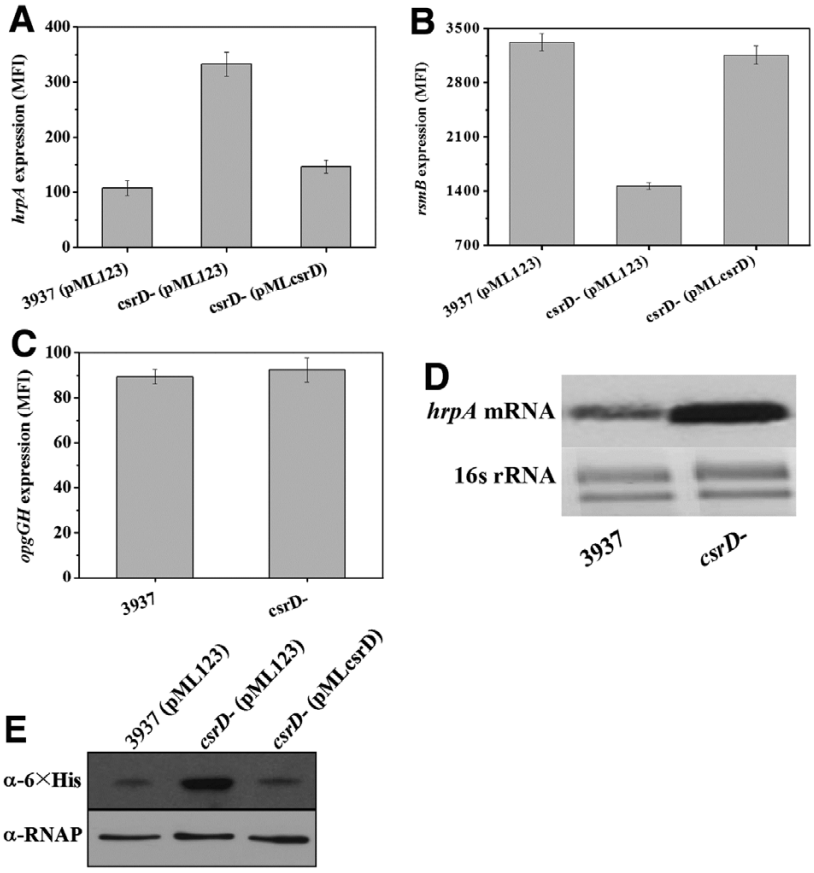

Fig. 5. Effect of CsrD on the expression of type III secretion system and *rsmB* in *Dickeya dadantii* 3937. In *D. dadantii* 3937 with vector pML123, or the Δ*csrD* mutant Ech193 carrying pML123 or pMLcsrD, the promoter activities of **A,** *hrpA*; **B,** *rsmB*; and **C,** *opgGH* were measured in each bacterial strain carrying plasmids pPhrpA, pPrsmB, and pPopgGH, respectively. Values of mean fluorescence intensity (MFI) are an average green fluorescent protein fluorescence intensity of total bacterial cells with standard deviations. **D,** Northern blot analysis of *hrpA* mRNA (10 μg of total cellular RNA was loaded onto each lane) in wild-type strain *D. dadantii* 3937 and the Δ*csrD* mutant Ech193. **E,** Analysis of His_6-OpgG levels in total crude lysates (containing 5 μg of total protein, as determined by the Bradford assay) prepared from the wild-type strain *D. dadantii* 3937 carrying empty vector pML123, the Δ*csrD* mutant Ech193 carrying empty vector pML123, and the Δ*csrD* mutant Ech193 with pMLcsrD by immunoblotting.

lates the T3SS through c-di-GMP, we further examined the *hrpL* promoter activity. Our result showed that *hrpL* promoter activity was increased when the expression of the *csrD* gene was induced (Fig. 6C).

Previous work showed that c-di-GMP levels affect biofilm formation and swarming motility (Jenal and Malone 2006; Yi et al. 2010). As expected, expression of *csrD* in *D. dadantii* 3937 increased swarming motility but decreased biofilm formation (Fig. 6D and E). Further quantification by liquid chromatography tandem mass spectrometry showed that the c-di-GMP levels in the Δ*ecpC* mutant were threefold higher than that of wild-type strain *D. dadantii* 3937. As expected, the c-di-GMP level in the Δ*csrD* mutant was 8.5 nM, which was significantly higher than the wild-type strain level of 5.1 nM (Fig. 6F). Together, these results indicate that CsrD is an active PDE. When the expression of *csrD* was induced, it regulated the T3SS through manipulating c-di-GMP levels in the bacterial cells.

Virulence of *D. dadantii* is associated with small RNA RsmB levels manipulated by OPG, CsrD, and the Rcs phosphorelay.

We predicted that OPG, the Rcs phosphorelay, and CsrD may regulate Pel by modulating RsmB RNA levels. Reduced Pel activity and RNA levels of RsmB were observed in the Δ*opgH* mutant compared with the wild type (Figs. 4A and 7). By deleting the *rcsB* gene in the Δ*opgH* mutant, the level of RsmB RNA was recovered to the wild-type strain (Fig. 4A). In accordance with the *rsmB* result, the Pel activity was restored to near wild-type levels by inactivation of the *rcsB* gene in the

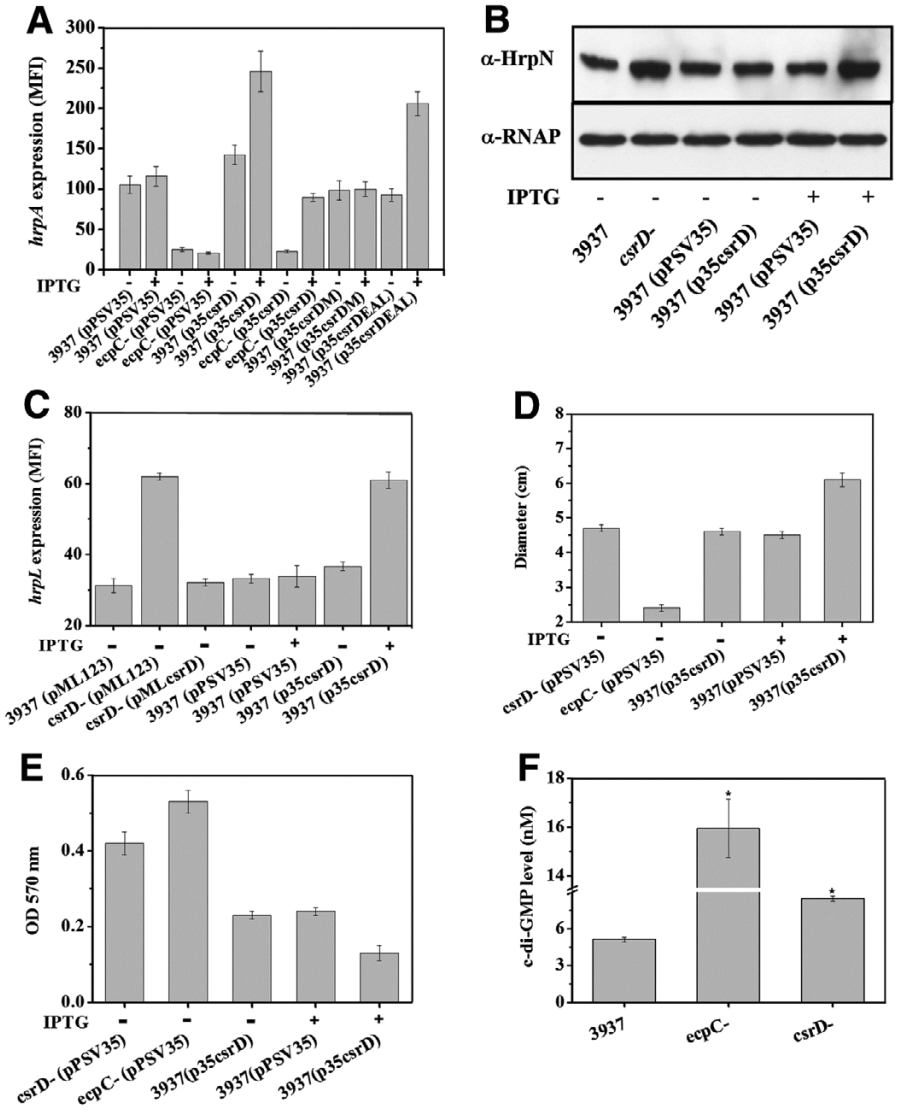

Fig. 6. CsrD controls the type III secretion system as a c-di-GMP phosphodiesterases. **A,** Promoter activities of *hrpA* were measured using plasmid pPhrpA in *Dickeya dadantii* 3937 carrying empty vector pPSV35, the Δ*ecpC* mutant Ech170 carrying empty vector pPSV35, *D. dadantii* 3937 carrying p35csrD, *D. dadantii* 3937 carrying p35csrDM, *D. dadantii* 3937 carrying p35csrDEAL, and the Δ*ecpC* mutant carrying p35csrD. Values of mean fluorescence intensity (MFI) are an average green fluorescent protein (GFP) fluorescence intensity of total bacterial cells with standard deviations (SD). **B,** Western blot analysis of total crude lysates (containing 5 μg of total protein, as determined by the Bradford assay) prepared from the wild-type strain *D. dadantii* 3937, the Δ*csrD* mutant Ech193, *D. dadantii* 3937 carrying empty vector pPSV35, and *D. dadantii* 3937 carrying p35csrD. **C,** The promoter activity of *hrpL* was measured using plasmid pPhrpL in strains *D. dadantii* 3937 carrying empty vector pML123, the Δ*csrD* mutant Ech193 carrying empty vector pML123, the Δ*csrD* mutant Ech193 carrying pMLcsrD, *D. dadantii* 3937 carrying empty vector pPSV35, and *D. dadantii* 3937 carrying p35csrD. Values of MFI are an average GFP fluorescence intensity of total bacterial cells with SD. **D,** Swarming motility was tested on mannitol-glutamate plates containing 0.4% agar. **E,** Biofilm formation phenotype of wild-type strain *D. dadantii* 3937 and its derivatives in minimal medium. OD = optical density at 570 nm. **F,** Intracellular c-di-GMP levels of *D. dadantii* were determined by liquid chromatography tandem mass spectrometry. Asterisks indicate $P < 0.05$ (Student's *t* test). Strain information and whether cultures were induced with isopropyl β-D-thiogalactoside (IPTG) (100 μM final concentration) are indicated below the graph.

Δ*opgH* mutant background (Fig. 7). OPG, the Rcs phosphorelay, and CsrD were found to control the T3SS of *D. dadantii* 3937 by manipulating the levels of RsmB RNA (Figs. 3, 4, and 5). Similar to the T3SS-*rsmB* gene expression patterns shown in the Δ*opgH,* Δ*rcsB*, and Δ*csrD* mutants (Figs. 3, 4, and 5), the Δ*opgH* mutant showed a reduction in Pel activity and the Δ*rcsB* and Δ*csrD* mutants showed an increase in Pel activity (Fig. 7). Thus, these results indicate that OpgH, RcsB, and CsrD co-regulate the T3SS and Pels by manipulating the levels of RsmB RNA.

It has been previously shown that OPG are required for *D. dadantii* pathogenicity (Page et al. 2001). Because RsmB RNA levels controlled the T3SS and Pel activity (Figs. 3, 4A, 5, and 7), we further hypothesized that OPG, Rcs phosphorelay, and CsrD coordinate bacterial virulence by manipulating regulatory small RNA levels. Compared with the wild-type strain, a significant reduction in RsmB RNA level and virulence was observed in the Δ*opgH* mutant (Figs. 4A and 8). The defect was caused by the loss of OPG, because introduction of a plasmid containing the *opgGH* operon restored pathogenicity (Fig. 8). Because an elevated level of RsmB RNA was observed in the Δ*rcsB* mutant (Fig. 4A), we examined whether the Δ*rcsB* mutant would result in a hypervirulent phenotype. As predicted, an increase in virulence was observed for the Δ*rcsB* mutant (Fig. 8). The Δ*opgH* Δ*rcsB* double mutant and wild-type strain that showed similar levels of RsmB RNA (Fig. 4A) displayed a similar virulence phenotype (Fig. 8). In addition, the virulence phenotype of the Δ*opgH* Δ*rcsB* double mutant was able to be restored to the Δ*opgH* and Δ*rcsB* mutant levels by placing pMLrcsB and pMLopgGH into the Δ*opgH* Δ*rcsB* double mutant, respectively (Fig. 8). Similar to the Δ*rcsB* mutant, the Δ*csrD* mutant that displayed higher levels of RsmB RNA was more virulent than the wild-type strain (Fig. 8). To further confirm that the virulence of *D. dadantii* is associated with RsmB RNA level, we overexpressed the *rsmB* gene in the wild-type strain. The overexpression of *rsmB* gene in wild-type strain *D. dadantii* 3937 increased the virulence compared with the control strain (Fig. 8). In summary, these results suggest that OPG, Rcs phosphorelay, and CsrD play major roles in the virulence of *D. dadantii* by modulation of T3SS expression and Pel production through manipulating the RsmB RNA levels.

DISCUSSION

In this study, we demonstrated that disparate regulatory elements such as OpgGH, Rcs phosphorelay, and CsrD coordinately regulate the two major virulence factors of *D. dadantii* (T3SS and Pel) through manipulating the expression of the regulatory small RNA RsmB. It is worth noting that CsrD is a dual-function protein; it is not only a regulator of *rsmB* but also a PDE. When *csrD* expression is induced, CsrD regulates T3SS and Pel activity by lowering the c-di-GMP levels in the bacterial cell (Fig. 1).

In *D. dadantii*, the *hrp* genes that encode structural and effector proteins of the T3SS such as *hrpA*, *hrpN*, and *dspE* are activated by the alternative σ factor HrpL (Tang et al. 2006; Yang et al. 2010). The expression of *hrpL* is controlled by HrpX/HrpY-HrpS at the transcriptional level and by GacS/GacA-RsmB at the post-transcriptional level (Yap et al. 2006; Yang et al. 2008) (Fig. 1). The RNA-binding protein RsmA binds to the *hrpL* mRNA in the 5′ untranslated region and induces its decay. In this study, OpgH influences the T3SS and Pel activity by modulating the level of RsmB RNA at the post-transcriptional level (Figs. 3 and 4). GacS/GacA regulates RsmB at the transcriptional level (Yang et al. 2008). Because OPG regulate *rsmB* at the post-transcriptional level (Fig. 4A and B), it is unlikely that OPG regulates RsmB via the GacS/GacA system. In species of Enterobacteria, products of the *opgGH* operon are responsible for the biosynthesis of the glucose backbone of OPG. OPG are general periplasmic constituents of the envelope of many gram-negative bacteria (Bohin and Lacroix 2006). OpgH is an inner-membrane glucosyltransferase and OpgG is predicted as a periplasmic protein. OpgG and OpgH catalyze the synthesis of linear glucans containing 5 to 13 glucose units joined by β (1→2) linkages and branched by β (1→6) linkages (Bohin and Lacroix 2006). Proteomic analysis data revealed that some genes related to carbohydrate metabolism and nutrient uptake were stimulated in response to OPG deficiency (Bouchart et al. 2007). The RsmA-RsmB system was first identified as a carbon storage regulatory system (Csr), and tricarboxylic acid cycle metabolic end products such as formate and acetate were reported to provide a physiological stimulus for the BarA/UvrY TCS (homolog of GacS/GacA), which stimulates the expression of regulatory

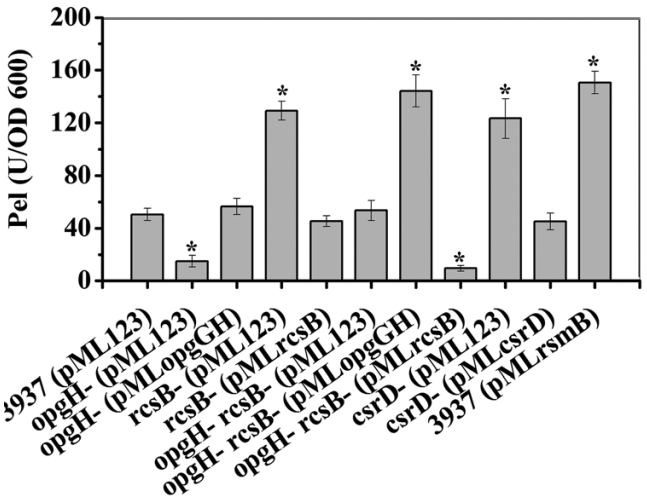

Fig. 7. Production of pectate lyases (Pel) in *Dickeya dadantii* 3937 and its derivatives. Spectrophotometric quantification of Pel activity was measured as described (Matsumoto et al. 2003). Data represent three biological replicates, and error bars indicate the standard errors of the means. Asterisks indicate statistically significant differences in Pel activities of the mutants compared with that of the wild type ($P < 0.05$; Student's *t* test).

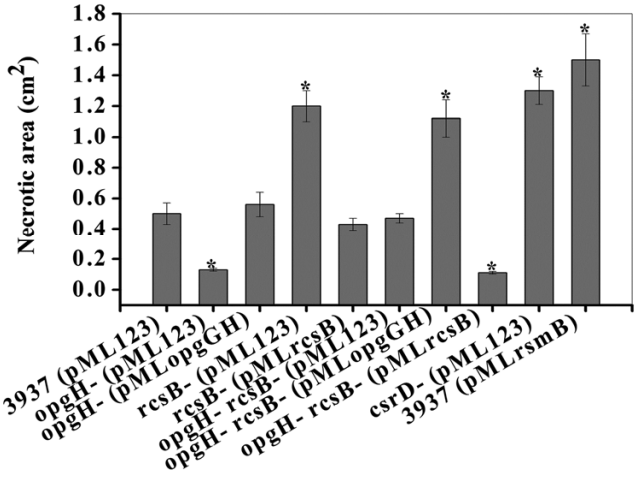

Fig. 8. Disease lesions caused by *Dickeya dadantii* 3937 and its derivatives in leaves of Chinese cabbage. Leaves were inoculated at small wounds with a 10-μl bacterial suspension at the concentration of 10^8 CFU/ml. Values are means of the necrotic area produced in Chinese cabbage leaves after 24 h of incubation at 28°C. Asterisks indicate statistically significant differences in necrotic areas of the mutants compared with that of the wild type ($P < 0.05$; Student's *t* test).

small RNA CsrB (Chavez et al. 2010; Romeo et al. 1993). Therefore, it is possible that OpgGH may influence the carbon availability in cells and, therefore, modulate the expression of the *rsmB* gene. A recent study showed that OpgH acted as a UDP-glucose-activated inhibitor of FtsZ ring formation in *E. coli* (Hill et al. 2013). OpgH interacts directly with FtsZ via its N-terminal domain to inhibit cell division independent from its role in OPG synthesis (Hill et al. 2013). Thus, the mechanism behind OpgH on gene regulation remains to be elucidated.

A previous study has shown that the virulence of the Δ*opgG* mutant was restored by inactivation of the RcsCD-RcsB phosphorelay, indicating the role of Rcs phosphorelay in regulation of virulence in *D. dadantii* (Bouchart et al. 2010). The data presented here suggest that restoration of virulence in the Δ*opgH* Δ*rcsB* double mutant is achieved by modulating the RsmB RNA level. RcsB negatively regulates the transcription of *rsmB*, whereas OPG influence the expression of *rsmB* at the post-transcriptional level. The different levels of *rsmB* regulation between RcsB and OPG are assumed to facilitate fine tuning of the RsmB-dependent regulon in response to cellular physiology and environmental stimuli in *D. dadantii*. In the related bacterium *Pectobacterium carotovorum*, RcsB is a transcriptional repressor that regulates the expression of the *rsmB* gene (Andresen et al. 2010). Although the DNA-binding site of RcsB was found within the *rsmB* promoter region of *P. carotovorum*, the regulation of *rsmB* by RcsB was mainly through modulating *flhDC* transcription (Andresen et al. 2010). Although the *rsmB* promoter region of *D. dadantii* 3937 also comprises an RcsB-like palindromic sequence (data not shown), it is uncertain how RcsB regulates *flhDC* and *rsmB* at this stage. A previous study has shown that IgaA, a homologue of YrfF, works as an intracellular-growth-attenuator protein and a positive regulator of bacterial virulence in *S. enterica* (Tierrez and Garcia-del Portillo 2004). IgaA repressed the RcsCD-RcsB phosphorelay at the post-transcriptional level. However, our data showed that YrfF had no effect on the expression of *rsmB* or *hrpA* genes, which suggested that, unlike the role of YrfF in *S. enterica,* YrfF isn't involved in regulation of RcsCD-RcsB phosphorelay in *D. dadantii* 3937.

Our results provide new insights into how the T3SS and other virulence factors are regulated by the GGDEF-EAL domain protein CsrD. In *E. coli*, small RNAs CsrB and CsrC counteract the activity of the RNA-binding protein CsrA (Romeo et al. 1993). An additional component of the Csr circuit is CsrD (Suzuki et al. 2006). CsrD binds to the CsrB and CsrC RNAs with high affinity and transformed these regulatory RNAs into substrates for RNase E degradation (Suzuki et al. 2006). In accordance with the phenotype reported in *E. coli*, a mutation of *csrD* increases the RsmB (CsrB homologue) RNA level in *D. dadantii*. Unexpectedly, we observed a reduced level of *rsmB* promoter activity in the Δ*csrD* mutant. To examine the inverse regulation of *rsmB* at transcriptional and post-transcriptional levels by CsrD, we examined the effect of CsrD on OpgGH. Our result showed a negative effect of CsrD on OpgH protein levels, which suggested that CsrD regulates the levels of RsmB RNA through the OpgGH (Figs. 1 and 5). Per the literature, an increased level of OpgG was also detected in a *pigX* mutant of *Serratia* sp. (Fineran et al. 2007).

The second messenger c-di-GMP controls multiple phenotypes in bacteria, including biofilm formation, motility, and virulence (Jenal and Malone 2006; Romling et al. 2013). The c-di-GMP is synthesized by diguanylate cyclase (DGC) enzymes encoding GGDEF domains, and is degraded by PDE enzymes encoding EAL or HD-GYP domains. The intracellular level of c-di-GMP is controlled by antagonistically acting DGC and PDE proteins (Romling et al. 2013). In *E. coli*, CsrD is involved in binding a nonencoding RNA CsrB but did not play a role in the c-di-GMP signaling pathway (Suzuki et al. 2006). PigX of *S. marcescens* is a CsrD ortholog. It was suggested that PigX functioned as a c-di-GMP PDE, because the EAL domain of PigX alone could complement the production of prodigiosin and swarming motility phenotypes in *S. marcescens* (Fineran et al. 2007). Sequence analysis showed that CsrD of *D. dadantii* 3937 contains a central GGDEF domain and a C-terminal EAL domain. In this study, the expression of *hrpA* was increased when CsrD was overexpressed, suggesting that CsrD is a PDE (Fig. 6A). Ultraperformance liquid chromatography (UPLC)-MS-MS assays further verified that the intracellular level of c-di-GMP was increased in the Δ*csrD* mutant (Fig. 6F). Genetic and biochemical evidence demonstrates that CsrD functions as a PDE in *D. dadantii* 3937. However, it needs to be mentioned that, although overexpression of *csrD* in *D. dadantii* 3937 caused the increase of *hrpA* expression and HrpN protein production, the transcription of *hrpA* and production of HrpN protein were enhanced in the Δ*csrD* mutant (Figs. 5 and 6). This suggests that, under our experimental conditions, CsrD might play a minor role as an EAL in controlling T3SS and Pel. Previously, a study showed that a mutation in the *ecpC* gene, encoding an EAL domain protein, resulted in reducing expression of *hrpA* which was due to the increase of c-di-GMP level in cells (Yi et al. 2010). Indeed, although a moderate increase in c-di-GMP levels was observed in the Δ*csrD* mutant compared with the wild-type strain *D. dadantii* 3937, the level of c-di-GMP of the Δ*csrD* mutant was much lower than the Δ*ecpC* mutant observed in this study (Fig. 6F).

In conclusion, we have shown that virulence factors of *D. dadantii* are controlled in a coordinated manner. CsrD regulates the expression and production of T3SS and Pel virulence factors by modulating levels of RsmB RNA and c-di-GMP. In addition, a sophisticated regulatory circuit consisting of CsrD, OpgGH, and the RcsCD-RcsB regulatory system controls the production of T3SS and Pel factors through manipulating the regulatory small RNA RsmB at the transcriptional and post-transcriptional levels.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

Bacterial strains and plasmids used in this study are listed in Table 2. *E. coli* was grown in Luria-Bertani (LB) medium at 37°C and *D. dadantii* was cultured in LB medium, minimal medium (MM) (Yang et al. 2007), or mannitol-glutamate (MG) medium (Bell 1990) at 28°C. When necessary, antibiotics were added at the following concentrations: ampicillin at 100 μg/ml, chloramphenicol at 20 μg/ml, kanamycin at 50 μg/ml, and gentamicin at 20 μg/ml.

DNA manipulations and sequence analyses.

Chromosomal DNA of *D. dadantii*, plasmid DNA extractions, and other molecular assays were performed according to standard procedures (Sambrook and Russell 2001). Electroporation of bacterial cells with plasmid DNA was performed as described previously (Choi et al. 2006). Nucleotide sequences were determined on an ABI-Prism 373 automatic sequencer (Applied Biosystems). Nucleotide and deduced amino acid sequences were analyzed with programs of the National Center for Biotechnology Information BLAST server (Altschul et al. 1997).

Construction of bacterial mutants.

To generate the Δ*opgH,* Δ*rcsB,* Δ*yrfF,* and Δ*csrD* mutants, two fragments flanking each target gene were amplified by

PCR. A kanamycin cassette, obtained from pKD4, was ligated with these two fragments and then cloned into pWM91 (Metcalf et al. 1996). The plasmid constructs were then transferred into *D. dadantii* 3937 by conjugation using *E. coli* S17-1, and colonies with sucrose and kanamycin resistance and ampicillin sensitivity were selected and confirmed by PCR and DNA sequencing (Li et al. 2010). The Δ*opgH* Δ*rcsB* double mutant was generated using the Δ*opgH* mutant background and the same methods and primers as described above. To construct the plasmids for complementation, the coding region of the *opgGH*, *rcsB*, and *csrD* genes were PCR-amplified and cloned into pML123 to yield pMLopgGH, pMLrcsB, and pMLcsrD, respectively. Plasmid p35csrD was constructed by PCR amplification of the *csrD* gene using the CsrDEcoRI and CsrDSalI primer pair (Table 2). The EcoRI-SalI restriction fragment of *csrD* was cloned into pPSV35 (Rietsch et al. 2005), resulting in the *csrD* overexpression vector p35csrD. To construct the plasmid for the overexpression of the EAL domain of CsrD, the EAL domain coding region was PCR amplified using the CsrDEALin35E and CsrDSalI primer pair and then cloned into pPSV35.

To construct a C-terminal 6×His tag-OpgG fusion, a PCR-generated fragment with the sequence 5′-CACCACCACCAC CACCAC-3′ inserted in-frame to the 3′ end of the *opgG* gene was cloned into pWM91. The resulting plasmid, p91opgGHis, was verified by sequencing and mobilized into wild-type strain *D. dadantii* 3937 and the *csrD* mutant to generate strains Ech192 and Ech194, respectively.

Site-directed mutagenesis of the EAL domain of CsrD.

To introduce the point-specific mutation into the EAL domain of CsrD, oligonucleotides containing the designed mutation (primer pairs ELLD5 and ELLD5R) were used to generate p35csrD derivative by inverse PCR (QuickChange Lightning site-directed mutagenesis kit; Stratagene). The specificity of the nucleotide sequence was confirmed by DNA sequencing.

Transposon mutagenesis and promoter activity assay.

A transposon mutagenesis combined with a GFP reporter FACS was used to screen for novel regulators of *hrpA*. *D. dadantii* 3937 carrying a *hrpA-gfp* transcriptional fusion in the pPhrpA vector was subject to random mini-*Himar* RB1 insertion mutagenesis using the suicide plasmid pMiniHimar RB1, following a method described by Bouhenni and associates (2005). *D. dadantii* colonies with mini-*Himar* RB1 insertions were transferred to LB broth and induced in MM broth for 12 h during the exponential growth phase and the *hrpA* promoter activity was monitored by FACS, as previously described (BD Biosciences) (Zeng et al. 2012). Mutants in which *hrpA-gfp* expression was significantly altered compared with the wild-type strain were picked and reconfirmed by FACS. The genomic DNA fragments flanking the mini-*Himar* RB1 in the

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

Strain or plasmid	Sequence (5′→3′)	Description[a]	Reference or source
Dickeya dadantii			
3937	…	Wild-type strain	Hugouvieux-Cotte-Pattat et al. 1996
Ech191	…	*opgH* deletion mutant	This study
Ech192	…	Km^r; 3937 with a 6×His epitope sequence tagged to the C terminus of OpgG	This study
Ech193	…	*csrD* deletion mutant	This study
Ech194	…	Km^r; Ech193 with a 6×His epitope sequence tagged to the C terminus of OpgG	This study
Ech195	…	*rcsB* deletion mutant	This study
Ech196	…	Km^r; *rcsB* and *opgH* double deletion mutant	This study
Ech197	…	Km^r; *yrfF* deletion mutant	This study
Ech170	…	Km^r; *ecpC* deletion mutant	Yi et al. 2010
Escherichia coli			
DH5α	…	*supE44 lac*U169 (ϕ80*lacZ* M15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Sambrook and Russell 2001
S17-1 λpir	…	λ(pir) *hsdR pro thi*; chromosomally integrated RP4-2 Tc::Mu Km::Tn*7*	Simon et al. 1983
Plasmids			
pMiniHimar RB1	…	Km^r; plasmid carrying mini-*Himar* RB1, *oriR6K oriT lacZ*	Bouhenni et al. 2005
pPROBE-AT	…	Ap^r; promoter-probe vector	Miller et al. 2000
pPdspE	…	Ap^r; pPROBE-AT containing a *dspE-gfp* transcriptional fusion	Zeng et al. 2010
pPhrpA	…	Ap^r; pPROBE-AT containing a *hrpA-gfp* transcriptional fusion	Yang et al. 2008
pPhrpL	…	Ap^r; pPROBE-AT containing a *hrpL-gfp* transcriptional fusion	Yang et al. 2008
pPhrpN	…	Ap^r; pPROBE-AT containing a *hrpN-gfp* transcriptional fusion	Yang et al. 2008
pPrsmB	…	Ap^r; pPROBE-AT containing a *rsmB-gfp* transcriptional fusion	Zeng et al. 2012
pPopgGH	…	Ap^r; pPROBE-AT containing a *opgGH-gfp* transcriptional fusion	This study
pWM91	…	Ap^r; sucrose-based counter-selectable plasmid	Metcalf et al. 1996
p91DopgH	…	Ap^r; plasmid pWM91 carrying a deleted *opgH* gene	This study
p91DrcsB	…	Ap^r; plasmid pWM91 carrying a deleted *rcsB* gene	This study
p91opgGHis	…	Ap^r; pWM91 with a 6×His epitope sequence tagged to the C terminus of OpgG	This study

(*continued on next page*)

[a] Km^r, Ap^r, and Gm^r indicate kanamycin, ampicillin, and gentamycin resistance respectively.

mutants were cloned by self-ligation and sequenced with primer himar1 and primer 615 (Bouhenni et al. 2005).

pPROBE-AT is a promoter probe vector which contains the ribosomal binding site upstream of the *gfp* gene (Miller et al. 2000). The reporter constructs pPhrpA, pPhrpL, pPhrpN, pPdspE, pPrsmB, and pPopgGH were used to monitor promoter activities of *hrpA, hrpL, hrpN, dspE, rsmB*, and *opgGH*, respectively, in *D. dadantii* by flow cytometry. The shuttle vectors pPSV35 (Rietsch et al. 2005) and pML123 (Labes et al. 1990) contain a pBR322 and RSF1010 replication origin, respectively. The promoter probe vector pPROBE-AT has a pBBR1 origin (Miller et al. 2000). These plasmids stably coreplicate in bacterial cells (Davison 2002).

qRT-PCR and Northern blot analysis.

Bacteria were cultured in LB broth overnight and then induced in MM broth at 28°C for 12 h during the exponential growth phase. Total bacterial RNA was isolated using RNeasy mini kit (Qiagen). Extracted RNA was treated with Turbo DNase I (Ambion). cDNA synthesis was performed with iScript cDNA synthesis kit (Bio-Rad). The iQ SYBR Green Supermix (Bio-Rad) was used for real-time PCR. Data were collected by the Opticon 2 system (Bio-Rad) and analyzed using the Relative Expression Software Tool (Pfaffl et al. 2002). The expression of *rplU* was used as an endogenous control for data analysis (Pfaffl et al. 2002).

For the Northern blot analysis, total RNA was prepared using TRI reagent (Sigma-Aldrich). Samples of RNA (10 μg for each lane) were analyzed by Northern blot using biotin-labeled *hrpA* or *rsmB* probes, and a biotin detection method provided by Ambion (BrightStar Psoralen-Biotin and Bright Star BioDetect). 16S rRNA was used as an internal control.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting.

D. dadantii cells were grown in MM broth at 28°C for 12 h during the exponential growth phase and 1-ml samples were taken. Cells were then suspended in phosphate-buffered saline (PBS) buffer and lysed by sonication. The protein in crude lysates was quantified using the Bradford protein assay (Bio-Rad). Samples were boiled before being loaded onto 12% sodium dodecyl sulfate polyacrylamide gels. Proteins were then transferred onto a polyvinylidene fluoride membrane (Millipore). Blots were washed with PBS containing 0.05% Tween-20 and probed with an anti-HrpN antibody (1:5,000) or anti-His polyclonal antibody (1:2,000) (SouthernBiotech). Anti-RNA polymerase monoclonal antibody (1:2,000) (Neoclone) was used as a control. The resulting blots were incubated for 1 min in enhanced chemiluminescence reagent (GE Healthcare) and detected using O-MAT X-ray film.

Determination of intracellular c-di-GMP concentration.

Quantitative analysis of intracellular c-di-GMP concentrations was performed using UPLC-MS-MS, as described previously (Edmunds et al. 2013; Massie et al. 2012) . Specifically, *D. dadantii* 3937 and its derivatives were cultivated with shak-

Table 2. (*continued from preceding page*)

Strain or plasmid	Sequence (5′→3′)	Description[a]	Reference or source
pML123	…	Gm^r; RSF1010-derived expression and *lac*-fusion broad-host-range vector	Labes et al. 1990
pMLopgGH	…	Gm^r; pML123 containing the *opgGH* genes	This study
pMLrsmB	…	Gm^r; pML123 containing the *rsmB* gene	Zeng et al. 2010
pMLrcsB	…	Gm^r; pML123 containing the *rcsB* gene	This study
pMLcsrD	…	Gm^r; pML123 containing the *csrD* gene	This study
pPSV35	…	Gm^r; huttle vector, *lacIq*, and the *lacUV5* promoter and MCS of pUC18	Rietsch et al. 2005
p35csrD	…	Gm^r; pPSV35 containing the *csrD* gene	This study
p35csrDEAL	…	Gm^r; pPSV35 containing the EAL domain of CsrD	This study
p35csrDM	…	Gm^r; p35csrD derivative with ^{443}ELL to ^{443}ALL point mutation	This study
Oligonucleotides			
rcsB430	TGGGATCCTGGGGTGCTGATGTCGCG	*rcsB* deletion	…
rcsB1080	GAAGCAGCTCCAGCCTACACATCGATTTGT TCCAGCGAC	…	…
rcsB1561	GGAATAGGAACTAAGGAGGATATTCATAT GATCGGCCATGACCA	…	…
rcsB2120	ATGGATCCTGCCGTCGGTCAGTTACAATC	…	…
opgH40SacI	ATGAGCTCCGCGAGTTCTGGATAG	*opgH* deletion	…
opgH1060EcoRI	TAGAATTCTCACGCAGGGCCTCTGCC	…	…
opgH3460EcoRI	ACGAATTCGCCTGCACCATCGGGTGTG	…	…
opgH4520XhoI	AGTCTCGAGGTTTCGTGCCTAACGCCAATG	…	…
yrfFUF	GCAGGGATCCACTGAATCAACAGCAG	*yrfF* deletion	…
yrfFUR	AAAAAATGCGGCCAATAGAAGACGAC	…	…
YrfFDF	TTTTTTTGCGGCCCTGATACTGAATC	…	…
yrfFDR	CGTCTCGAGGACAACGAATGCCAAAC	…	…
MLrcsBXbaI	AGTCTAGAGACTATATACTGAGACCCAAG	*rcsB* complementation	…
MLrcsBamHI	ATGGATCCAAACCGGTGACGCTGGATACG	…	…
MLcsrDXbaI	CGTCTAGAAGGCCATTTTGTTGCCTGAGC	*csrD* comlementation	…
MLcsrDEcoRI	CGAATTCATCAAACATACGTGCGTGGTG	…	…
MLopgGHXbaI	GATCTAGATATCCGTTATGGTTTTTCATCCTG	*opgGH* complementation	…
MLopgGHEcoRI	TCGAATTCCTGAGACGCGGAAAATTTCTC	…	…
CsrDEcoRI	ACGAATTCAGGCCATTTTGTTGCCTGAGCAC	Clone *csrD* gene into pPSV35	…
CsrDSalI	CAGTCGACATCAAACATACGTGCGTGGTGAA	…	…
CsrDEALin35E	ACGAATTCATGCTGGAACATACCCTGTTGCG	Clone the EAL domain of *csrD* gene into pPSV35	…
CsrDSalI	CAGTCGACATCAAACATACGTGCGTGGTGAA	…	…
ELLD5	TGATGGCACCCAGGCGTTGCTGGAGGCG	Site-directed mutagenesis of the EAL domain of CsrD	…
ELLD5R	CGCCTCCAGCAACGCCTGGGTGCCATCA	…	…

ing in 40 ml of LB liquid media at 28°C for 12 h. The cells were harvested by centrifugation in 50-ml polystyrene centrifuge tubes at 4°C for 15 min at 4,000 × *g*. The supernatant was removed, and the pellet was resuspended in 1 ml of extraction buffer (40% acetonitrile and 40% methanol in 0.1 N formic acid) and incubated at –20°C for 1 h, the lysate was then centrifuged at 4°C for 5 min at 15,000 × *g*, and the debris-free liquid was collected and stored at –80°C. Prior to analysis, a 200-µl aliquot was taken and the extraction buffer was evaporated using a vacuum manifold. The pellet was rehydrated in 100 µl of water and analyzed by UPLC-MS-MS using an Acquity UPLC system (Waters) coupled with a Quattro Premier XE mass spectrometer (Waters). The c-di-GMP concentration of the sample was determined using an eight-point standard curve of chemically synthesized c-di-GMP (Biolog) ranging from 1.9 to 250 nM. The c-di-GMP measurements were normalized to the optical density at 600 nm values of the respective cultures used for extraction.

Phenotypic and virulence assays.

The biofilm growth assay and swarming motility assay were performed as previously described (Yi et al. 2010). Pel activity was determined by monitoring spectrophotometrically the formation of unsaturated products from polygalacturonate at 230 nm, as described previously (Matsumoto et al. 2003). The assay mixture consisted of 0.1 M Tris-HCl (pH 8.5), 0.1 mM $CaCl_2$, and 0.5% polygalacturonate. For virulence assays, *D. dadantii* 3937 and its derivatives were grown in LB broth at 28°C for 12 h. Cells were harvested and resuspended in PBS to 10^8 CFU/ml. Chinese cabbage leaves were used for the maceration assay. For each Chinese cabbage leaf, two wounds were punched on each half, and dried to remove moisture. Maceration symptoms were documented at 30 h postinoculation, as described previously (Li et al. 2010). Virulence assays were performed in triplicate.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

NCBI BLAST server: www.ncbi.nlm.nih.gov/BLAST
University of Wisconsin-Madison ASAP database:
asap.ahabs.wisc.edu/asap/ASAP1.htm