

# The diguanylate cyclase GcpA inhibits the production of pectate lyases via the H-NS protein and RsmB regulatory RNA in *Dickeya dadantii*

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## SUMMARY

*Dickeya dadantii* 3937 secretes pectate lyases (Pels) to degrade plant cell walls. Previously, we have demonstrated that EGcPB and EpcC function as bis-(3',5')-cyclic dimeric guanosine monophosphate (c-di-GMP)-specific phosphodiesterases (PDEs) to positively regulate Pel production. However, the diguanylate cyclase (DGC) responsible for the synthesis of c-di-GMP and the dichotomous regulation of Pel has remained a mystery. Here, we identified GcpA as the dominant DGC to negatively regulate Pel production by the specific repression of *pelD* gene expression. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assays revealed that the expression levels of histone-like, nucleoid-structuring protein encoding gene *hns* and post-transcriptional regulator encoding genes *rsmA* and *rsmB* were significantly affected by GcpA. Deletion of *hns* or *rsmB* in the *gcpA*<sup>D418A</sup> site-directed mutant restored its Pel production and *pelD* expression, demonstrating that H-NS and RsmB contribute to the GcpA-dependent regulation of Pel in *D. dadantii*. In addition, RsmB expression was subject to positive regulation by H-NS. Thus, we propose a novel pathway consisting of GcpA–H-NS–RsmB–RsmA–*pelD* that controls Pel production in *D. dadantii*. Furthermore, we showed that H-NS and RsmB are responsible for the GcpA-dependent regulation of motility and type III secretion system (T3SS) gene expression, respectively. Of the two PDEs involved in the regulation of Pels, only EGcPB regulates *pelD* expression through the same pathway as GcpA.

**Keywords:** c-di-GMP, motility, pectate lyase, soft-rot pathogen, type III secretion system, virulence.

## INTRODUCTION

*Dickeya dadantii* 3937 is an Enterobacterium that causes soft-rot disease in a wide range of economically important crops (Czajkowski *et al.*, 2011; Ma *et al.*, 2007). Pectate lyases (Pels), which degrade the plant cell wall, are one of the major virulence factors that contribute to the pathogenesis of *D. dadantii* (Collmer and Keen, 1986). The production of Pel is controlled by a sophisticated regulatory mechanism that includes modifications of DNA topology, quorum sensing and other regulatory systems associated with bacterial physiological status or environmental stimuli (Charkowski *et al.*, 2012; Hugouvieux-Cotte-Pattat *et al.*, 1996; Reverchon and Nasser, 2013). During the early stages of infection, several transcriptional repressors, such as FIS, H-NS, KdgR, PecS, PecT, Fur and the PhoP/Q two-component regulatory system, negatively regulate the expression of *pel* genes in response to initially encountering the oxidative and acidic environment in plant intercellular spaces (Franza *et al.*, 2002; Héroult *et al.*, 2014; Llama-Palacios *et al.*, 2005; Ouafa *et al.*, 2012; Reverchon *et al.*, 1991). In addition, the post-transcriptional regulator RsmA/RsmB inhibits Pel via an unknown mechanism (Wu *et al.*, 2014; Yang *et al.*, 2008). RsmA facilitates specific mRNA degradation, whereas RsmB is an untranslated regulatory RNA that binds to RsmA and neutralizes its effect on target gene expression (Liu *et al.*, 1997). After adaptation to the intracellular spaces, *D. dadantii* secretes a massive amount of Pel into the plant apoplast following the inactivation of the previously mentioned Pel repressors and the activation of Pel inducers, which include the GacS/A two-component system, MfbR, CRP and the Vfm quorum sensing system (Charkowski *et al.*, 2012; Franza *et al.*, 2002; Hugouvieux-Cotte-Pattat *et al.*, 1996; Nasser *et al.*, 2013; Reverchon and Nasser, 2013; Reverchon *et al.*, 2010; Yang *et al.*, 2008).

Bis-(3',5')-cyclic dimeric guanosine monophosphate (c-di-GMP) is a common bacterial second messenger found in most major bacterial phyla (Römling *et al.*, 2013). It was first discovered as an

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allosteric activator for cellulose synthase in *Gluconacetobacter xylinus* (Ross *et al.*, 1987). It is now established that c-di-GMP is involved in the regulation of many cellular activities, including biofilm formation, motility, the cell cycle, antibiotic production and virulence (Cotter and Stibitz, 2007; Hengge, 2009; Jenal *et al.*, 2017; Tamayo *et al.*, 2007). The synthesis and hydrolysis of c-di-GMP are catalyzed by diguanylate cyclase (DGC) and c-di-GMP-specific phosphodiesterase (PDE) enzymes, respectively. DGC activity is associated with the GGDEF domain, which converts two molecules of guanosine-5'-triphosphate (GTP) to one molecule of c-di-GMP (Paul *et al.*, 2004; Whiteley and Lee, 2015). PDE activity is associated with either an EAL or HD-GYP domain, which degrades c-di-GMP to 5'-phosphoguanylyl-(3'-5')-guanosine (pGpG) or two molecules of guanosine monophosphate (GMP) (Ryan *et al.*, 2006; Schmidt *et al.*, 2005; Tamayo *et al.*, 2005). The sophisticated c-di-GMP-mediated signalling network includes transcriptional, post-transcriptional and post-translational regulation. The regulatory function of c-di-GMP is exerted through the binding of c-di-GMP to a variety of cellular effectors, such as PilZ domain proteins, transcription factors, enzymatically inactive GGDEF, EAL or HD-GYP domain proteins and RNA riboswitches (Jenal *et al.*, 2017; Orr *et al.*, 2016; Römling *et al.*, 2013; Ryan *et al.*, 2012).

It has been revealed that GGDEF and EAL domain proteins are abundantly present in many Gram-negative bacteria. For example, *Escherichia coli* K-12 contains 29 genes and *Vibrio cholerae* contains 53 (Povolotsky and Hengge, 2012; Waters *et al.*, 2008). In *D. dadantii*, we found 12 *gcp* (GGDEF domain-containing protein), four *ecp* (EAL domain-containing protein) and two *egcp* (EAL-GGDEF domain-containing protein) genes in the genome using the Pfam program (pfam.xfam.org) (Fig. S1, see Supporting Information). Our previous studies have demonstrated that two c-di-GMP-specific PDEs, EGcpB and EcpC, positively regulate swimming motility, Pel production and type III secretion system (T3SS) gene expression, but negatively regulate biofilm formation (Yi *et al.*, 2010; Yuan *et al.*, 2015). EGcpA, a homologue of *E. coli* CsrD, negatively regulates Pel production and T3SS gene expression by modulating the expression of RsmB (Wu *et al.*, 2014). Nevertheless, the function of other Gcp and Ecp proteins in *D. dadantii* and the molecular mechanism of c-di-GMP signalling in the regulation of diverse virulence factors remain unclear.

In this study, we first analysed the regulatory roles of 18 GGDEF and/or EAL domain proteins in Pel production. GcpA was identified to be the major DGC that negatively regulated Pel production by repression of the expression of the *pelD* gene. We then demonstrated that GcpA regulates *pelD* through the H-NS–RsmB–RsmA pathway. Although EGcpB and EcpC are the two major PDEs that up-regulate Pel production, only EGcpB positively regulates *pelD* gene expression through the same regulatory pathway as GcpA. Furthermore, we demonstrated that GcpA was involved in the regulation of swimming motility and T3SS gene expression

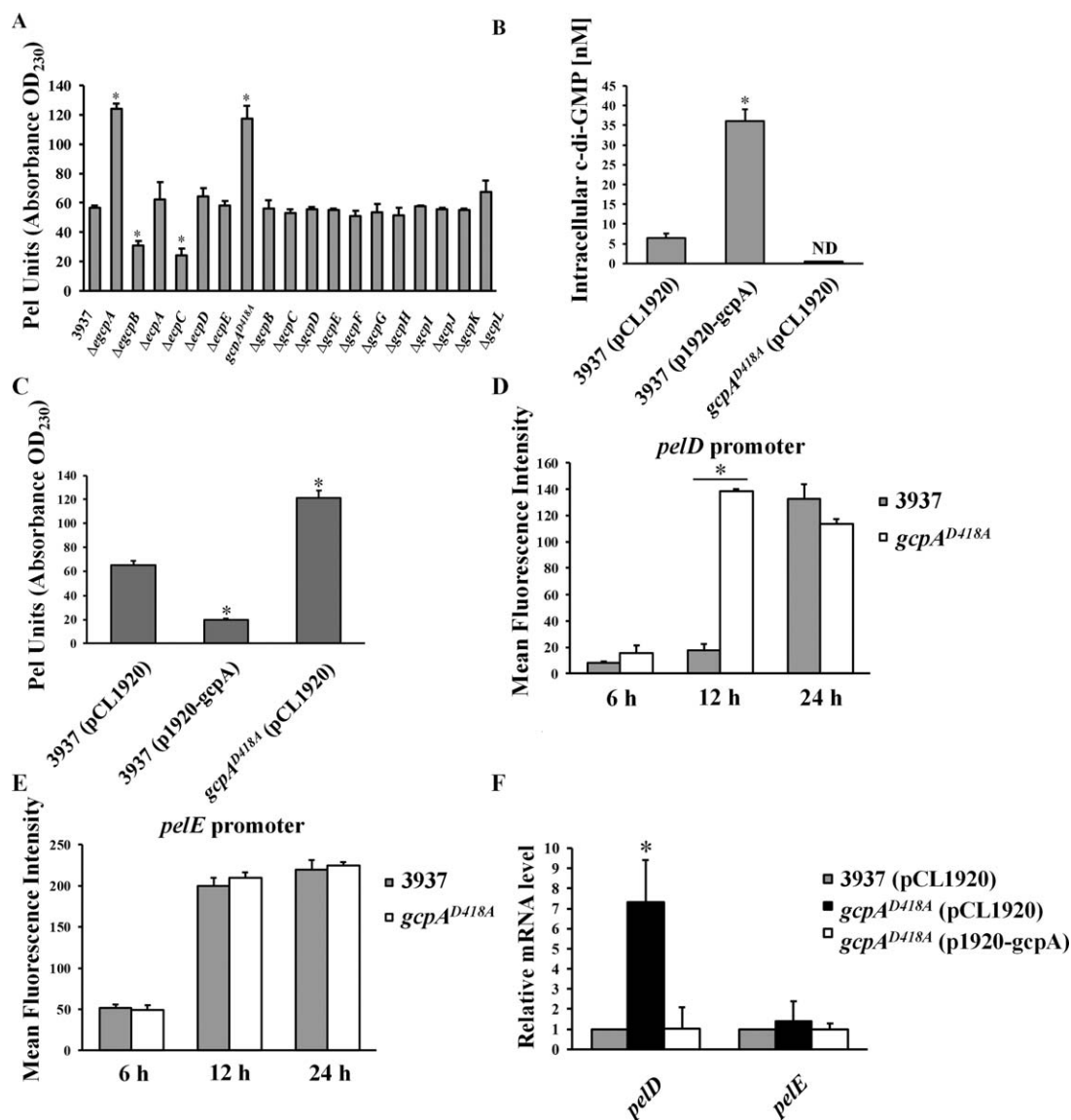
through diverse mechanisms that were independent of its regulation of Pel. Together, our studies define a comprehensive signalling network that links c-di-GMP signalling and multiple virulence factors in *D. dadantii*.

## RESULTS

### The GGDEF domain protein GcpA negatively regulates Pel production in *D. dadantii*

GGDEF and EAL domains are responsible for the enzymatic activities of DGCs and PDEs, respectively. In *D. dadantii*, 18 genes were found to encode proteins containing putative GGDEF and/or EAL domains at their C-terminal regions (Fig. S1A), implying that a complicated c-di-GMP signalling network exists for the regulation of diverse cell behaviour. Our results showed that four proteins, GcpA, GcpD, GcpF and EGcpB, contained two types of sensory domain, GAF (cGMP phosphodiesterase, Adenyl cyclase, FhIA domain) and PAS (Per/Arnt/Sim), at their N-terminus (Fig. S1A). Nine proteins (GcpB, GcpC, GcpG, GcpH, GcpJ, GcpK, GcpL, EGcpA and EcpD) contained one or multiple N-terminal transmembrane domains (Fig. S1A). Amino acid sequence alignments between the known GGDEF and EAL domains from *Caulobacter crescentus*, *V. cholerae*, *Pseudomonas aeruginosa* and *D. dadantii* revealed that most of the GGDEF domains in *D. dadantii* contained an active site (A-site) that is involved in GTP binding (Römling *et al.*, 2013). Eight GGDEF domains from GcpA–H were annotated with an inhibition site or I-site (RxxD motif), a secondary c-di-GMP binding site that represses the cyclase activity of DGC enzymes (Christen *et al.*, 2006) (Fig. S1B).

To fully investigate the network of c-di-GMP signalling in *D. dadantii* involved in Pel regulation, the impacts of each Gcp and Ecp protein on Pel were investigated. Nine *gcp* and *ecp* gene deletion mutants were newly constructed (Table S1, see Supporting Information). However, several attempts to delete *gcpA* were not successful, suggesting that *gcpA* might be essential for the viability of *D. dadantii*. We then disrupted the predicted A-site motif (SGDEF) in the GGDEF domain of GcpA by replacing the essential aspartic acid residue with alanine (SGAEF), resulting in a *gcpA*<sup>D418A</sup> site-directed mutant. This mutant was not defective for growth when compared with the wild-type strain (data not shown). Therefore, together with eight mutants that had been constructed previously, we expanded the mutant library to cover each individual *gcp*, *ecp* or *egcp* gene (Table S1). Pel activity was measured in the 18 mutants and the wild-type strain. As reported previously, deletion of *egcpB* and *ecpC* led to reduced Pel activities, whereas  $\Delta$ *egcpA* showed enhanced Pel activity relative to the wild-type strain (Wu *et al.*, 2014; Yi *et al.*, 2010) (Fig. 1A). No difference in Pel activity was observed in  $\Delta$ *ecpA*,  $\Delta$ *ecpD* or  $\Delta$ *ecpE* (Fig. 1A). Interestingly, amongst the 12 *gcp* gene deletion mutants, only *gcpA*<sup>D418A</sup> exhibited increased Pel activity compared



**Fig. 1** GcpA synthesizes bis-(3',5')-cyclic dimeric guanosine monophosphate (c-di-GMP) to negatively regulate pectate lyase (Pel) production and *pelD* gene expression in *Dickeya dadantii*. (A) Pel production of wild-type *D. dadantii* and GGDEF and/or EAL deletion mutant strains cultured in minimal medium (MM) + 0.1% polygalacturonic acid (PGA) for 12 h at 28 °C. OD<sub>230</sub>, optical density at 230 nm. Measurement of intracellular c-di-GMP (B) and Pel (C) production in wild-type *D. dadantii* harbouring empty vector pCL1920, wild-type harbouring pCL1920-*gcpA* and *gcpA*<sup>D418A</sup> harbouring pCL1920-*gcpA*. *pelD* (D) and *pelE* (E) promoter activities were measured in the parental strain *D. dadantii* and *gcpA*<sup>D418A</sup>. Cells cultured in MM + 0.1% PGA were harvested at 6, 12 and 24 h to measure the mean fluorescence intensity (MFI) by flow cytometry. (F) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of mRNA levels of *pelD* and *pelE* in *D. dadantii* strains. The data represent the expression levels of each gene relative to that in the wild-type, which was mathematically designated as unity. The *rplU* gene was used as an endogenous control for the calculation. All results are from one representative experiment. Three independent experiments were performed and three replicates were used for each experiment. Error bars indicate standard errors of the means. ND represents not detectable. Asterisks indicate statistically significant differences of the means ( $P < 0.05$  by Student's *t*-test).

with the wild-type strain (Fig. 1A). Complementation assays confirmed that the *in trans* expression of *gcpA* drastically reduced Pel activity in *gcpA*<sup>D418A</sup> (Fig. S2, see Supporting Information). These findings suggest that GcpA negatively regulates Pel production in *D. dadantii*.

### The DGC activity of GcpA is essential for its regulation of Pel production

As mutation of the GcpA A-site enhanced the production of Pel and a functional A-site is required for GGDEF domain activity, we hypothesized that GcpA is an active DGC and regulates Pel

production through c-di-GMP signalling. To test this, the intracellular concentrations of c-di-GMP were compared in the wild-type strain, *gcpA*<sup>D418A</sup> and wild-type expressing *gcpA* using ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS-MS). The results showed that the c-di-GMP concentration in the strain over-expressing *gcpA* was about six-fold higher than that in the same strain carrying the empty vector (Fig. 1B). Alternatively, the c-di-GMP concentration was below the detection limit in *gcpA*<sup>D418A</sup> (Fig. 1B). These data confirm that GcpA is an active DGC. We also observed that the over-expression of *gcpA* drastically reduced Pel activity in the wild-type strain (Fig. 1C). Together, these results support the conclusion that GcpA synthesizes c-di-GMP in *D. dadantii* to negatively regulate the production of Pel.

### The expression of *pelD* is enhanced in the *gcpA*<sup>D418A</sup> A-site mutant

To reveal the underlying mechanism of the inhibition of Pel production by GcpA, we analysed the effects of GcpA on two major Pel genes, *pelD* and *pelE*. In the presence of polygalacturonic acid (PGA), the promoter activities of *pelD* and *pelE* were induced by 22- and 4.5-fold, respectively, at 24 h (Fig. S3, see Supporting Information), suggesting that these *pel*<sub>promoter</sub>-GFP transcriptional fusions are sensitive to the addition of pectin catabolic products. Next, the promoter activities of *pelD* and *pelE* were determined in wild-type and *gcpA*<sup>D418A</sup> strains cultured in the presence of PGA for 6, 12 and 24 h. At 12 h, a significant increase in *pelD* promoter activity was observed in the *gcpA*<sup>D418A</sup> mutant compared with the wild-type strain (Fig. 1D). Interestingly, no significant change was observed for *pelE* promoter activity (Fig. 1E). To confirm the negative effect of GcpA on *pelD*, the mRNAs of *pelD* and *pelE* were measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in wild-type, *gcpA*<sup>D418A</sup> and the complemented strain containing the p1920-*gcpA* plasmid. Consistent with the change in *pelD* promoter activity, *pelD* transcript levels increased by seven-fold in *gcpA*<sup>D418A</sup> relative to the wild-type strain (Fig. 1F). The elevated mRNA level of *pelD* in *gcpA*<sup>D418A</sup> was restored to wild-type levels by the complementation plasmid p1920-*gcpA* (Fig. 1F). In contrast, *pelE* transcript levels were not altered in the tested strains (Fig. 1F). Thus, these results suggest that GcpA negatively regulates Pel production by repression of the expression of *pelD* in *D. dadantii*.

### The expression of Pel regulators in the *gcpA*<sup>D418A</sup> mutant

To assess whether the regulation of Pel by GcpA is through any known Pel regulators, we determined the expression of six negative regulators and three positive regulators in wild-type and *gcpA*<sup>D418A</sup> strains using qRT-PCR assays (Fig. 2A). Mutation of *gcpA* resulted in increased RNA levels of *rsmB*, *hns*, *fur*, *pecS* and

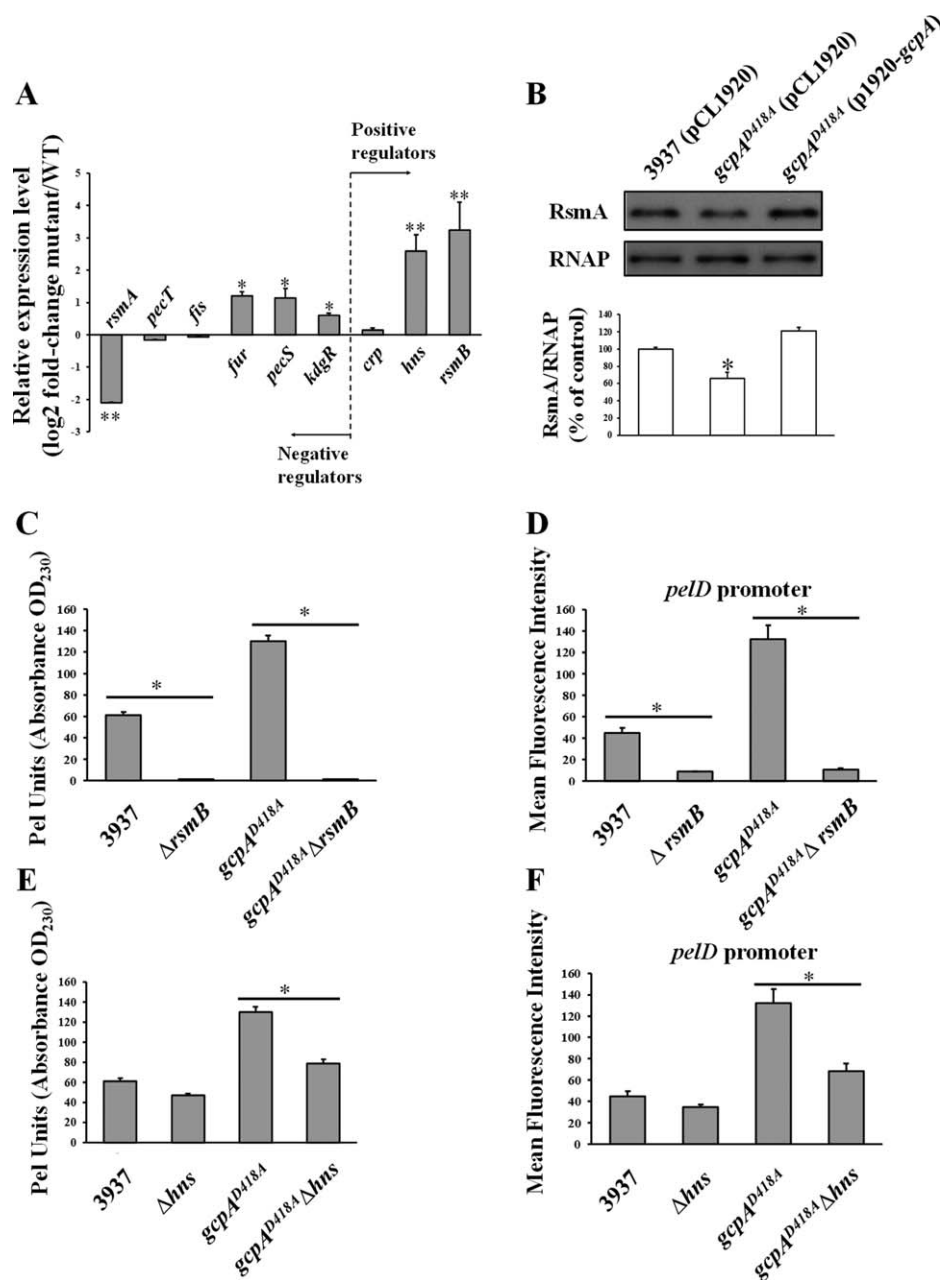
*kdgR*, and decreased RNA levels of *rsmA*. No significant difference was observed for *pecT*, *fis* and *crp*. Given that Fur, PecS and KdgR are *pel* gene repressors, we reasoned that the enhanced Pel activity in *gcpA*<sup>D418A</sup> was not caused by the increased expression of these genes. As *hns*, *rsmA*, and *rsmB* showed the highest fold changes by qRT-PCR analysis, we questioned whether there were any changes at the level of promoter activity. These promoters were examined using transcriptional fusions to a green fluorescent protein (GFP) reporter in wild-type and *gcpA*<sup>D418A</sup> strains. None of the promoter activities were significantly influenced in the *gcpA*<sup>D418A</sup> mutant (Fig. S4, see Supporting Information), suggesting that GcpA regulates the expression of these genes at the post-transcriptional level. A Western blot assay confirmed that the protein levels of RsmA were reduced in the *gcpA*<sup>D418A</sup> mutant (Fig. 2B). Together, these results imply that the control of Pel production by GcpA might rely on its positive regulation of RsmA and negative regulation of RsmB and H-NS at the post-transcriptional level.

### GcpA regulates Pel through the Rsm system

A significant increase in *rsmB* and decrease in *rsmA* in *gcpA*<sup>D418A</sup> strongly suggest that the Rsm system may play an important role in the GcpA-dependent signalling pathway for Pel regulation. To investigate this hypothesis, we constructed an *rsmB* deletion in the wild-type and *gcpA*<sup>D418A</sup> strains, and tested the Pel-related phenotypes. We found that deletion of *rsmB* resulted in severe defects in Pel production and *pelD* promoter activity in both backgrounds (Fig. 2C,D). These results were in agreement with previous studies showing that RsmB is a positive regulator of Pel (Yang *et al.*, 2008). More importantly, deletion of *rsmB* in *gcpA*<sup>D418A</sup> (*gcpA*<sup>D418A</sup>Δ*rsmB*) drastically reduced both Pel production and *pelD* promoter activity to a level similar to that of the Δ*rsmB* mutant (Fig. 2C,D). This result suggests that RsmB plays a predominant role in controlling Pel in *D. dadantii*, and that the repression of Pel production by GcpA is possibly through the regulation of RsmB. Considering that RsmB functions mainly by titration of the effect of the RNA-binding protein RsmA, we speculated that the over-expression of *rsmA* might result in similar phenotypes. To test this hypothesis, we constructed a plasmid to express *rsmA* *in trans* and transformed it into the *gcpA*<sup>D418A</sup> mutant. As expected, the over-expression of *rsmA* reduced Pel production and *pelD* promoter activity to near wild-type levels in the *gcpA*<sup>D418A</sup> mutant (Fig. S5, see Supporting Information). Therefore, we conclude that the RsmA/RsmB system plays an important role in the c-di-GMP signalling pathway mediated by GcpA to regulate Pel activity.

### H-NS is involved in the GcpA–Rsm pathway

It has been shown previously that H-NS positively modulates Pel synthesis in *D. dadantii* (Nasser and Reverchon, 2002).

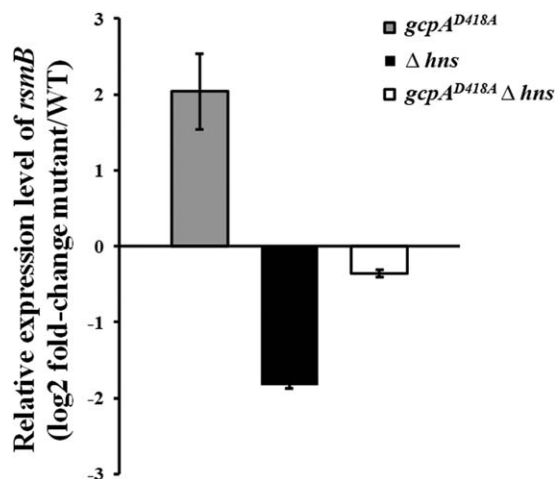


**Fig. 2** RsmB and H-NS play important roles in GcpA-dependent pectate lyase (Pel) regulation. (A) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of RNA levels of *rsmA*, *pecT*, *fis*, *fur*, *pecS*, *kdgR*, *crp*, *hns* and *rsmB* in *Dickeya dadantii* wild-type (WT) and *gcpA*<sup>D418A</sup>. The mutant/WT ratio for each gene expression was calculated as described in Experimental procedures. (B) Western blot analysis of RsmA protein in *D. dadantii* strains. Pel production (C, E) and *pelD* promoter activities (D, F) were tested in *D. dadantii* strains. Values are representative of three independent experiments. Three replicates were used in each experiment. Error bars indicate standard errors of the means. Asterisks indicate statistically significant differences of the means (\* $P < 0.05$  or \*\* $P < 0.01$  by Student's *t*-test). OD<sub>230</sub>, optical density at 230 nm.

Interestingly, we did not observe a significant reduction in Pel production or *pelD* promoter transcription when *hns* was deleted in the wild-type strain (Fig. 2E,F). However, when *hns* was deleted in the *gcpA*<sup>D418A</sup> mutant, Pel production and *pelD* promoter activity were dramatically reduced (Fig. 2E,F). These results, together with the observation that the expression of *hns* was elevated in the *gcpA*<sup>D418A</sup> mutant (Fig. 2A), suggest that GcpA also represses Pel through H-NS.

As we have shown that both H-NS and the Rsm system are involved in GcpA-dependent Pel regulation, we hypothesized that there might be a genetic link between these two regulators. To

address this hypothesis, the RNA levels of *rsmB* were determined in wild-type, *gcpA*<sup>D418A</sup>,  $\Delta$ *hns* and the *gcpA*<sup>D418A</sup>  $\Delta$ *hns* double mutant using qRT-PCR. In contrast with the approximate four-fold increase in *rsmB* in the *gcpA*<sup>D418A</sup> mutant, the relative level of *rsmB* was decreased by about four-fold in the  $\Delta$ *hns* mutant (Fig. 3). More importantly, *rsmB* expression was recovered to near wild-type levels in the *gcpA*<sup>D418A</sup>  $\Delta$ *hns* double mutant. The difference in *rsmB* RNA levels in the different strains was further confirmed by Northern blot analysis (data not shown). Together, our data indicate that GcpA regulates Pel through the GcpA–H-NS–RsmB–RsmA–*pelD* pathway.



**Fig. 3** H-NS is involved in the GcpA-dependent regulation of *rsmB*. *rsmB* RNA levels were examined in *Dickeya dadantii* using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The mutant/wild-type (WT) ratio for *rsmB* gene expression was calculated as described in Experimental procedures. One representative experiment was chosen, and three independent experiments were performed. Error bars indicate standard errors of the means. Asterisks indicate statistically significant differences of the means ( $P < 0.05$  by Student's *t*-test).

### EGcpB and EcpC differentially affect Pel production

Previously, we have shown that the PDEs EGcpB and EcpC increase Pel production in *D. dadantii* by reducing c-di-GMP, which is in contrast with GcpA, which synthesizes c-di-GMP to inhibit Pel production (Yi *et al.*, 2010; Yuan *et al.*, 2015). Therefore, we asked whether these two PDEs control Pel through the same regulatory pathways as GcpA. To address this question, we first examined the promoter activities of *pelD* in Δ*egcpB* and Δ*ecpC*. As expected, deletion of either *egcpB* or *ecpC* in the wild-type strain strongly reduced the expression of *pelD* (Fig. 4A). Next, we compared the RNA levels of *hns* and *rsmB* in the Δ*egcpB* and Δ*ecpC* mutants with those in the wild-type strain using qRT-PCR. Interestingly, although a strong reduction in *rsmB* transcripts was detected in both Δ*egcpB* and Δ*ecpC*, the *hns* transcript levels were only reduced in Δ*egcpB*, but not in Δ*ecpC* (Fig. 4B). These data suggest that EGcpB might regulate Pel through the H-NS–RsmB–RsmA–*pelD* pathway, whereas EcpC might regulate *rsmB* expression through a different mechanism. To further investigate their genetic interactions, the *egcpB* or *ecpC* gene was deleted in *gcpA*<sup>D418A</sup>, resulting in the double mutants *gcpA*<sup>D418A</sup>Δ*egcpB* and *gcpA*<sup>D418A</sup>Δ*ecpC*. We then detected *rsmB* transcripts in the wild-type strain and the single and double mutants by Northern blots. Consistent with the qRT-PCR results, both Δ*egcpB* and Δ*ecpC* mutants showed reduced levels of *rsmB* (Fig. 4C). Interestingly, *rsmB* expression was recovered to near wild-type level in the *gcpA*<sup>D418A</sup>Δ*egcpB* double mutant, whereas it appeared to be even more reduced in the *gcpA*<sup>D418A</sup>Δ*ecpC* double mutant than in the

Δ*ecpC* mutant. Moreover, the expression levels of *pelD* in various mutants were detected by qRT-PCR assays. The results showed that the expression of *pelD* was down-regulated in both Δ*egcpB* and Δ*ecpC* (Fig. 4D), which is consistent with the reduced *rsmB* levels in these mutants. As expected, *pelD* expression was recovered to near wild-type levels in the *gcpA*<sup>D418A</sup>Δ*egcpB* double mutant, but remained low in *gcpA*<sup>D418A</sup>Δ*ecpC* (Fig. 4D). In summary, our results suggest that GcpA and EGcpB inversely regulate *pelD* gene expression through the same regulatory pathway, whereas EcpC utilizes a different mechanism.

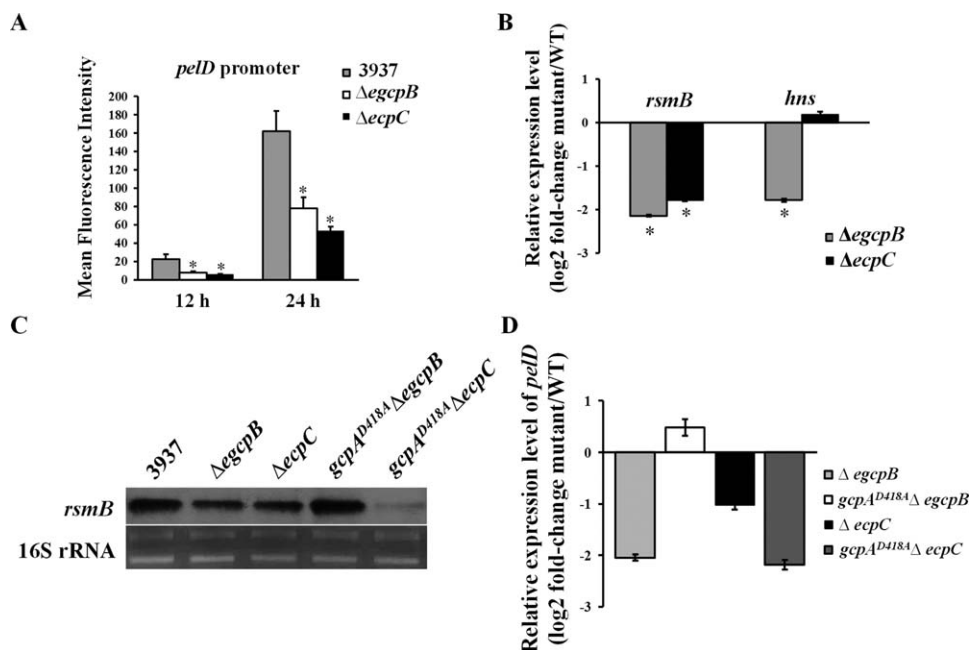
### GcpA negatively regulates the virulence, swimming motility and T3SS gene expression of *D. dadantii*

As the mutation of *gcpA* showed an opposite effect on Pel production to the mutation of either *egcpB* or *ecpC*, and the virulence of Δ*egcpB* and Δ*ecpC* on host plants was reduced, we further determined whether the virulence of *gcpA*<sup>D418A</sup> was altered. As shown in Fig. 5A, *gcpA*<sup>D418A</sup> exhibited a two-fold increase in maceration area relative to the wild-type strain in the leaves of the host plant Chinese cabbage (*Brassica campestris*). Furthermore, in comparison with the reduced maceration areas caused by Δ*egcpB* and Δ*ecpC*, double mutants of both *gcpA*<sup>D418A</sup>Δ*egcpB* and *gcpA*<sup>D418A</sup>Δ*ecpC* partially restored the virulence phenotype, but not to the extent of the wild-type strain (Fig. 5A). This result was noteworthy for *gcpA*<sup>D418A</sup>Δ*ecpC*, as its *pelD* expression was not restored at all (Fig. 4D), indicating that GcpA might regulate other virulence factors in addition to Pel in *D. dadantii*.

Thus, we further tested the swimming motility and expression of the T3SS gene, *hrpA*, in the *gcpA*<sup>D418A</sup> mutant, both of which are virulence factors previously shown to be regulated by c-di-GMP signalling in *D. dadantii* (Yi *et al.*, 2010). The results showed that the swimming motility and *hrpA* promoter activity were significantly enhanced in the *gcpA*<sup>D418A</sup> mutant compared with the wild-type strain (Fig. 5B,C). More interestingly, mutation of *gcpA* in the Δ*egcpB* mutant fully recovered its swimming motility (Fig. 5B), but not its *hrpA* promoter activity (Fig. 5C). The *gcpA*<sup>D418A</sup>Δ*ecpC* mutant exhibited low *hrpA* promoter activity that was equivalent to that of the Δ*ecpC* mutant (Fig. 5C), whereas its swimming motility was partially restored from Δ*ecpC* (Fig. 5B). These results could explain the partial restoration of virulence in the *gcpA*<sup>D418A</sup>Δ*egcpB* and *gcpA*<sup>D418A</sup>Δ*ecpC* double mutants, suggesting that all three virulence factors, including Pel production, swimming motility and T3SS, are essential for the full virulence of *D. dadantii* in host plants.

### H-NS and Rsm play different roles in the GcpA-dependent regulation of swimming motility and T3SS

As we have proposed a novel regulatory pathway in which GcpA controls Pel production via H-NS–RsmB–RsmA, we asked whether the same regulatory pathway was involved in the



**Fig. 4** EGcpB and EcpC positively regulate *pelD* gene expression via different pathways. (A) The promoter activity of *pelD* was examined in *Dickeya dadantii*. (B) RNA levels of *rsmB* and *hns* were examined using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The mutant/wild-type (WT) ratio of each gene was calculated as described in Experimental procedures. (C) Northern blot analysis of *rsmB* mRNA in *D. dadantii* strains. (D) mRNA levels of *pelD* in mutant strains relative to that in the wild-type strain. Values are representative of three independent experiments. Three replicates were used in each experiment. Error bars indicate standard errors of the means. Asterisks indicate statistically significant differences of the means ( $P < 0.05$  by Student's *t*-test).

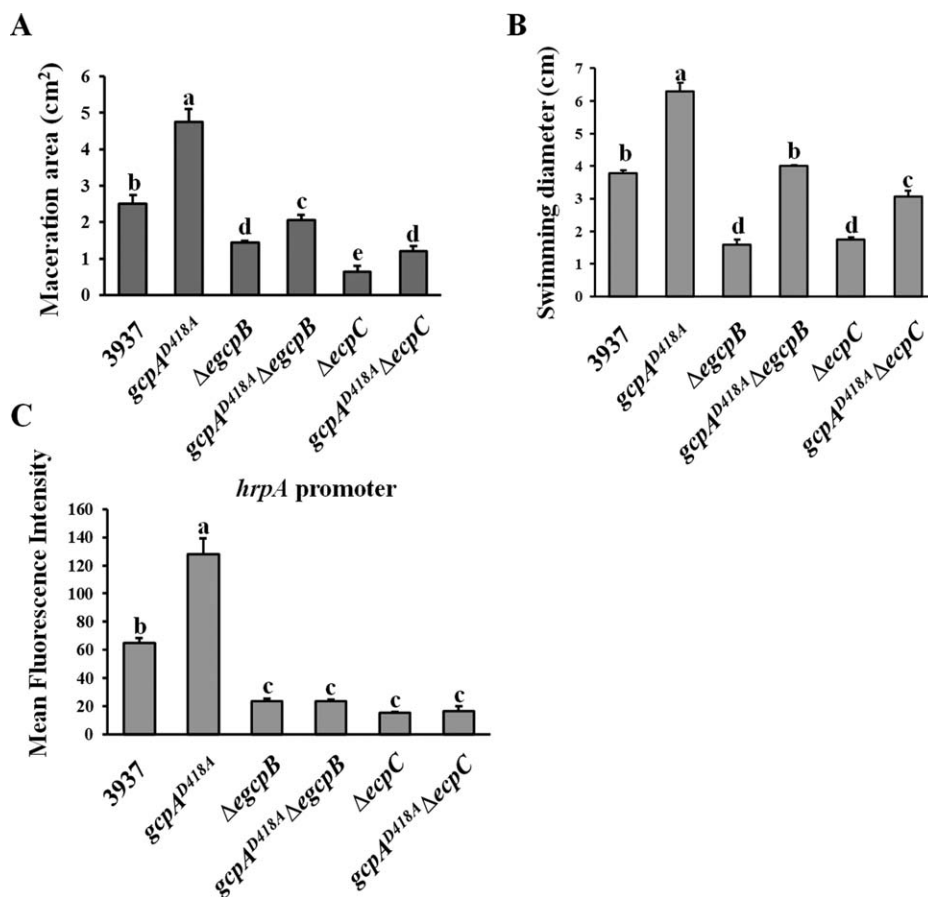
GcpA-dependent regulation of swimming motility and T3SS gene expression. As shown in Fig. 6A,  $\Delta hns$  and  $\Delta rsmB$  mutants exhibited wild-type levels of swimming motility; however, deletion of *hns*, but not *rsmB*, in the *gcpA*<sup>D418A</sup> mutant fully recovered its swimming motility to the wild-type level. This result, together with the observation that GcpA negatively regulates H-NS (Fig. 2A), suggests that H-NS is essential for GcpA to control swimming motility. Next, *hrpA* promoter activity was determined, and the results indicated that H-NS is not involved in the regulation of T3SS in either the *D. dadantii* wild-type or *gcpA*<sup>D418A</sup> strain (Fig. 6B). Deletion of *rsmB* drastically decreased the promoter activity of *hrpA* relative to the wild-type, and deletion of *rsmB* in *gcpA*<sup>D418A</sup> reduced its *hrpA* promoter activity to the  $\Delta rsmB$  level (Fig. 6B). To investigate how H-NS and RsmB contribute to virulence through various GcpA-regulated virulence factors, a virulence assay was performed in the host plant *B. campestris*. The maceration ability of  $\Delta hns$  was reduced compared with that of the wild-type strain. In addition, deletion of *hns* in *gcpA*<sup>D418A</sup> significantly reduced its maceration ability to nearly wild-type levels (Fig. 6C). However, despite the hyperswimming motility phenotype observed in the *gcpA*<sup>D418A</sup> $\Delta rsmB$  double mutant (Fig. 6A), deletion of *rsmB* in either the wild-type strain or the *gcpA*<sup>D418A</sup> mutant resulted in a non-pathogenic phenotype (Fig. 6C). Together, we conclude that the molecular mechanisms of GcpA that control various virulence factors are diverse; H-NS

and RsmB are essential for the GcpA-dependent regulation of swimming motility and T3SS, respectively. However, the H-NS–RsmB–RsmA pathway is not a major component modulating these two virulence factors in a GcpA-dependent manner.

## DISCUSSION

*Dickeya dadantii* produces Pels to degrade plant cell walls, and the production of this virulence factor is negatively regulated by c-di-GMP (Yi *et al.*, 2010; Yuan *et al.*, 2015). In this study, we propose a unique regulatory model that connects c-di-GMP regulation of bacterial virulence to both the global transcriptional and post-transcriptional regulatory systems in *D. dadantii*. To our knowledge, this is the first report implicating the H-NS–Rsm systems in the c-di-GMP signalling network for the negative regulation of *pelD*.

We confirmed that GcpA, which contains conserved A- and I-sites, is a genuine DGC (Fig. 1B). More importantly, our findings clearly demonstrated that GcpA relies on its DGC activity to regulate Pel (Fig. 1B,C). Moreover, the unsuccessful attempt to delete *gcpA* suggests that GcpA may be essential for bacterial viability and may play an additional role to its DGC activity. Interestingly, no other Gcp or Ecp proteins were shown to affect Pel production, suggesting that there might be specificity in different c-di-GMP signalling pathways.



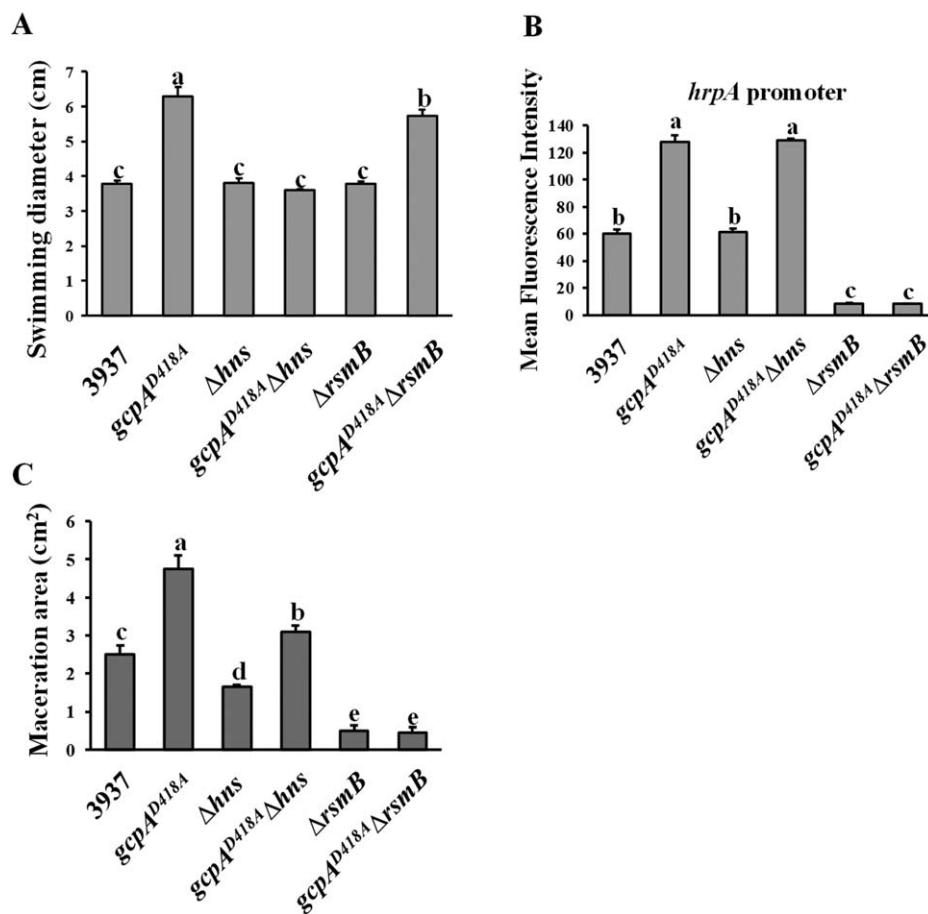
**Fig. 5** Effects of GcpA on swimming motility, type III secretion system (T3SS) gene expression and virulence. (A) Bacterial cells of *Dickeya dadantii* were inoculated in the leaves of Chinese cabbage (*Brassica campestris*). The maceration areas were measured at 16 h post-inoculation. The swimming motility (B) and T3SS gene *hrpA* promoter activity (C) were examined. Values are representative of three independent experiments. Three replicates were used in each experiment. Error bars indicate standard errors of the means. Different lowercase letters above the bars indicate statistically significant differences between treatments [Fisher's least significant difference (LSD),  $P < 0.05$ ].

PelD and PelE, which share high homology (89% similarity in amino acid sequence), are the most important Pels for the virulence of *D. dadantii* (Boccard *et al.*, 1988). The expression of *pelD* and *pelE* is differentially regulated in both plant tissues and media (Hugouvieux-Cotte-Pattat *et al.*, 1992; Masclaux *et al.*, 1996; Tardy *et al.*, 1997). Here, we observed that the expression of *pelD* was much more significantly induced (22-fold) than that of *pelE* (4.5-fold) when PGA was supplied in minimal medium (MM), probably because the basal expression level of *pelE* is higher than that of *pelD* (Fig. S3). These results are in agreement with previous studies, which indicate that a high basal level of *pelE* expression is essential to initiate rapid pectin degradation, whereas a high induced expression of *pelD* is necessary for the maximum production of Pels during infection in the plant (Ouafa *et al.*, 2012; Robert-Baudouy *et al.*, 2000). Furthermore, the transcription of *pelD* was monitored together with growth in MM supplemented with PGA, and the results showed that the expression of the *pelD* gene was only strongly induced in the mid-logarithmic, but not in the early logarithmic, phase of growth (Fig. S6, see Supporting Information). This result suggests that the transcription of the *pelD* gene is growth phase dependent. Interestingly, we observed that the expression of *pelD* at both transcriptional and

post-transcriptional levels was significantly enhanced in the *gcpA<sup>D418A</sup>* A-site mutant relative to the wild-type at 12 h, which corresponded to the early logarithmic phase, whereas the expression level of *pelE* was unaltered (Fig. 1D–F). A recent study has demonstrated that the transcriptional start site shift after gene duplication might be one of the reasons for the different expression patterns of *pelD* and *pelE* (Duprey *et al.*, 2016). However, the upstream regulatory mechanism remains unclear. Our results imply that the GcpA-mediated c-di-GMP signalling pathway is involved in the mechanism of differential expression of *pelD* and *pelE*.

The post-transcriptional regulatory system, RsmA/RsmB, has been shown to regulate several virulence factors, including Pel, in the soft-rot pathogens *D. dadantii* and *Pectobacterium carotovorum*, but how RsmA/RsmB regulates *pel* gene expression remains unclear (Chatterjee *et al.*, 1995; Mukherjee *et al.*, 1996; Yang *et al.*, 2008). Deletion of *rsmA* was lethal to *D. dadantii* 3937, unlike previous results with *P. carotovorum*. Therefore, we were unable to generate a *rsmA* mutant to examine its direct effect on Pel production. Instead, we observed that the over-expression of *rsmA* significantly repressed *pelD* expression and Pel production (data not shown). Nevertheless, our results demonstrate that





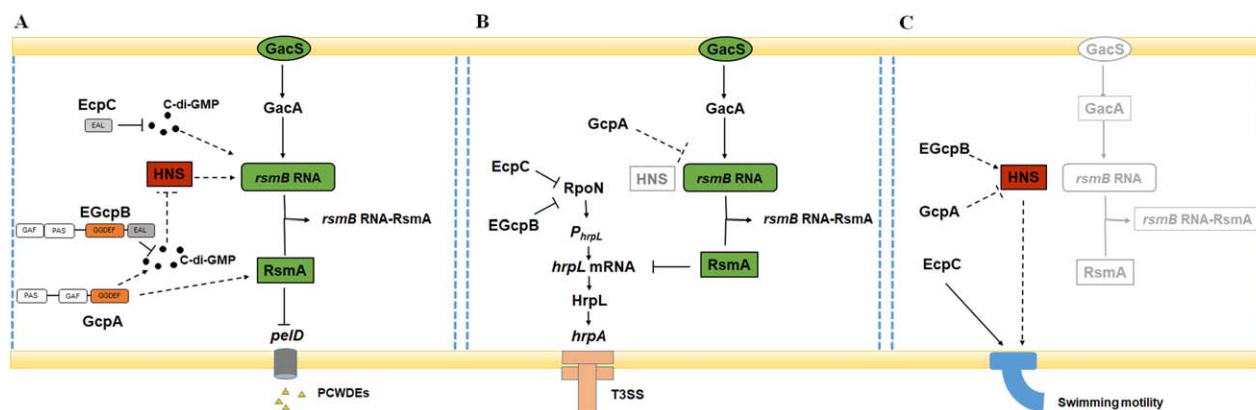
**Fig. 6** Effects of H-NS and RsmB on swimming motility, type III secretion system (T3SS) gene expression and virulence. The swimming motility (A), T3SS gene *hrpA* promoter activity (B) and maceration area on the leaves of Chinese cabbage (C) were examined. Values are representative of three independent experiments. Three replicates were used in each experiment. Error bars indicate standard errors of the means. Different lowercase letters above the bars indicate statistically significant differences between treatments [Fisher's least significant difference (LSD),  $P < 0.05$ ].

GcpA regulates the expression of RsmB and RsmA (Fig. 2). This regulation was further determined to be essential for GcpA-dependent Pel regulation, as either deletion of *rsmB* or overexpression of *rsmA* in the *gcpA<sup>D418A</sup>* mutant drastically reduced *pelD* promoter activity and Pel production (Figs 2C,D and S5). In *P. aeruginosa*, the RsmY and RsmZ sRNAs sequester the mRNA-binding protein RsmA in a mechanism similar to that of the RsmA/RsmB system (Lapouge *et al.*, 2008). Several studies have revealed that different DGCs negatively regulate RsmA activity through RsmY or RsmZ to control biofilm formation (Colley *et al.*, 2016; Moscoso *et al.*, 2014; Valentini *et al.*, 2016). Thus, our findings strongly suggest that the regulation of c-di-GMP signalling through the Rsm system might be common in different bacterial species.

H-NS is a nucleoid-associated protein that functions as a global transcriptional regulator in many Gram-negative bacteria (Castang *et al.*, 2008; Falconi *et al.*, 1998; Yu and DiRita, 2002). In *D. dadantii*, it has been shown that H-NS positively regulates swimming motility and Pel production, but negatively regulates EPS synthesis (Nasser *et al.*, 2001). Our findings demonstrated that GcpA negatively regulates *hns* expression at the post-transcriptional level (Figs 2A and S4). Strikingly, deletion of *hns* in

the *gcpA<sup>D418A</sup>* mutant not only restored its *pelD* promoter activity and Pel production (Fig. 2E,F), but also restored *rsmB* RNA to the wild-type level (Fig. 3). Taken together, we propose a regulatory pathway in which GcpA represses *pelD* gene expression via H-NS–RsmB–RsmA. In addition to its direct function as a repressor of *pelD* gene expression, H-NS is also known to be a positive regulator of Pel synthesis because of its negative impacts on PecT production (Nasser and Reverchon, 2002). As we did not observe significant changes in *pecT* expression in the *gcpA<sup>D418A</sup>* mutant (Fig. 2A), this regulatory pathway might not be related to PecT.

It is of interest to note that bacteria use multiple GGDEF and/or EAL domain proteins to regulate the same cellular behaviour in a sophisticated manner (Lindenberg *et al.*, 2013; Valentini *et al.*, 2016). Our findings demonstrated that the Rsm system plays an essential role in c-di-GMP signalling and the regulation of Pel production in *D. dadantii*. Although EGcpB, EcpC and GcpA have been shown to modulate the expression of *rsmB* at a post-transcriptional level (Figs 2A and 4C), our results indicate that their regulatory mechanisms might be different. GcpA and EGcpB may respond to similar environmental signals via their GAF and PAS sensory domains (Fig. S1A), and modulate the same c-di-GMP pool to control *pelD* gene expression through the



**Fig. 7** Working model for the bis-(3',5')-cyclic dimeric guanosine monophosphate (c-di-GMP) signalling pathway in *Dickeya dadantii*. (A) The regulation of c-di-GMP signalling on pectate lyase (Pel) production in *D. dadantii* is complex, involves several Gcp and Ecp proteins, and takes place at both transcriptional and post-transcriptional levels. The Rsm system is a central component in c-di-GMP-related Pel regulation. GcpA and EGcpB modulate the same c-di-GMP pool to control *pelD* gene expression through the H-NS–*rsmB*–RsmA pathway. EcpC modulates a different c-di-GMP pool that directly targets RsmB, bypassing H-NS. GcpA positively regulates RsmA at the post-transcriptional level. PCWDE, plant cell wall degrading enzyme. (B) The regulation of GcpA on type III secretion system (T3SS) gene expression is dependent on its impact on RsmB, which controls the expression of *hrpL* at the post-transcriptional level. This regulation is different from that of EGcpB and EcpC, which regulate *hrpL* at the transcriptional level via the RpoN–*hrpL* pathway. (C) GcpA and EGcpB regulate swimming motility through H-NS, whereas EcpC is different. ⊥, negative control; →, positive control. The broken lines indicate the regulatory mechanisms identified in this study.

H-NS–RsmB–RsmA pathway (Fig. 7A). In *Acetobacter xylinum*, Qi *et al.* (2009) have reported that the PAS domain of the DGC AxDGC2 enhances its cyclase activity by binding to the flavin adenine dinucleotide (FAD) cofactor under redox conditions. Similarly, oxygen levels may play a role in the modulation of c-di-GMP metabolism in *D. dadantii*. EcpC, the sole-EAL domain protein, probably modulates a different c-di-GMP pool that targets RsmB directly, bypassing H-NS. This convergence and divergence in c-di-GMP signalling are also supported by the regulation of swimming motility and T3SS gene expression in *D. dadantii* (Fig. 7B,C). We have shown here that GcpA and EGcpB inversely modulate swimming motility through H-NS, which is different from EcpC. It is worth noting that our data indicate that the regulatory mechanism of GcpA on T3SS gene expression might be different from that of EGcpB and EcpC, for which previous reports have indicated that these two PDEs positively regulate RpoN at the post-transcriptional level to control the T3SS master regulator HrpL. Indeed, we observed that only the transcript of *hrpL*, not *rpoN*, was increased in the *gcpA*<sup>D418A</sup> A-site mutant compared with the wild-type strain (Fig. S7, see Supporting Information). Finally, our virulence assay further confirmed that swimming motility, T3SS gene expression and Pel production are essential for *D. dadantii* to express full virulence in plants. Nevertheless, the environmental signals triggering c-di-GMP-dependent regulation, the expression patterns and localization of different DGCs and PDEs, and the c-di-GMP effectors that contribute to the signalling specificity of diverse cellular behaviours remain to be determined.

Overall, this multilevel regulation of c-di-GMP signalling of diverse virulence factors ensures the accurate control of the

virulence process for the infection of *D. dadantii*. It provides evidence of the complexity and specificity of c-di-GMP signalling in bacteria and sheds light on the understanding of *D. dadantii* infection strategies under various environmental conditions or during different infection status.

## EXPERIMENTAL PROCEDURES

### Bacterial strains, plasmids, primers and media

The bacterial strains and plasmids used in this study are listed in Table S1. *Dickeya dadantii* strains were grown in Luria–Bertani (LB) medium (1% tryptone, 0.5% yeast extract and 1% NaCl), mannitol–glutamic acid (MG) medium (1% mannitol, 0.2% glutamic acid, 0.05% potassium phosphate monobasic, 0.02% NaCl and 0.02% MgSO<sub>4</sub>) or M9 minimal medium (MM) supplemented with 0.1% PGA at 28 °C (Yang *et al.*, 2007). *Escherichia coli* strains were grown in LB at 37 °C. Antibiotics were added to the media at the following concentrations: ampicillin (100 µg/mL), kanamycin (Km, 50 µg/mL), chloramphenicol (20 µg/mL) and spectinomycin (100 µg/mL). The *D. dadantii* 3937 genome sequence was retrieved from a systematic annotation package for community analysis of genomes (ASAP) (<https://asap.ahabs.wisc.edu/asap/home.php>). Primers used for cloning and qPCR in this study are listed in Table S2 (see Supporting Information).

### Mutant construction and complementation

The GGDEF and/or EAL domain encoding genes, *hns*, *rsmB* and *pepC*, were deleted from the genome by allelic exchange mutagenesis (Yang *et al.*, 2002). In brief, upstream and downstream fragments flanking each target gene were amplified by PCR with specific primers (Table S2). The Km cassette was amplified from the pKD4 plasmid (Datsenko and

Wanner, 2000) and cloned between two flanking regions using three-way cross-over PCR. The PCR construct was inserted into the suicide plasmid pWMM91, and the resulting plasmid was transformed into *D. dadantii* 3937 by conjugation using *E. coli* strain S17-1  $\lambda$ -pir. Recombinants that grew on Km medium were plated onto 10% sucrose plates to select strains with chromosomal deletions. Cells that were resistant to sucrose because of the loss of SacB-mediated toxicity were then plated onto an ampicillin plate, and the ampicillin-sensitive cells were confirmed by PCR using outside primers. Mutations were confirmed by sequencing.

To construct the site-specific point mutation in the GGDEF motif of GcpA, single nucleotide substitution was performed using the QuikChange XL Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA, USA). Briefly, a primer set, *gcpA*-D418A-1 and *gcpA*-D418A-2 (Table S2), was used to generate *gcpA*<sup>D418A</sup>, in which the SGDEF motif was changed to SGAEF. Substitution was confirmed by sequencing. The *gcpA*<sup>D418A</sup> fragment was then amplified using the primer set, *gcpA*-A-SacI and *gcpA*-B (Table S2), and cloned upstream of the Km cassette, followed by the downstream fragment flanking *gcpA*, using three-way cross-over PCR. The construct was inserted into pWMM91, and the resulting plasmid was transferred into *D. dadantii* by conjugation using *E. coli* strain S17-1  $\lambda$ -pir. The above described allelic exchange mutagenesis was conducted to replace wild-type *gcpA* with *gcpA*<sup>D418A</sup>. Mutation was confirmed by sequencing using outside primers.

To construct double mutants, *rsmB*, *hns*, *egcpB* and *ecpC* were allelically exchanged in a *gcpA*<sup>D418A</sup> unmarked mutant strain. In brief, the pFLP2 plasmid encoding the FLP (flipase) recombinase enzyme was transferred into the *gcpA*<sup>D418A</sup>::Km strain by conjugation using *E. coli* S17-1  $\lambda$ -pir. Two FLP recombinase target (*FRT*) sites flanking the Km cassette allowed for flipase-mediated excision of Km. Transconjugants that were sensitive to Km and a high concentration of sucrose were then confirmed using outside primers and sequencing. To generate complemented strains, the promoter and open reading frame (ORF) regions of target genes were amplified and cloned into the low-copy-number plasmid pCL1920 (Table S1). The resulting plasmids were then confirmed by sequencing and electroporated into mutant strains.

### Swimming motility assay

Swimming motility was examined by inoculation of 10  $\mu$ L of overnight bacterial culture [optical density at 600 nm ( $OD_{600}$ ) = 1.0] onto the centre of MG plates containing 0.2% agar. The inoculated plates were incubated at 28 °C for 16 h. The diameter of radial growth was measured (Antúnez-Lamas *et al.*, 2009).

### Pel activity assay

Extracellular Pel activity was measured by spectrometry as described previously (Matsumoto *et al.*, 2003). Bacterial cells were cultured in MM supplemented with 0.1% PGA at 28 °C for 16 h; 1 mL of bacterial culture was then centrifuged at 13 000 *g* for 2 min, and the supernatant was collected; 10  $\mu$ L of supernatant were added to 990  $\mu$ L of reaction buffer [0.05% PGA, 0.1 M Tris-HCl (pH 8.5) and 0.1 mM CaCl<sub>2</sub>, prewarmed to 30 °C]. Pel activity was monitored at  $A_{230}$  for 3 min and calculated on the basis of one unit of Pel activity being equal to an increase of  $1 \times 10^{-3}$   $OD_{230}$  in 1 min.

### GFP reporter plasmid construction and flow cytometry assay

To generate the reporter plasmids pAT-*pelE*, pAT-*rsmA* and pAT-*hns*, the promoter regions of each gene were PCR amplified and cloned into the promoter probe vector pPROBE-AT, which contains a ribosomal binding site upstream of the *gfp* gene (Leveau and Lindow, 2001; Miller *et al.*, 2000). The reporter plasmids pAT-*pelD*, pAT-*hnpA* and pAT-*rsmB* had been constructed previously following the same procedure (Li *et al.*, 2015; Peng *et al.*, 2006; Yang *et al.*, 2007). Promoter activity was monitored by measurement of the GFP intensity through flow cytometry (BD Biosciences, San Jose, CA, USA), as described previously (Peng *et al.*, 2006). Briefly, bacterial cells with the reporter plasmid were grown in LB medium overnight and inoculated 1 : 100 into MM with or without 0.1% PGA. Samples were collected at 6, 12 and 24 h, and promoter activity was quantified by detection of the GFP intensity using flow cytometry.

### Determination of the intracellular c-di-GMP concentration

Intracellular c-di-GMP concentrations were determined using UPLC-MS-MS as described previously (Massie *et al.*, 2012). Briefly, overnight bacterial cultures were inoculated 1 : 1000 into 50 mL of LB medium in a flask. After  $OD_{600}$  of the bacterial culture had reached about 0.8, corresponding to mid- to late-exponential growth, all cells were centrifuged in 50-mL polystyrene centrifuge tubes for 30 min at 1500 *g*. The supernatant was then removed, and the pellet was resuspended in 1.5 mL of extraction buffer (40% acetonitrile and 40% methanol in 0.1 M formic acid). To lyse the cells and release intracellular c-di-GMP, cells resuspended in extraction buffer were dried by speed-vac, resuspended in 100  $\mu$ L of HPLC grade water, centrifuged for 5 min at 21 000 *g* in a tabletop centrifuge to pellet insoluble debris, filtered through a Titan syringe filter [polyvinylidene difluoride (PVDF), 0.45  $\mu$ m, 4 mm] and analysed by UPLC-MS-MS.

### Western blot analysis

*Dickeya dadantii* cells were grown in MM broth supplemented with 0.1% PGA at 28 °C for 12 h during the exponential growth phase, and 1-mL samples were taken. Cells were then resuspended in phosphate-buffered saline (PBS) and lysed by sonication. The protein in crude lysates was quantified using the Bradford protein assay (Bio-Rad, Hercules, CA, USA). Samples were boiled before loading onto 12% sodium dodecyl sulfate polyacrylamide gels. Proteins were then transferred onto a PVDF membrane (Millipore, Bedford, MA, USA). Blots were washed with PBS containing 0.05% Tween-20 and probed with an anti-RsmA antibody (Proteintech, Rosemont, IL, USA). Anti-RNA polymerase monoclonal antibody (Neoclone, Madison, WI, USA) was used as a control. The resulting blots were incubated for 1 min in enhanced chemiluminescence reagent (GE Healthcare, Chicago, IL, USA) and detected using O-MAT X-ray film.

### Northern blot analysis

To measure the RNA levels of *rsmB* in *D. dadantii* strains, bacterial cells grown in MM supplemented with 0.1% PGA for 12 h were harvested, and total RNA was isolated using TRI reagent (Sigma-Aldrich, St Louis, MO, USA). The residual DNA was removed with a Turbo DNA-free DNase kit

(Invitrogen, Austin, TX, USA). Northern blot analysis was performed using biotin-labelled probe and a biotin detection system (BrightStar Psolaren-Biotin and Bright Star BioDetect, Ambion, Carlsbad, CA, USA). 16S rRNA was used as an internal control.

### qRT-PCR analysis

The mRNA levels of *pelD*, *pelE*, *rsmA*, *rsmB*, *pecT*, *pecS*, *fis*, *fur*, *kdgR*, *crp*, *hns*, *hrpL* and *rpoN* were measured by qRT-PCR. Briefly, bacterial cells cultured in MM broth supplemented with 0.1% PGA for 12 h were harvested, and total RNA was extracted using a PureLink RNA Mini Kit (Ambion) according to the manufacturer's instructions. On-column DNase treatment (Invitrogen, Carlsbad, CA, USA) was performed. cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). The cDNA level of different samples was quantified by real-time PCR using a PowerUp SYBR Green Master Mix (Life Technologies, Carlsbad, CA, USA). The relative levels of gene expression were determined using the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen, 2001), with the *rplU* gene as the internal control (Mah *et al.*, 2003). Three technical replicates were used each time.

### Virulence assay

The local leaf maceration assay was performed using the leaves of Chinese cabbage (*B. campestris*), as described previously (Yuan *et al.*, 2015). In brief, 10  $\mu$ L of bacterial suspension at  $10^7$  colony-forming units (CFU)/mL were inoculated into the wounds punched with a sterile pipette on the leaves. Five leaves were used for each strain. Inoculated Chinese cabbage leaves were kept in a growth chamber at 28 °C with 100% relative humidity for 16 h before photographs were taken. To evaluate disease symptoms, American Physiological Society ASSESS 1.0 software (Image Analysis Software for Plant Disease Quantification) was used to determine the leaf maceration areas.

### Statistical analysis

Means and standard deviations of experimental results were calculated using Excel, and the statistical analysis was performed using a two-tailed Student's *t*-test (Microsoft, Redmond, WA, USA) or Fisher's least significant difference (LSD) test with a DPS data processing system ([http://www.dpsw.cn/dps\\_eng](http://www.dpsw.cn/dps_eng)).

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

**Fig. S1** Analysis of GGDEF and/or EAL domain proteins in *Dickeya dadantii*. (A) Summary of GGDEF and/or EAL domain proteins. Proteins are shown with the encoded gene names and protein length. Protein domains were predicted by the simplified modular architecture research tool (SMART). ORF, open reading frame. (B) Amino acid sequence alignment for the GGDEF domains in *D. dadantii*. PleD and WspR are two active diguanylate cyclases (DGCs) from *Caulobacter crescentus* and *Pseudomonas aeruginosa*, respectively. The inhibition site RxxD motif (I-site) and enzymatic activity site GGDEF motif (A-site) are marked. (C) Amino acid sequence alignment for the EAL domains in *D. dadantii*. VC1086 is an active phosphodiesterase (PDE) from *Vibrio cholerae*. The arrow indicates the glutamate residue in the EAL motif. '\*\*', residues identical in all sequences in the alignment; '.', conserved substitutions; '.', semi-conserved substitutions.

**Fig. S2** Effect of complementation of *gcpA*<sup>D418A</sup> on pectate lyase (Pel) production. Values are representative of three independent experiments. Three replicates were used in each experiment. Error bars indicate standard errors of the means. Asterisks indicate statistically significant differences of the means ( $P < 0.05$  by Student's *t*-test). OD<sub>230</sub>, optical density at 230 nm.

**Fig. S3** Polygalacturonic acid (PGA) induces the transcription of *pelD* and *pelE* genes. (A, B) The promoter activities of *pelD* and *pelE* in *Dickeya dadantii* were examined at 12 and 24 h. The experiments were repeated three independent times with similar results. The figure represents the results from one experiment which includes three technical replicates. Error bars indicate standard errors of the means. Asterisks indicate statistically significant differences of the means ( $P < 0.05$  by Student's *t*-test).

**Fig. S4** Promoter activities of *rsmA*, *rsmB* and *hns* are not affected in *gcpA*<sup>D418A</sup>. (A–C) The promoter activities of *rsmA*, *rsmB* and *hns* in *Dickeya dadantii* strains. One representative experiment was chosen, and three independent experiments were performed. Assays were performed as described in Experimental procedures. Error bars indicate standard errors of the means.

**Fig. S5** Expression of *rsmA* restores pectate lyase (Pel) in the *gcpA*<sup>D418A</sup> A-site mutant. (A, B) Pel production and *pelD* promoter activity were measured in *Dickeya dadantii*. Three independent experiments were performed with three replicates in each experiment. Values are from one representative experiment. Error bars indicate standard errors of the means. Asterisks indicate statistically significant differences of the means ( $*P < 0.05$  by Student's *t*-test). OD<sub>230</sub>, optical density at 230 nm.

**Fig. S6** *pelD* gene transcription is growth phase dependent. The promoter activity of the *pelD* gene was determined throughout growth in minimal medium (MM) supplemented with 0.1% polygalacturonic acid (PGA) at 28 °C. The experiments were repeated twice independently with similar results. Three replicates were used for each experiment. Error bars indicate standard errors of the means. Open squares, OD<sub>600</sub> (optical density at 600 nm). Filled triangles, mean fluorescence intensity.

**Fig. S7** Impact of GcpA on the expression of *hrpL* and *rpoN*. mRNA levels of *hrpL* and *rpoN* in *gcpA*<sup>D418A</sup> relative to that in the wild-type, which was mathematically designated as unity. All results are from one representative experiment. Three independent experiments were performed and three replicates were used for each experiment. Error bars indicate standard errors of the means. Asterisk indicates statistically significant difference of the means ( $P < 0.05$  by Student's *t*-test).

**Table S1** Strains and plasmids used in this study.

**Table S2** Primers used in this study.