Molecular Microbiology (2010) 77(3), 787–800 ■
doi:10.1111/j.1365-2958.2010.07246.x
First published online 23 June 2010

Genetic analysis of two phosphodiesterases reveals cyclic diguanylate regulation of virulence factors in *Dickeya dadantii*

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Summary

Cyclic diguanylate (c-di-GMP) is a second messenger implicated in the regulation of various cellular properties in several bacterial species. However, its function in phytopathogenic bacteria is not yet understood. In this study we investigated a panel of GGDEF/EAL domain proteins which have the potential to regulate c-di-GMP levels in the phytopathogen *Dickeya dadantii* 3937. Two proteins, EcpB (contains GGDEF and EAL domains) and EcpC (contains an EAL domain) were shown to regulate multiple cellular behaviours and virulence gene expression. Deletion of *ecpB* and/or *ecpC* enhanced biofilm formation but repressed swimming/swarming motility. In addition, the *ecpB* and *ecpC* mutants displayed a significant reduction in pectate lyase production, a virulence factor of this bacterium. Gene expression analysis showed that deletion of *ecpB* and *ecpC* significantly reduced expression of the type III secretion system (T3SS) and its virulence effector proteins. Expression of the T3SS genes is regulated by HrpL and possibly RpoN, two alternative sigma factors. *In vitro* biochemical assays showed that EcpC has phosphodiesterase activity to hydrolyse c-di-GMP into linear pGpG. Most of the enterobacterial pathogens encode at least one T3SS, a major virulence factor which functions to subvert host defences. The current study broadens our understanding of the interplay between c-di-GMP, RpoN and T3SS and the potential role of c-di-GMP in T3SS regulation among a wide range of bacterial pathogens.

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Introduction

Cyclic diguanylate (c-di-GMP) [bis(3′,5′)-cyclic diguanylic acid] is a bacterial second messenger that has been implicated in the regulation of many bacterial behaviours, including biofilm, motility and virulence of pathogens (D'Argenio and Miller, 2004; Dow *et al.*, 2006; Jenal and Malone, 2006; Kolter and Greenberg, 2006; Römling and Amikam, 2006; Ryan *et al.*, 2006b; Hengge, 2009). It was first identified in *Gluconacetobacter xylinus* as an allosteric activator of cellulose synthesis (Ross *et al.*, 1987). GGDEF, EAL and HD-GYP domains (named after their conserved amino acids) are known to be involved in c-di-GMP turnover (Hengge, 2009). The GGDEF domain functions as a diguanylate cyclase that synthesizes c-di-GMP from two molecules of GTP (Paul *et al.*, 2004; Solano *et al.*, 2009). The EAL and HD-GYP domains act as phosphodiesterases (PDE) that hydrolyse c-di-GMP into linear nucleotide pGpG or two molecules of GMP (Schmidt *et al.*, 2005; Tamayo *et al.*, 2005; Ryan *et al.*, 2006a). Many bacterial genomes encode a number of proteins with GGDEF, EAL and HD-GYP domains (Galperin, 2005). For example, *Escherichia coli* encodes 36 such proteins (Jenal and Malone, 2006), *Vibrio cholerae* encodes 72 (Galperin *et al.*, 2001), *Pseudomonas aeruginosa* encodes 41 (Galperin *et al.*, 2001), and *Dickeya dadantii* 3937 (formerly known as *Erwinia chrysanthemi*) encodes 18 (ASAP website; https://asap.ahabs.wisc.edu/asap/home.php). The abundance of these proteins indicates the importance of the second messenger c-di-GMP in various bacteria.

In recent years, c-di-GMP has been shown to be involved in the pathogenesis of many animal pathogens (Tamayo *et al.*, 2007), including *Salmonella enterica* (Hisert *et al.*, 2005), *P. aeruginosa* (Kulasakara *et al.*, 2006) and *V. cholerae* (Tischler and Camilli, 2005), and one plant pathogen *Xanthomonas campestris* pv. *campestris* (Ryan *et al.*, 2006a). In a recent study by Kulasakara and colleagues, the effect of c-di-GMP on type III secretion system (T3SS)-mediated cytotoxicity was investigated in *P. aeruginosa* by deletion and transposon mutagenesis of genes encoding GGDEF and EAL domain proteins (Kulasakara *et al.*, 2006). Based on

results from these and other reports, the effect of c-di-GMP on virulence in different bacteria seems to be complex.

Although several studies have demonstrated a role for c-di-GMP as a common virulence regulator for many animal bacterial pathogens, little is known about its function in the disease process of phytopathogenic bacteria. *D. dadantii* is the causal agent of soft rot disease on cash crops worldwide including potato, carrot, celery and Chinese cabbage (Bradbury, 1986; Yap *et al*., 2005; Peng *et al*., 2006). Pectinases and T3SS are two major virulence factors that contribute to the pathogenesis of *D. dadantii* strain 3937. *D. dadantii* 3937 produces and secretes a large amount of pectinases through the type II secretion system to break down the plant cell wall and cause maceration of plant tissue (Boccara and Chatain, 1989; Kazemi-Pour *et al*., 2004). The T3SS delivers a set of bacterial effector proteins into host cells to promote parasitism and suppress host defences (Alfano and Collmer, 1997; 2004; Galán and Collmer, 1999). The T3SS is conserved among various plant pathogens, such as *Pseudomonas syringae*, *X. campestris* and *Erwinia* species (Tang *et al*., 2006), and is also known as the Hrp system. This highly specialized translocation system is encoded by the *hrp* (hypersensitive response and pathogenicity) gene cluster, and is essential for the disease symptoms in host plants and the hypersensitive response in non-hosts (Yang *et al*., 2002).

In *D. dadantii* 3937, the *hrp* genes encode several structural and effector proteins such as HrpA, HrpN and DspE (Yap *et al*., 2006). The expression of these *hrp* genes is activated by HrpL (Yap *et al*., 2005; Peng *et al*., 2006), an alternative sigma factor that binds to the promoter region of the *hrp* genes (Wei and Beer, 1995; Frederick *et al*., 2001; Fouts *et al*., 2002). *hrpL* expression is controlled by a sophisticated regulatory cascade (Yap *et al*., 2005; Yang *et al*., 2008), and its transcription is activated by the binding of RpoN (σ^{54}) to the *hrpL* promoter region (Chatterjee *et al*., 2002) (Fig. 7). HrpS acts as a σ^{54} enhancer in conjunction with RpoN to stimulate *hrpL* expression (Chatterjee *et al*., 2002; Yap *et al*., 2005) (Fig. 7). Furthermore, *hrpS* expression is activated by a two-component system, HrpX/Y (Yap *et al*., 2005) (Fig. 7). In addition, *hrpL* is regulated at the mRNA level by the RsmA/*rsmB* RNA-mediated post-transcriptional pathway (Chatterjee *et al*., 2002; Yang *et al*., 2008) (Fig. 7). RsmA is a small RNA-binding protein that accelerates the decay of *hrpL* mRNA upon binding (Chatterjee *et al*., 1995). However, *rsmB* is a regulatory RNA that binds to and sequesters the negative effect of RsmA on *hrpL* mRNA by forming an inactive ribonucleoprotein RsmA–*rsmB* complex (Liu *et al*., 1998; Chatterjee *et al*., 2002). Expression of *rsmB* is upregulated by another two-component system GacS/A (Yang *et al*., 2008) (Fig. 7).

In this study, the relationship between the second messenger c-di-GMP and the expression of virulence factors was examined. A total of 18 GGDEF/EAL domain proteins were identified in the *D. dadantii* 3937 genome. A panel of deletion mutants was constructed and evaluated for their impact on c-di-GMP levels. This work demonstrates that c-di-GMP negatively regulates T3SS expression in *D. dadantii* 3937. Genetic evidence suggests that this regulation is mediated by the alternative sigma factor HrpL and possibly involves RpoN (σ^{54}). In addition, we show that c-di-GMP acts as a global regulator of biofilm formation, cell motility and pectate lyase (Pel) production.

Results

Mutant construction

The *D. dadantii* 3937 genome encodes 12 GGDEF domain proteins, four EAL domain proteins and two GGDEF-EAL domain proteins (ASAP website), whose potential functions are related to c-di-GMP turnover. To study the role of c-di-GMP in *D. dadantii* 3937, nine of these proteins were selected for mutagenesis (Table 1 and Table S1), based on the presence of conserved GGDEF/EAL domains which suggests functional diguanylate cyclases or phosphodiesterases. These proteins were designated as GcpA-GcpF (GGDEF domain-containing protein) and EcpA-EcpC (EAL domain-containing protein). It is noted that EcpB was named due to its function as an active phosphodiesterase in the genetic analysis, even though it contains both a GGDEF and an EAL domain. Deletion mutations of these genes were constructed as described in *Experimental procedures* except *gcpA* (ABF-20368) and *gcpE* (ABF-19019), which could not be deleted using this method.

EcpB and EcpC negatively affect biofilm formation

In several bacteria, c-di-GMP levels have been shown to positively affect biofilm formation (Jenal and Malone, 2006; Römling and Amikam, 2006; Tamayo *et al*., 2007). To evaluate the function of c-di-GMP in *D. dadantii* 3937, we assayed biofilm formation of wild-type and mutant strains in T3SS-inducing minimal medium (MM) (Yang *et al*., 2007). Deletion of either *ecpB* or *ecpC* enhanced biofilm formation in MM (fivefold increase) compared with wild type, whereas deletion of the other genes encoding GGDEF or EAL domain-containing proteins had no significant effect (Fig. 1A). A double-deletion mutant, Δ*ecpB*Δ*ecpC*, showed more than a 10-fold increase in biofilm formation compared with wild type (Fig. 1A), and produced more biofilm than either of the single mutants. Complementation analysis was per-

Table 1. Bacterial strains and plasmids.

Strains/plasmids	Characteristics	References or source
E. coli		
DH5α	High-efficiency transformation	Invitrogen
S17-1 *pir+*	λ-*pir* lysogen of S17-1	Victor de Lorenzo
BL21 (DE3)	Protein expression host	Novagen
D. dadantii		
3937	Wild type, *Santpaulia* (African violet) isolate	Hugouvieux-Cotte-Pattat, N
Δ*gcpB*	Δ*gcpB::Km*; Km^r, ABF-16029 deletion mutant	This study
Δ*gcpC*	Δ*gcpC::Km*; Km^r, ABF-19499 deletion mutant	This study
Δ*gcpD*	Δ*gcpD::Km*; Km^r, ABF-14719 deletion mutant	This study
Δ*gcpF*	Δ*gcpF::Km*; Km^r, ABF-16283 deletion mutant	This study
Δ*ecpA*	Δ*ecpA::Km*; Km^r, ABF-15066 deletion mutant	This study
Δ*ecpB*	Δ*ecpB::Km*; Km^r, ABF-20123 deletion mutant	This study
Δ*ecpC*	Δ*ecpC::Km*; Km^r, ABF-20364 deletion mutant	This study
Δ*ecpBC*	Δ*ecpB::Km* Δ*ecpC::Cm*; Km^r Cm^r, ABF-20123 and ABF-20364 double mutant	This study
Plasmids		
pKD3	Cm^r, template plasmid carrying Cm resistance cassette	Datsenko and Wanner (2000)
pKD4	Km^r, template plasmid carrying Km resistance cassette	Datsenko and Wanner (2000)
pWM91	Suicide vector, Ap^r	Metcalf *et al.* (1996)
pML122	Low-copy-number plasmid, Gm^r	Labes *et al.* (1990)
pML*ecpB*	Complementation, *ecpB* cloned in pML122, Gm^r	This study
pML*ecpC*	Complementation, *ecpC* cloned in pML122, Gm^r	This study
pML*ecpB*^{E528A}	*ecpB* with point mutation (E528A) cloned in pML122, Gm^r	This study
pML*ecpC*^{E56A}	*ecpC* with point mutation (E56A) cloned in pML122, Gm^r	This study
pML*rsmB*	*rsmB* overexpression, *rsmB* cloned in pML123, Gm^r	This study
pPROBE-AT	Promoter-probe vector, promoterless *gfp*, Ap^r	Miller *et al.* (2000)
pAT–*ecpC*	*ecpC* promoter–*gfp* transcriptional fusion in pPROBE-AT, Ap^r	This study
pAT–*ecpB*	*ecpB* promoter–*gfp* transcriptional fusion in pPROBE-AT, Ap^r	This study
pAT–*hrpA*	*hrpA* promoter–*gfp* transcriptional fusion in pPROBE-AT, Ap^r	Yang *et al.* (2008)
pAT–*hrpN*	*hrpN* promoter–*gfp* transcriptional fusion in pPROBE-AT, Ap^r	Yang *et al.* (2008)
pAT–*dspE*	*dspE* promoter–*gfp* transcriptional fusion in pPROBE-AT, Ap^r	Yang *et al.* (2008)
pAT–*hrpL*	*hrpL* promoter–*gfp* transcriptional fusion in pPROBE-AT, Ap^r	Yang *et al.* (2008)
pAT–*hrpS*	*hrpS* promoter–*gfp* transcriptional fusion in pPROBE-AT, Ap^r	This study
pAT–*rpoN*	*rpoN* promoter–*gfp* transcriptional fusion in pPROBE-AT, Ap^r	This study
pAT–*rsmA*	*rsmA* promoter–*gfp* transcriptional fusion in pPROBE-AT, Ap^r	This study
pET21b	Overexpression and purification vector, Ap^r	Novagen
pET*ecpC*	*ecpC* cloned in pET21b, Ap^r	This study

Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance.

formed using pML122 containing the constitutive *nptII* promoter. Expression of EcpB and EcpC (pML*ecpB* and pML*ecpC*) repressed the hyper-biofilm phenotype in Δ*ecpB* and Δ*ecpC*, respectively, whereas expression of EcpB and EcpC with a point mutation in the EAL motif [pML*ecpB*^{E528A} (EAL to AAL) and pML*ecpC*^{E56A} (ELL to ALL)] did not suppress the biofilm phenotype in the single mutants (Fig. 1B). The EAL motif is essential for PDE activity (Tamayo *et al.*, 2005; Ferreira *et al.*, 2008). Alteration of the EAL motif in EcpB or EcpC resulted in failure to suppress the hyper-biofilm phenotype in the corresponding mutants. This suggests that PDE activity is important for regulation of biofilm formation in *D. dadantii*. In addition, cross-complementation of Δ*ecpC* and Δ*ecpB* by expression of *ecpB* and *ecpC in trans* restored biofilm formation to wild-type levels (Fig. 1B). These results show that EcpB and EcpC negatively regulate biofilm formation and that the EAL motif is required for this regulation.

Mutation of ecpB *or* ecpC *reduces motility*

In several bacteria c-di-GMP suppresses swimming, swarming and twitching motility (Jenal and Malone, 2006; Römling and Amikam, 2006; Ryan *et al.*, 2006b). For this reason, the GGDEF and EAL mutants were tested for swimming and swarming motility. All mutants except Δ*ecpA*, Δ*ecpB* and Δ*ecpC* displayed swimming and swarming motility similar to wild type on MG plates (Fig. 1C and D). Mutations in *ecpB* or *ecpC* resulted in reduction of swimming motility and a substantial decrease in swarming motility compared with wild type (Fig. 1C and D). No significant difference was observed in swimming motility in an *ecpB* and *ecpC* double mutant, whereas the double mutation led to a greater deficiency in swarming motility compared with the single mutants (Fig. 1C and D). In addition, although swimming motility of Δ*ecpA* was slightly reduced, swarming motility was indistinguishable from the wild type. These results are consistent with the

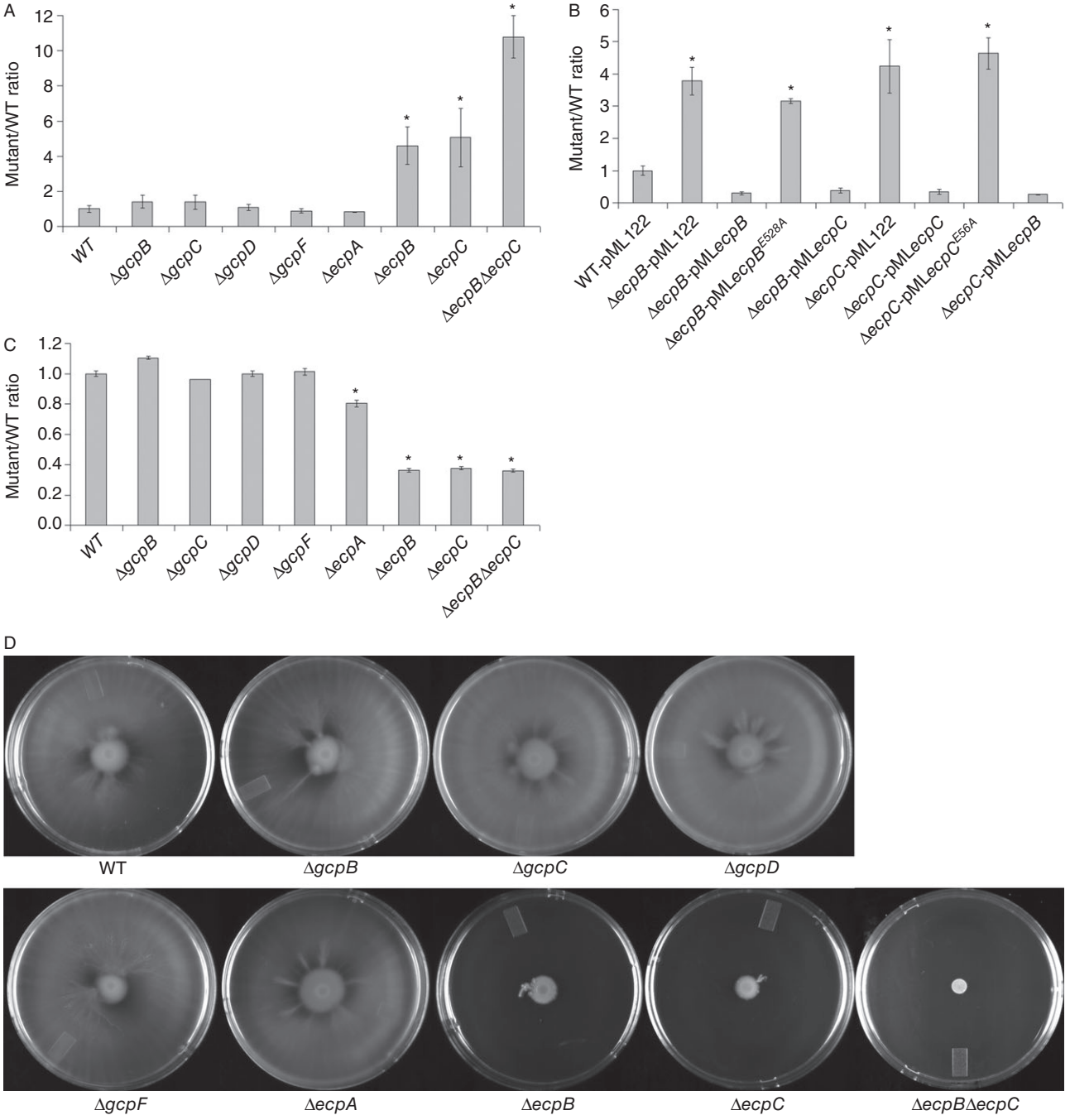

Fig. 1. Phenotypic analysis of *D. dadantii* mutants of GGDEF and EAL domain proteins. Biofilm production was quantified using crystal violet staining.

A. Biofilm formation phenotype of wild-type and GGDEF/EAL mutant strains in MM.

B. Biofilm formation of wild type, Δ*ecpB*, Δ*ecpC* and complemented strains.

C. Swimming motility was measured on MG plates containing 0.2% agar. The biofilm formation and swimming motility were expressed as mutant/wild type ratio, with wild type being 1. The ratio for swimming refers to the relative diameter of the radial growth.

D. Swarming motility was tested on MG plates containing 0.4% agar.

These experiments were repeated at least three times. Three technical replicates were used in each experiment. The values are representatives of one experiment. Asterisks indicate $P < 0.05$ (Student's *t*-test).

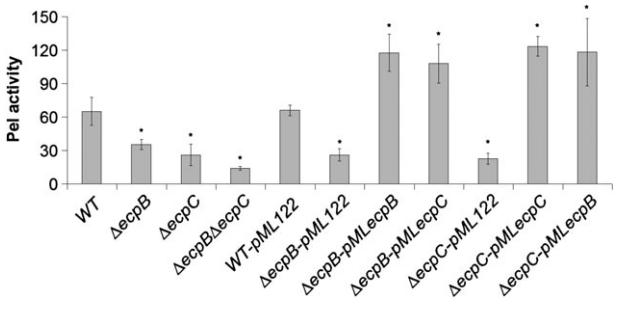

Fig. 2. EcpB and EcpC effect on extracellular Pel synthesis. Pel activity in wild type, Δ*ecpB*, Δ*ecpC*, Δ*ecpB*Δ*ecpC* and complemented strains. Pel activity was measured after 12 h induction in MM containing 1% polygalacturonic acid. This experiment was repeated three times. Three technical replicates were used in each experiment. The values are representatives of one experiment. Asterisks indicate $P < 0.05$ (Student's *t*-test).

role of c-di-GMP in suppressing motility in other organisms (Ryan *et al*., 2006b; Tamayo *et al*., 2007; Wolfe and Visick, 2008) and suggests that c-di-GMP negatively regulates motility in *D. dadantii* 3937. Since deletion of *ecpB* or *ecpC* caused an increase in biofilm and a reduction in motility, the following characterization focused on these two mutants.

Mutation of ecpB *or* ecpC *reduces pectate lyase activity*

Extracellular pectate lyase (Pel) activity is an important virulence factor of *D. dadantii* (Kazemi-Pour *et al*., 2004). Pels are secreted through the type II secretion system. To examine the effect of EcpB and EcpC on extracellular Pel production, supernatants from wild type, Δ*ecpB*, Δ*ecpC* and Δ*ecpB*Δ*ecpC* cultures were assayed for Pel activity. Compared with the wild type, the single mutations resulted in a considerable loss of Pel enzyme activity which was reduced even further in the double mutant (Fig. 2). Complementation and cross-complementation of Δ*ecpB* or Δ*ecpC* with *ecpB* or *ecpC in trans* restored Pel production to wild-type levels (Fig. 2). These results show that EcpB and EcpC affect extracellular Pel production and suggest a link between c-di-GMP levels and Pel production in *D. dadantii* 3937.

EcpB and EcpC positively regulate T3SS expression

C-di-GMP has been implicated in the regulation of flagellum-related motility (D'Argenio and Miller, 2004; Ryan *et al*., 2006b). Considering the co-evolutionary relationship between T3SS and the flagella system, we speculated that c-di-GMP may affect T3SS expression. To investigate this possibility, the expression of three representative *hrp* genes, *hrpA*, *hrpN* and *dspE*, was examined using promoter–*gfp* transcriptional fusions (plasmids pAT–*hrpA*, pAT–*hrpN* and pAT–*dspE*). Promoter activity was measured by detecting the intensity of green fluorescence protein (GFP). A dramatic reduction in *hrpA*, *hrpN* and *dspE* expression was observed in Δ*ecpB* and Δ*ecpC* (Fig. 3A). Moreover, a double mutation in *ecpB* and *ecpC* led to an even greater loss of *hrp* gene expression (Fig. 3A). Complemention of Δ*ecpB* and Δ*ecpC* with a wild-type copy of *ecpB* or *ecpC* restored *hrpA* expression close to wild-type level (Fig. 3B). The amount of *hrpA* transcript was also measured in wild type, Δ*ecpB* and Δ*ecpC* by Northern blot (Fig. 3C) and real-time RT-PCR (Fig. 4B). In both Δ*ecpB* and Δ*ecpC* abundance of *hrpA* transcript was considerably less compared with wild type (Figs 3C and 4B). These results show that EcpB and EcpC are required for T3SS expression at the transcriptional level.

Complementation and cross-complementation by expression of the intact EcpB and EcpC restored *hrpA* expression (Fig. 3B). However, introduction of pML*ecpB*^{E528A} or pML*ecpC*^{E56A} into Δ*ecpB* and Δ*ecpC*, respectively, did not recover *hrpA* expression (Fig. 3B). These results demonstrate that the EAL motif in EcpB and EcpC is essential for the regulation of T3SS gene expression. Furthermore, it strongly suggests that elevated levels of c-di-GMP negatively regulate T3SS expression.

EcpB and EcpC affect transcription of hrpL

Previous studies indicate that HrpL regulates T3SS genes (Yap *et al*., 2005; Peng *et al*., 2006). HrpL is an alternative sigma factor that activates the expression of many *hrp* genes (including *hrpA*, *hrpN* and *dspE*) by binding to their promoter regions (Wei and Beer, 1995; Frederick *et al*., 2001; Fouts *et al*., 2002). EcpB and EcpC may affect *hrp* gene expression by regulating *hrpL* transcription. To test this, a *hrpL* promoter–*gfp* reporter fusion (pAT–*hrpL*, transcriptional fusion) was introduced into the wild-type, Δ*ecpB* and Δ*ecpC* backgrounds, and *hrpL* promoter activity was assessed by measuring GFP intensity. At least a twofold decrease in *hrpL* promoter activity was detected in Δ*ecpB* and Δ*ecpC* compared with wild type (Fig. 4A). To confirm that *hrpL* expression is positively regulated by EcpB and EcpC, the *hrpL* transcript level in wild type and the mutants was assessed by real-time RT-PCR. In comparison with wild type, at least a sixfold decrease in *hrpL* transcript was detected in Δ*ecpB* and Δ*ecpC* (Fig. 4B). These results suggest that EcpB and EcpC are required for maximum expression of *hrpL*, which in turn activates expression of *hrp* genes in the HrpL regulon.

EcpB and EcpC regulation of hrp *gene expression may involve RpoN*

EcpB and EcpC may affect the expression of *hrpL* through the known *hrpL* regulators RpoN, HrpS, RsmA or *rsmB*

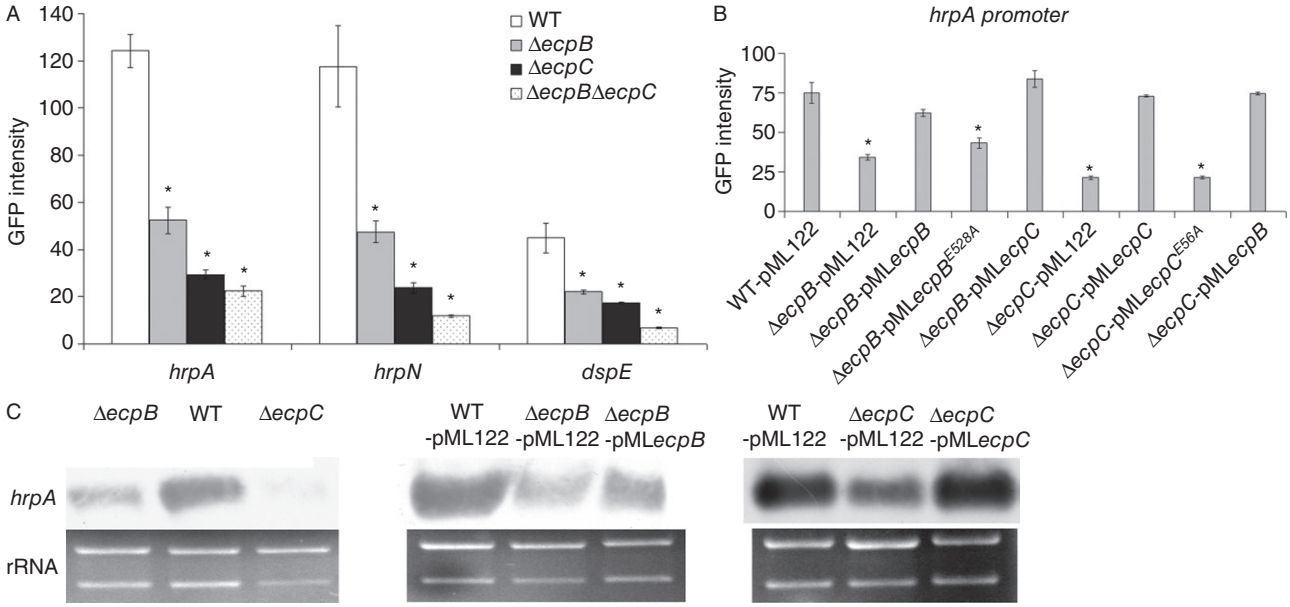

Fig. 3. Promoter activity and Northern blot assays of T3SS expression in *D. dadantii*. Cells were grown in MM for 12 h.
A. Promoter activity of *hrpA*, *hrpN* and *dspE* in wild type, Δ*ecpB*, Δ*ecpC* and Δ*ecpB*Δ*ecpC* strains. Values (Mean Fluorescence Intensity) of GFP were measured by flow cytometry.
B. Promoter activity of *hrpA* in wild type, Δ*ecpB*, Δ*ecpC* and complemented strains.
C. Northern blot analysis using *hrpA* as a probe, showing *hrpA* expression in wild type, Δ*ecpB*, Δ*ecpC* and complemented strains.
Promoter activity assays were repeated three times. Three technical replicates were used in each experiment. The values are representatives of one experiment. Asterisks indicate $P < 0.05$ (Student's *t*-test). Northern blot assay was repeated three times.

(Fig. 7). The promoter activity of *hrpS*, *rpoN* and *rsmA* was assessed in wild type, Δ*ecpB* and Δ*ecpC* using promoter–*gfp* reporter fusions. Similar promoter activities were detected in the wild type and the *ecpB* and *ecpC* mutants (Fig. 4A). In addition, the mRNA levels of *hrpS*, *rpoN*, *rsmA* and *rsmB* were measured by real-time RT-PCR. A fivefold and a sixfold reduction of the *rpoN* mRNA was observed in Δ*ecpB* and Δ*ecpC*, respectively, compared with wild type (Fig. 4B). The amount of *hrpS*, *rsmA* and *rsmB* transcripts in Δ*ecpB* and Δ*ecpC* was similar to that in wild type (Fig. 4B). These results suggest that EcpB and EcpC regulate *hrpL* expression by altering the *rpoN* mRNA level. The mRNA stability assay showed no significant difference in the rate of *rpoN* mRNA degra-

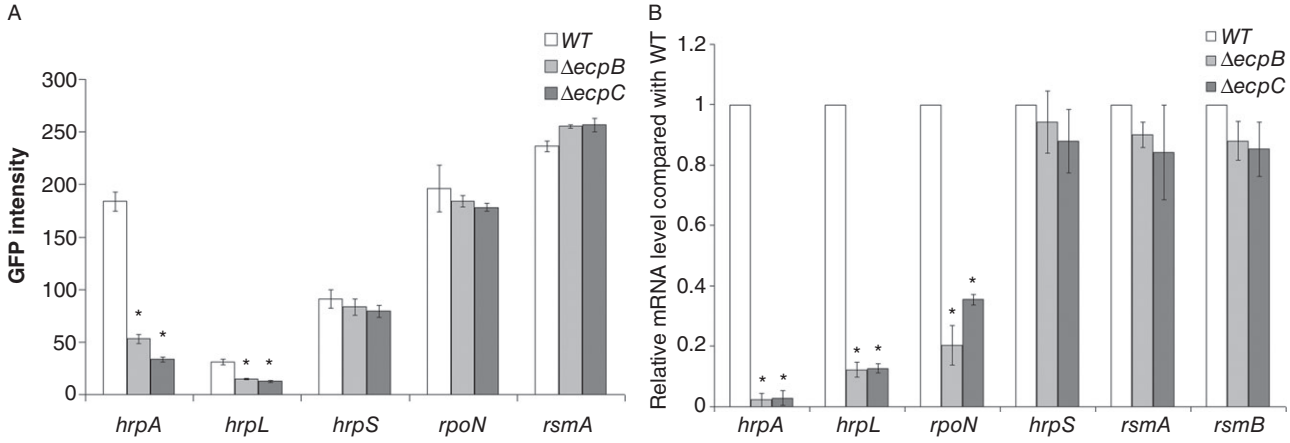

Fig. 4. Effect of EcpB and EcpC on expression of *hrp* gene regulators, HrpL, HrpS, RpoN and RsmA.
A. Promoter activity of *hrpA*, *hrpL*, *rpoN*, *hrpS* and *rsmA* in wild-type *D. dadantii* 3937, Δ*ecpB* and Δ*ecpC* strains.
B. Real-time RT-PCR quantification of *hrpA*, *hrpL*, *rpoN*, *hrpS*, *rsmA* and *rsmB* gene expression in wild type, Δ*ecpB* and Δ*ecpC*. *rplU* was used as an endogenous control for data analysis (Mah *et al*., 2003).
Quantitative RT-PCR assays were repeated three times. Three technical replicates were used in each experiment. The values are representatives of one experiment. Asterisks indicate $P < 0.05$ (Student's *t*-test).

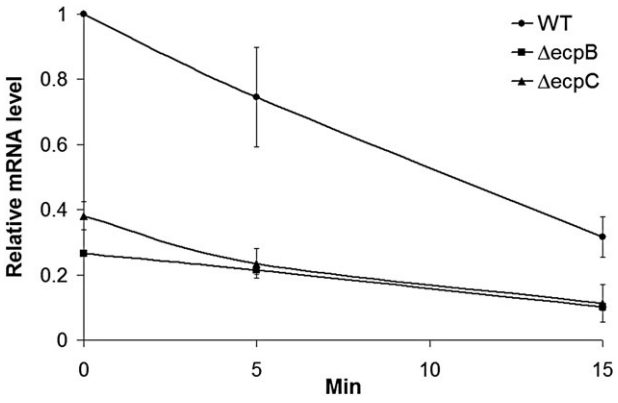

Fig. 5. *rpoN* mRNA stability assay. RNA synthesis was stopped by adding rifampicin to a final concentration of 500 μg ml^{-1}. Samples were removed from cultures at 0, 5 and 15 min to measure the *rpoN* transcript level by qRT-PCR. The relative mRNA levels of *rpoN* were plotted. The mean *rpoN* transcript degradation rate (% min^{-1}) was calculated during 5–15 min after adding rifampicin in wild type (5.75 ± 0.08), Δ*ecpB* (5.28 ± 0.30) and Δ*ecpC* (5.42 ± 1.44) strains. No significant difference in the *rpoN* transcript degradation rate was observed among wild type, Δ*ecpB* and Δ*ecpC* (Student's *t*-test).

dation among Δ*ecpB*, Δ*ecpC* and wild type (Fig. 5A and B), suggesting that the decreased levels of *rpoN* mRNA in Δ*ecpB* and Δ*ecpC* were not due to changes in the rate of *rpoN* mRNA degradation in these two mutants.

The rsm *regulatory pathway has no effect on* ecpB *and* ecpC *expression*

The effect of RsmA on *ecpB* and *ecpC* expression was also examined. RsmA was depleted by overexpressing *rsmB* (pML*rsmB*) in the wild-type strain. In principle, if *rsmB* RNA is in excess, formation of RsmA–*rsmB* complexes will increase and less unbound RsmA will be available to suppress *hrpL* and HrpL-regulated genes. To validate the effect of the *rsmB*-overexpression construct, the *hrpA* mRNA level was measured as a positive control in the *rsmB*-overexpression strain. Based on the increase in *hrpA* expression, we found that introduction of pML*rsmB* into the wild-type strain was indeed effective in depletion of RsmA (Fig. S1). The *ecpB* and *ecpC* promoter–*gfp* fusions (pAT–*ecpB*, pAT–*ecpC*) were then introduced into wild type and the *rsmB*-overexpression strain. Assessment of promoter activity showed similar results to real-time RT-PCR demonstrating that overexpression of *rsmB* (depletion of RsmA) did not affect the transcription of *ecpB* and *ecpC* (Fig. S2A and B). Taken together, these results suggest that the *rsm* pathway does not regulate *ecpB* and *ecpC* transcription and mRNA steady-state level.

Biochemical studies showed that EcpC is an active PDE

EcpC was purified and its PDE activity was assessed by two *in vitro* assays, a colorimetric assay and reverse-phase high-performance liquid chromatography (HPLC) assay. The colorimetric assay measures the hydrolysis of colourless bis(*p*-nitrophenyl) phosphate into yellow *p*-nitrophenol (Bobrov *et al*., 2005; Ryan *et al*., 2006a) which can be detected spectrophotometrically at 410 nm. Incubation of EcpC with bis(*p*-nitrophenyl) phosphate for 1.5 h resulted in production of a significant amount of *p*-nitrophenol (A_{410} = 0.76 ± 0.02), while the negative control using the eluate of cells containing pET21b (vector control) from His spin column purification did not convert bis(*p*-nitrophenyl) phosphate into *p*-nitrophenol (A_{410} = 0.04 ± 0.01), suggesting the PDE activity of EcpC.

The enzymatic ability of EcpC was further evaluated by reverse-phase HPLC. EcpC showed phosphodiesterase activity against c-di-GMP (Fig. 6). The major product of the c-di-GMP hydrolysis had a retention time that was distinct from c-di-GMP and GMP (Fig. 6C). Electrospray ionization-mass spectrometry (ESI-MS) of the fraction showed a $[M+H]^+$ *m/z* at 708, which corresponds to the molecular mass of linear pGpG (Fig. 6E), and suggests that c-di-GMP was hydrolysed into pGpG. In the EcpC PDE reaction, a small amount of GMP was also detected, with a $[M+H]^+$ *m/z* at 363 (Fig. 6C and F). This may be due to the minor activity of pGpG or the secondary activity of EcpC. The GMP level in the reaction remained low even after 24 h of incubation (data not shown), indicating the conversion of pGpG to GMP was slow and negligible, and probably not relevant to EcpC activity. Both *in vitro* biochemical assays indicate that EcpC is a c-di-GMP PDE.

Mutation of EcpB and EcpC reduces virulence in vivo

Both pectinolytic enzymes and the T3SS play major roles in the pathogenicity of *D. dadantii* (Beaulieu and Van Gijsegem, 1990; Yang *et al*., 2002). To assess the effect of elevated cellular levels of c-di-GMP on pathogenesis, virulence assays were performed with Δ*ecpB* and Δ*ecpC* in the leaves of African violet (*Saintpaulia ionantha*) and Chinese cabbage (*Brassica campestris*). Compared with wild type, both Δ*ecpB* and Δ*ecpC* showed a significant reduction in plant tissue maceration in both hosts (Table 2) implicating a role for c-di-GMP in virulence.

Discussion

The *D. dadantii* 3937 genome encodes 18 GGDEF/EAL domain proteins. A subset of these proteins was investigated by mutagenesis. Deletion of *ecpB* (GGDEF-EAL domains) and *ecpC* (EAL domain), two putative PDE-encoding genes, resulted in increased biofilm formation and reduced motility, phenotypes that have been associated with increased c-di-GMP levels. Deletion of *ecpA*, another gene encoding an EAL domain protein, resulted in a slight reduction in swimming motility but did not have a significant effect on swarming motility or biofilm forma-

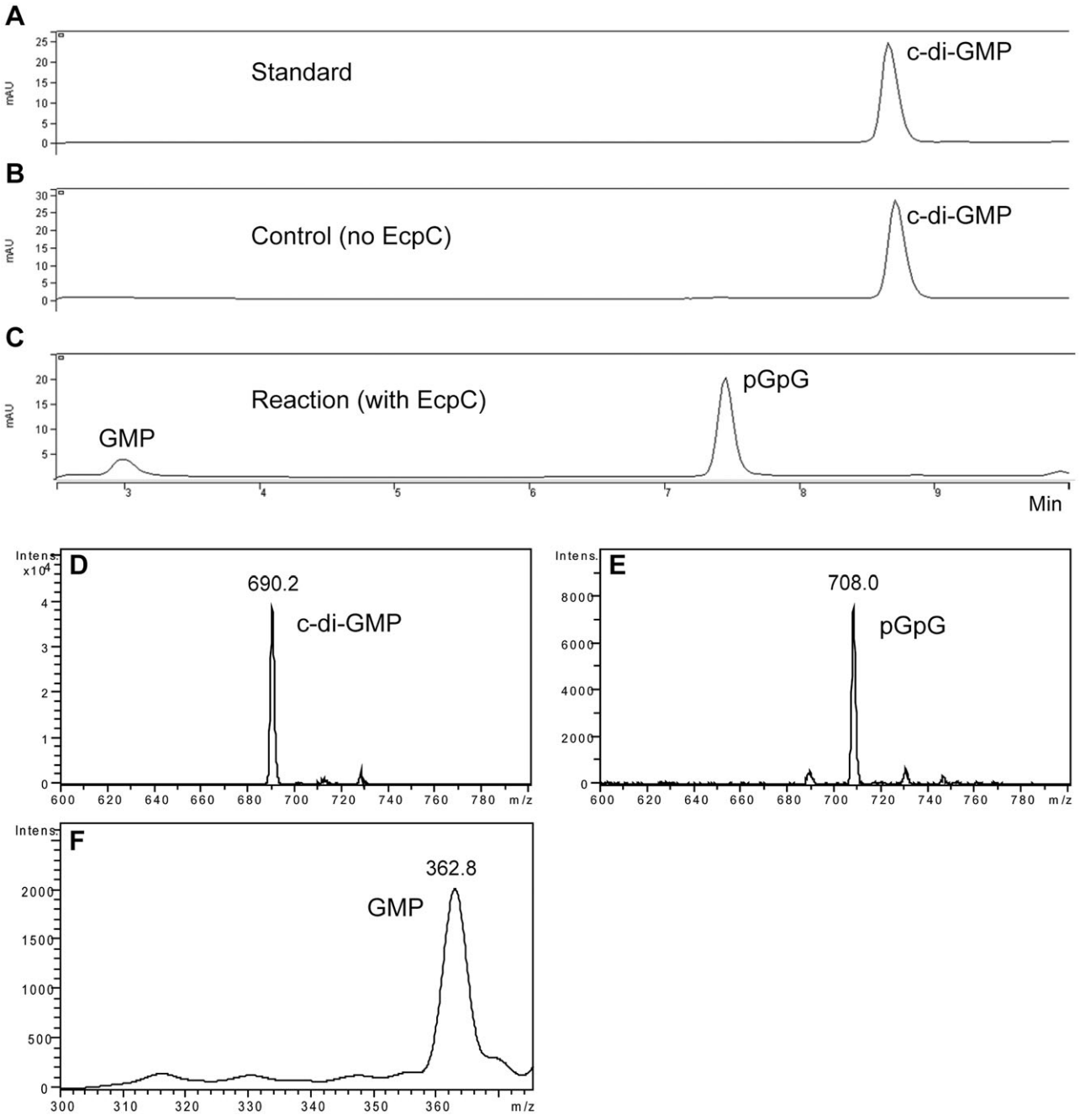

Fig. 6. EcpC PDE activity assays.
A–C. HPLC analysis of products in c-di-GMP PDE reaction. Purified EcpC was tested for PDE activity using c-di-GMP as substrate. (A) C-di-GMP standard. (B) Reaction control without EcpC. C-di-GMP was stable and no degradation was detected. (C) Purified EcpC incubated with c-di-GMP for 1 h. C-di-GMP was hydrolysed by EcpC. The most abundant product is linear pGpG, although a small amount of GMP was also detected.
D–F. Mass spectrometry, operated at positive ion mode, was used to confirm the identity of HPLC fractions. (D) C-di-GMP peak was detected by ESI-MS at *m/z* of 690. (E) pGpG peak was detected at *m/z* of 708. (F) GMP peak was detected at *m/z* of 363. HPLC and ESI-MS analyses were repeated three times.

tion (Fig. 1C). It is possible that EcpA is specifically involved in mechanisms regulating swimming motility. Alternatively, EcpA may regulate biofilm and swarming motility under conditions other than those tested in this study. Interestingly, deletion of genes encoding four GGDEF domain proteins had no significant effect on biofilm formation or motility. The four GGDEF domain proteins may not be involved in these processes or may

Table 2. Virulence assay of African violet and Chinese cabbage.

	African violet[a]	Chinese cabbage[b]
Wild type	2.5	7/8
Δ*ecpB*	0.8*	1/8*
Wild type	2.6	7/8
Δ*ecpC*	1.05*	2/8*

a. African violet leaves were evaluated for symptom development using the following scale: 0 = no symptoms, 1 = 1–10 mm lesion, 2 = 11–20 mm lesion, 3 = 21–30 mm lesion, 4 = lesion size larger than 30 mm. The number indicates the mean disease rating.
b. The number represents the number of Chinese cabbage leaves that developed symptoms out of the total number of plant leaves tested.
Asterisks indicate the Δ*ecpB* and Δ*ecpC* mutants were significantly different from the wild type ($P < 0.001$). This experiment was repeated two times.

be active in specific environmental niches not tested in this work. This may also be a result of redundancy of these proteins in *D. dadantii* 3937, given that 12 putative GGDEF proteins were identified in the genome. In other bacteria, such as *S. enterica*, the loss of one diguanylate cyclase can be compensated by overexpressing other diguanylate cyclases (García *et al*., 2004).

Deletion of *ecpB* and *ecpC* had similar effects on biofilm, motility, extracellular enzyme production and T3SS. Complementation and cross-complementation restored Δ*ecpC* and Δ*ecpB* phenotypes to wild-type levels. In addition, the EAL motif in EcpB and EcpC plays a crucial role in regulating biofilm formation and T3SS gene expression (Figs 1B and 3B). These results strongly suggest that both EcpB and EcpC possess enzymatic activity to degrade c-di-GMP, as deletion of *ecpB*, *ecpC* or both resulted in phenotypes associated with elevated c-di-GMP levels. Despite similar functions, the *ecpB* and *ecpC* genes are not, or at least not completely redundant. This outcome was supported by the double deletion of *ecpB* and *ecpC*, which resulted in an even more dramatic effect on biofilm formation (Fig. 1A), swarming motility (Fig. 1D), Pel activity (Fig. 2) and T3SS expression (Fig. 3A), compared with either of the single mutants Δ*ecpB* or Δ*ecpC*. Interestingly, there was no significant difference in swimming motility among the single-deletion mutants and the double-deletion mutant (Fig. 1C). It is possible that c-di-GMP levels regulate swimming and swarming through distinctive pathways. In fact, ScrG (a phosphodiesterase containing both a GGDEF and an EAL domain) of *Vibrio parahaemolyticus* regulates swarming but not swimming motility by modulation of c-di-GMP levels (Kim and McCarter, 2007). It is prudent to mention that the cross-complementation between *ecpB* and *ecpC* may be due to the dosage effect as a result of the copy number of the plasmid pML122 and/or the constitutive *nptII* promoter used for complementation.

EcpC is predicted to be a cytoplasmic protein that contains an EAL domain. The EAL signature motif (QP-X_{16}-ELLTA-X_{52}-N-X_{27}-ELVE-X_{21}-DDFGCGVAN-X_{16}-KIAREL-X_{30}-EGVE-X_{16}-QG, underlined sequence represents the EAL motif) is conserved in EcpC. In addition, EcpC shows homology (53% identity, 69% similarity) to *E. coli* YhjH. YhjH is a PDE with a class 2 EAL signature motif and was shown to degrade c-di-GMP (Pesavento *et al*., 2008; Römling, 2009). EcpB is predicted to have one transmembrane domain (TMHMM prediction program), a GGDEF and an EAL domain, and additional regulatory/sensory domains (GAF and PAS) (Pfam). The GGDEF and EAL domains of EcpB contain the conserved signature motifs, GGDEF and EAL respectively. In many proteins which contain both GGDEF and EAL domains, only one domain is catalytically active and the other domain either is inactive or has a regulatory function (Paul *et al*., 2004; Schmidt *et al*., 2005; Kim and McCarter, 2007). *In vitro* biochemical assays indicate that EcpC is an active PDE because it hydrolyses the second messenger c-di-GMP into linear pGpG (Fig. 6). Although there is no direct biochemical evidence, the genetic analysis including the point mutation approach strongly suggests that EcpB acts as a PDE.

The expression of type III secretion system gene in *D. dadantii* 3937 is tightly controlled by a sophisticated regulatory cascade (Fig. 7). In this study, we have demonstrated that in addition to its known regulation of cellular behaviours, the second messenger c-di-GMP negatively regulates transcription of three representative T3SS genes, *hrpA*, *hrpN* and *dspE* encoding the T3SS pilus, a T3SS harpin and a T3SS effector respectively (Fig. 4). To the best of our knowledge, these results represent the first report which investigates the role of c-di-GMP in T3SS regulation in detail. We also demonstrated that c-di-GMP negatively regulates *hrpL* expression. This regulation is likely mediated by RpoN, as the *rpoN* mRNA level was considerably lower in Δ*ecpB* and Δ*ecpC*. However, the promoter–*gfp* fusion assay suggests there is no difference in the *rpoN* promoter activity among wild type, Δ*ecpB* and Δ*ecpC*. It is unlikely that the reduction in *rpoN* mRNA levels in the mutants is due to the change in the rate of mRNA degradation, as the *rpoN* mRNA degradation rate was similar among wild type, Δ*ecpB* and Δ*ecpC* mutants (Fig. 5). The manner in which c-di-GMP affects the *rpoN* mRNA level is unclear and further studies are needed to elucidate the c-di-GMP regulatory mechanism of T3SS in *D. dadantii* 3937.

The second messenger also has a negative effect on Pel production, as both Δ*ecpB* and Δ*ecpC* displayed reduced Pel activity compared with wild type (Fig. 2). C-di-GMP regulation on extracellular enzyme production was also reported in *X. campestris* and *V. cholera* (Tischler *et al*., 2002; Tischler and Camilli, 2004; Ryan *et al*.,

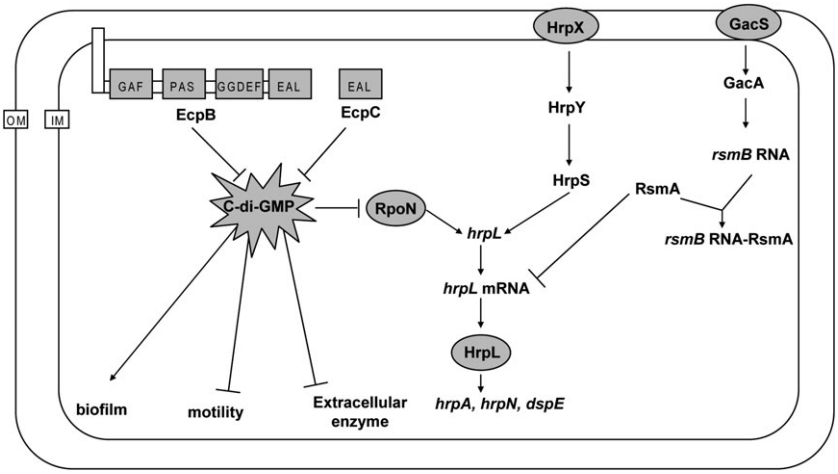

Fig. 7. Model of c-di-GMP regulatory cascade in *Dickeya dadantii* 3937. EcpB and EcpC modulate the c-di-GMP level by their c-di-GMP PDE activity. Expression of *ecpB* and *ecpC* is stimulated in *hrp*-inducing MM. Expression of *ecpB* and *ecpC* is under different regulation to adapt to diverse environment. EcpB contains GAF, PAS and TM domains which may be used to respond to environmental and/or intracellular signals. Similar to its well-established role, c-di-GMP stimulates biofilm formation, and suppresses motility in *D. dadantii* 3937. C-di-GMP also suppresses synthesis of virulence factors, including extracellular enzymes and T3SS. The molecular mechanism by which c-di-GMP regulates virulence factors is not clear. However, RpoN and HrpL play a major role in the pathway of c-di-GMP regulation on T3SS.

2006a). In *X. campestris*, mutation of RpfG, a PDE containing an HD-GYP domain, impaired the synthesis of various extracellular enzymes (Ryan *et al*., 2006a). In *V. cholera*, the PDE protein VieA positively regulates the expression of *ctxAB*, which encodes a cholera toxin (Tischler *et al*., 2002; Tischler and Camilli, 2004). This suggests that the effect of c-di-GMP on extracellular enzyme synthesis is not unique to *D. dadantii* but may be common in diverse bacteria. Pectinolytic enzymes and the T3SS play major roles in the pathogenicity of *D. dadantii* 3937 (Beaulieu and Van Gijsegem, 1990). Both Δ*ecpB* and Δ*ecpC* showed reduced maceration ability in plants (Table 2). This may be due to a decrease in Pel production and T3SS expression in these two mutants.

A CrsA/RsmA family protein in *E. coli* has been shown to control c-di-GMP levels by regulating expression of GGDEF, GGDEF/EAL and EAL proteins (Jonas *et al*., 2008). However, no significant difference was observed in the promoter activity or mRNA level of *ecpB* and *ecpC* regardless of the presence or absence of *rsmB* overexpression in the *D. dadantii* 3937 wild-type strain (Fig. S2A and B). This suggests that the *rsm* regulatory pathway in *D. dadantii* 3937 does not regulate *ecpB* and *ecpC* mRNA steady-state levels.

In this study, the c-di-GMP regulatory network was explored and a model of the c-di-GMP regulatory cascade is proposed (Fig. 7). The c-di-GMP levels in *D. dadantii* 3937 are modulated by two PDE proteins, EcpB and EcpC. Transcription of both *ecpB* and *ecpC* is stimulated by growth in MM, which mimics the nutrient-limited conditions within the plant host (data not shown). This suggests that EcpB and EcpC are active upon entry into the plant host. The activity of the membrane-bound EcpB may be modulated by environmental and/or intracellular signals which in turn are transmitted to the GGDEF-EAL domain, resulting in changes of c-di-GMP levels. This is supported by the presence of GAF and PAS domains (regulatory/sensory modules) in EcpB. The GAF domain functions as a small ligand-binding sensory domain, and the PAS domain is a sensory domain capable of sensing many environmental signals including redox changes (Taylor and Zhulin, 1999; Ho *et al*., 2000).

GGDEF, EAL and HD-GYP domain proteins are widely distributed in many bacterial pathogens. It is probable that c-di-GMP regulates T3SS in other bacterial species. For example, in DNA microarray studies of *P. aeruginosa*, the genes required for type III secretion are regulated by the SadARS system, of which the SadR protein contains an EAL domain (Kuchma *et al*., 2005). As a final point, the suppressive effect of c-di-GMP on the synthesis of virulence factors in *D. dadantii* makes this second messenger a potential target for drug development.

Experimental procedures

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. *D. dadantii* 3937 wild type and its mutant strains were grown in Luria–Bertani (LB) medium or T3SS-inducing minimal medium (MM) at 28°C (Yang *et al*., 2007). When required, antibiotics were added at the following concentrations: kanamycin 50 μg ml^{-1}; ampicillin 100 μg ml^{-1}; and gentamicin 20 μg ml^{-1}. The *D. dadantii* 3937 genome sequence can be retrieved from ASAP (https://asap.ahabs.wisc.edu/asap/home.php).

Mutant construction and complementation

Mutations of genes encoding GGDEF and/or EAL domain proteins were generated by allelic exchange (Metcalf *et al*., 1996). The flanking regions were amplified by PCR with specific primers (Table S2). The chloramphenicol and kanamycin cassettes were amplified from pKD3 and pKD4 respectively (Datsenko and Wanner, 2000). Three-way cross-over PCR was performed using the flanking regions and either the

kanamycin or chloramphenicol cassette as templates. The PCR product was then digested with XhoI and NotI (New England BioLabs, MA), and cloned into pWM91 digested with the same enzymes. The resulting plasmid was transformed into *E. coli* S17-1 λ-*pir*, and then mobilized into *D. dadantii* 3937 by conjugation. Recombinants resulting from double-cross-over events were selected by *sacB* and sucrose positive selection. Mutations were confirmed by PCR and sequencing.

To construct plasmids for complementation and overexpression, the coding regions of *ecpB* and *ecpC* were cloned into a low-copy-number plasmid, pML122, at XbaI and BamHI sites. In the resulting plasmids, pML*ecpB* and pML*ecpC*, the expression of *ecpB* and *ecpC* was controlled by the *nptII* promoter.

Point mutation in EAL motif-coding sequence

Single nucleotide substitutions in the EAL motif-coding sequence were performed using the QuikChange XL Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA). Primer sets C2-E-f3/C2-E-r3 and P3-E-f/P3-E-r were used to generate $ecpB^{E528A}$ and $ecpC^{E56A}$ respectively (Table S2). These mutations altered the EAL motif to AAL (EcpB) or ALL (EcpC). Substitutions were confirmed by DNA sequencing.

*Promoter–*gfp *fusion plasmid construction and promoter activity assay*

The GFP reporter plasmid pPROBE-AT was used to construct transcriptional fusions with the *ecpB* and *ecpC* promoters. The promoter regions of *ecpB* and *ecpC* were amplified by PCR using forward and reverse primers incorporating BamHI and EcoRI sites respectively (Table S2). The DNA fragments were digested with BamHI and EcoRI, and cloned into similarly digested pPROBE-AT. The resulting plasmids, pAT–*ecpB* and pAT–*ecpC*, were mobilized into *D. dadantii* by conjugation using *E. coli* S17-1 as the donor strain. The promoter activity was evaluated by measuring GFP intensity using flow cytometry (Becton Dickinson, San Jose, CA) as previously described (Peng *et al*., 2006).

Bacterial motility assay

Swimming and swarming were examined on MG (mannitol 10 g l^{-1}, glutamic acid 2 g l^{-1}, KH_2PO_4 0.5 g l^{-1}, NaCl 0.2 g l^{-1}, $MgSO_4$ 0.2 g l^{-1}, pH 7.2) plates containing 0.2% or 0.4% agar. The centre of the plates was inoculated with 10 µl of overnight bacterial cultures. All plates were incubated at 28°C, and the diameter of the radial growth was measured (Antúnez-Lamas *et al*., 2009).

Quantification of biofilm formation

Quantification of biofilm formation was adapted from the method described by O'Toole and Kolter (1998). Briefly, overnight bacterial cultures were inoculated 1:100 in MM in 1.5 ml polypropylene tubes and incubated at 28°C for 24 h. Cells were stained with crystal violet (CV) for 15 min. The planktonic cells were removed by several rinses with H_2O. The CV-stained bound cells were solubilized in 90% ethanol, and the absorbance was measured at A_{590} to quantify biofilm formation.

Pel activity assay

Extracellular pel activity of cells grown in MM supplemented with 1% polygalacturonic acid was measured by spectrometry (Matsumoto *et al*., 2003). Bacterial cultures were centrifuged at 15 800 *g* for 1 min and 10 µl of the supernatant was added to 990 µl of Pel reaction buffer (Matsumoto *et al*., 2003). Pel activity was monitored at A_{230} over a period of 3 min. Pel activity was calculated as previously described (Matsumoto *et al*., 2003). The assay was performed at least three times in triplicate.

RNA isolation and Northern blot analysis

Bacterial cells grown in MM for 12 h were harvested and total RNA was isolated using TRI reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions, followed by DNase treatment with a Turbo DNA-free DNase kit (Ambion, Austin, TX). Northern blots were performed using a NorthernMax kit (Ambion, Austin, TX) and detected by BrightStar BioDetect (Ambion, Austin, TX).

cDNA synthesis and real-time RT-PCR analysis

One microgram of DNase-treated RNA was used to synthesize cDNA with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). The RealMasterMix (Eppendorf, Westbury, NY) was used in real-time RT-PCR reactions to quantify the *hrpL* and *rpoN* transcript levels. *rplU* was chosen as the internal reference for data analysis (Mah *et al*., 2003). Assays were performed in triplicate in 20 µl reactions using the DNA Engine Opticon 2 system (Bio-Rad Laboratories, Hercules, CA). The amount of cDNA was calculated and analysed as previously described (Venkatesh *et al*., 2006).

rpoN *mRNA stability assay*

To suppress RNA synthesis and evaluate RNA stability, rifampicin (500 µg ml^{-1}) was added to cells grown in MM for 12 h. Incubation was continued and culture aliquots were withdrawn at the times indicated. RNA*later* Solution (Ambion, Austin, TX) was added to the culture aliquots according to manufacturer's instructions, followed by total RNA isolation using TRI reagent. Total RNA was treated with DNase and reverse transcribed into cDNA for real-time qRT-PCR analysis as described in previous sections.

EcpC expression and purification

A DNA fragment encoding the EcpC protein was amplified from *D. dadantii* 3937 chromosomal DNA by PCR using primers that incorporated NdeI (forward) and XhoI (reverse) sites (Table S2). The fragment was digested with NdeI and XhoI, and cloned into the pET21b expression vector (Merck

KGaA, Darmstadt, Germany) digested with the same enzymes. The resulting plasmid, pET*ecpC*, encoded EcpC with a C-terminal His_6-tag. The construct was confirmed by sequencing, and transformed into the expression host *E. coli* BL21 (DE3). BL21 cells were grown in LB medium at 37°C until reaching an OD_{600} of 0.5–0.8, and protein expression was induced upon addition of 1 mM IPTG. Proteins were extracted using bacterial protein extraction reagent (B-PER; Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's protocol, and purified from the soluble fraction using His SpinTrap columns (GE Healthcare, Waukesha, WI). The His_6-tagged proteins were washed and eluted as directed by the manufacturer.

PDE colorimetric assay

EcpC was assayed for PDE activity by incubation with bis(*p*-nitrophenyl) phosphate substrate (Bobrov *et al*., 2005). Standard assays were performed by incubating 20 μg of EcpC and 5 mM bis(*p*-nitrophenyl) phosphate at 37°C for 1.5 h in assay buffer (50 mM Tris-HCl, 1 mM $MnCl_2$, pH 8.5). The production of *p*-nitrophenol was quantified at A_{410} (Bobrov *et al*., 2005). Determination of enzyme activity was performed in duplicate from at least two independent assays. Controls without enzyme were used to measure non-enzymatic hydrolysis of the substrate.

C-di-GMP PDE enzyme assay

EcpC was tested for PDE activity against c-di-GMP using a modified method of HPLC developed by Ryan *et al*. (2006a). C-di-GMP was purchased from GL Synthesis (Worcester, MA) and GMP was purchased from Sigma (St. Louis, MO). The PDE reaction mixture contained 20 μg of protein, 100 μM c-di-GMP, 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 10 mM $MnCl_2$, 0.5 mM EDTA and 50 mM NaCl in a total volume of 200 μl. The mixture was incubated at 37°C for 1 h. The reaction was stopped by placing samples in a boiling water bath for 3 min. After centrifugation at 15 000 *g* for 2 min, the supernatant was filtered through a 0.22 μm filter and analysed by HPLC.

HPLC analysis and ESI-MS mass spectrometry

PDE reaction products were analysed by reverse-phase HPLC. Twenty microlitres of each sample was injected into a Hypersil C18 150 × 4.6 mm column (Thermo Fisher Scientific, Waltham, MA) at a flow rate of 1 ml min^{-1} and a linear gradient from 0–20% methanol in buffer A (20 mM potassium phosphate buffer, pH 5.8, containing 1% methanol) during 20 min. Nucleotides were detected at 254 nm. Nucleotide identity was confirmed using 1100 LC/MSD trap, an ESI-MS (Agilent, Santa Clara, CA), operated in positive mode with a 1 ml min^{-1} flow rate and a linear gradient of 0–20% buffer B (acetonitrile containing 0.01% formic acid) in buffer A (0.1% ammonium formate, pH 3.7) in 20 min. Commercially available c-di-GMP and GMP were used as reference standards.

Virulence assay

The local maceration assay was performed using the leaves of African violet cv. Gauguin (*S. ionantha*) and Chinese cabbage (*B. campestris*). For African violet, 50 μl of wild type and mutant cell suspensions at 10^6 colony-forming units (cfu) ml^{-1} were syringe infiltrated into opposite sides of the same leaf. Five pots of plants were used for each mutant with four leaves inoculated in each pot (total of 20 leaves). For Chinese cabbage, 10 μl of wild type and mutant cell suspensions at 10^7 cfu ml^{-1} were inoculated into the slits made with a sterile pipette tip on opposite sides of the same leaf. Eight leaves were used for each mutant. Inoculated leaves were incubated in a chamber with 100% humidity at 28°C. To evaluate disease symptoms, African violet plants were rated using the following scale: 0 = no symptoms, 1 = 1–10 mm lesion, 2 = 11–20 mm lesion, 3 = 21–30 mm lesion, 4 = lesion size larger than 30 mm. For Chinese cabbage, the number of leaves that developed symptoms was recorded. This experiment was repeated two times.

Statistical analysis

Means and standard deviations of experimental results were calculated using Excel (Microsoft, Redmond, WA) and the statistical analysis was performed using a two-tailed *t*-test.

Acknowledgements

This work is dedicated to Professor Noel T. Keen. We thank Nicole Perna of the University of Wisconsin for providing access to the annotated *D. dadantii* genome sequences (https://asap.ahabs.wisc.edu/asap/ASAP1.htm); Mary Lynne P. Collins and Nicholas C. Butzin for critical discussions and reading of the manuscript; Cheng Wang and Patrick Anderson for technical support on HPLC and ESI-MS. This project was supported by grants from the National Science Foundation (award No. EF-0332163) and the Research Growth Initiative of the University of Wisconsin-Milwaukee.

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