HHS Public Access

Author manuscript

Mol Plant Pathol. Author manuscript; available in PMC 2019 August 01.

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 the plant cell wall. Previously, we have demonstrated that EGcpB and EcpC function as cyclic-di-GMP (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 dichotomously regulation of Pel has remained a mystery. Here, we identified GcpA is the dominant DGC to negatively regulate Pel production by specifically repressing *pelD* gene expression. Quantitative real-time PCR (qRT-PCR) assays revealed that the expression levels of histone-like nucleoid-structuring protein encoding gene *hns* and the post-transcriptional regulator encoding genes *rsmA* and *rsmB* were significantly affected by GcpA. Deletion of *hns* or *rsmB* in the $gcpA^{D418A}$ site-directed mutant restored its Pel production and *pelD* expression, demonstrating that H-NS and RsmB contribute to 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 T3SS gene expression, respectively. Among the two PDEs involved in the regulation of Pels, only EGcpB regulates *pelD* expression through the same pathway as GcpA.

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Keywords

soft-rot pathogen; pectate lyase; c-di-GMP; virulence; type III secretion system; motility

Introduction

Dickeya dadantii 3937 is an Enterobacterium that causes soft-rot disease in a wide range of economically important crops (Ma *et al.*, 2007, Czajkowski *et al.*, 2011). Pectate lyases, which degrade the plant cell wall, are one of the major virulence factors that contribute to the pathogenesis of *D. dadantii* (Collmer & 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 (Hugouvieux-Cotte-Pattat *et al.*, 1996, Charkowski *et al.*, 2012, Reverchon & 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 environments in the plant intercellular spaces (Reverchon *et al.*, 1991, Franza *et al.*, 2002, Llama-Palacios *et al.*, 2005, Ouafa *et al.*, 2012, Hérault *et al.*, 2014). In addition, the post-transcriptional regulator RsmA/RsmB inhibits Pel via an unknown mechanism (Yang *et al.*, 2008, Wu *et al.*, 2014). RsmA facilitates specific mRNA degradation while 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 Vfm quorum sensing system (Reverchon *et al.*, 2010, Franza et al., 2002, Reverchon & Nasser, 2013, Charkowski et al., 2012, Yang et al., 2008, Hugouvieux-Cotte-Pattat et al., 1996, Nasser *et al.*, 2013).

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 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, cell cycle, antibiotic production, and virulence (Cotter & Stibitz, 2007, Tamayo *et al.*, 2007, Hengge, 2009, Jenal *et al.*, 2017). 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 & Lee, 2015). PDE activity is associated with either an EAL or HD-GYP domain, which degrade c-di-GMP to 5’-phosphoguanylyl-(3’–5’)-guanosine (pGpG) or two molecules of guanosine monophosphate (GMP) (Tamayo *et al.*, 2005, Schmidt *et al.*, 2005, Ryan *et al.*, 2006). The sophisticated c-di-GMP-mediated signaling 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 (Römling et al., 2013, Ryan *et al.*, 2012, Jenal et al., 2017, Orr *et al.*, 2016).

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 & Hengge, 2012, Waters *et al.*, 2008). In *D. dadantii*, we found 12 *gcp* (GGDEF-domain-containing protein), 4 *ecp* (EAL-domain-containing protein) and 2 *egcp* (EAL-GGDEF-domains-containing protein) genes in the genome using Pfam program (pfam.xfam.org) (Fig. S1). Our previous studies demonstrated that two c-di-GMP specific PDEs, EGcpB and EcpC, positively regulate swimming motility, Pel production and T3SS gene expression, while negatively regulating 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 signaling in the regulation of diverse virulence factors remain unclear.

In the present study, we first analyzed the regulatory roles of eighteen GGDEF and/or EAL domain proteins on Pel production. GcpA was identified to be the major DGC that negatively regulated Pel production by repressing the expression of *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, it appeared that only EGcpB positively regulated *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 from its regulation on Pel. Together, our studies defined a comprehensive signaling network that links c-di-GMP signaling 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*, eighteen genes were found to encode proteins that contain putative GGDEF and/or EAL domains at their C-terminal regions (Fig. S1A), implying a complicated c-di-GMP signaling network exists for regulating diverse cell behaviors. Our results showed that four proteins, GcpA, GcpD, GcpF, and EGcpB, contained two types of sensory domains, GAF (cGMP phosphodiesterase, Adenyl cyclase, FhlA domain) and PAS (Per/Arnt/Sim), at their N-terminus (Fig. S1A). Nine proteins including 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*, *Vibrio cholerae*, *Pseudomonas aeruginosa*, and those from *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 signaling 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). 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 to alanine (SGAEF), resulting in a $gcpA^{D418A}$ 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 were previously constructed, we expanded the mutant library to cover each individual *gcp*, *ecp* or *egcp* gene (Table S1). The Pel activity was measured in the eighteen mutants and the wild-type strain. As previously reported, deletion of *egcpB* and *ecpC* showed reduced Pel activities while Δ*egcpA* showed enhanced Pel activity relative to the wild-type strain (Yi et al., 2010, Wu et al., 2014) (Fig. 1A). No difference in Pel activity was observed in Δ*ecpA*, Δ*ecpD*, or Δ*ecpE* (Fig. 1A). Interestingly, among the twelve *gcp* gene deletion mutants, only $gcpA^{D418A}$ exhibited increased Pel activity compared with the wild-type strain (Fig. 1A). Complementation assays confirmed that in *trans* expression of *gcpA* drastically reduced Pel activity in $gcpA^{D418A}$ (Fig. S2). These findings suggested that GcpA negatively regulates Pel production in *D. dadantii*.

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

Since mutation of the GcpA A-site enhanced the production of Pel and a functional A-site is required for the GGDEF domain activity, we hypothesized that GcpA is an active DGC and it regulates Pel production through c-di-GMP signaling. To test this, the intracellular concentrations of c-di-GMP were compared in the wild-type strain, $gcpA^{D418A}$, and wild-type expressing *gcpA*, using ultra performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS-MS) approach. The results showed that the c-di-GMP concentration in the strain over-expressing *gcpA* was about 6-fold higher than the same strain carrying the empty vector (Fig. 1B). Alternatively, the c-di-GMP concentration was below detection in $gcpA^{D418A}$ (Fig. 1B). These data confirmed that GcpA is an active DGC. We also observed overexpression of *gcpA* drastically reduced the Pel activity in the wild-type strain (Fig. 1C). Altogether, these results supported the conclusion that GcpA synthesizes c-di-GMP in *D. dadantii* to negatively regulate the production of Pel.

Expression of *pelD* is enhanced in $gcpA^{D418A}$ A-site mutant

To reveal the underlying mechanism of GcpA to inhibit Pel production, we analyzed the effects of GcpA on two major Pel genes, *pelD* and *pelE*. In the presence of polygalacturonate (PGA), the promoter activities of *pelD* and *pelE* were induced by 22- and 4.5-fold at 24 h, respectively (Fig. S3), suggesting that these $pel_{promoter}$-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 $gcpA^{D418A}$ strains cultured in the presence of PGA for 6, 12 and 24 h, respectively. At 12 h, a significant increase of the *pelD* promoter activity was observed in the $gcpA^{D418A}$ mutant compared with the wild-type

strain (Fig. 1D). Interestingly, no significant change was observed for the *pelE* promoter activity (Fig. 1E). To confirm the negative effect of GcpA on *pelD*, the mRNA of *pelD* and *pelE* were measured by quantitative real-time PCR (qRT-PCR) in wild-type, $gcpA^{D418A}$, and the complemented strain containing p1920-gcpA plasmid. Consistent with the change in the *pelD* promoter activity, *pelD* transcript levels increased by 7-fold in $gcpA^{D418A}$ relative to the wild-type strain (Fig. 1F). The elevated mRNA level of *pelD* in $gcpA^{D418A}$ 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 suggested that GcpA negatively regulates Pel production by repressing the expression of *pelD* in *D. dadantii*.

Expression of Pel regulators in the $gcpA^{D418A}$ 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 $gcpA^{D418A}$ 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 the *pel* gene repressors, we reasoned that the enhanced Pel activity in $gcpA^{D418A}$ was not due to increased expression of these genes. Since *hns*, *rsmA*, and *rsmB* showed the highest fold changes by the qRT-PCR analysis, we asked whether there were any changes at the level of promoter activity. These promoters were examined using transcriptional fusions to a GFP reporter in wild-type and $gcpA^{D418A}$ strains. None of the promoter activities were significantly influenced in the $gcpA^{D418A}$ mutant (Fig. S4), suggesting that GcpA regulates expression of these genes at the post-transcriptional level. A western blot assay confirmed that the protein levels of RsmA were reduced in the $gcpA^{D418A}$ mutant (Fig. 2B). Altogether, these results implied 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 of *rsmB* and decrease of *rsmA* in $gcpA^{D418A}$ strongly suggested that the Rsm system may play an important role in the GcpA-dependent signaling pathway for Pel regulation. To investigate this hypothesis, we constructed a *rsmB* deletion in the wild-type and $gcpA^{D418A}$ strains, respectively, and tested the Pel related phenotypes. We found that deletion of *rsmB* resulted in severe defects in Pel production and *pelD* promoter activities in both backgrounds (Fig. 2C and 2D). These results were in agreement with previous studies showing that RsmB is a positive regulator for the Pel (Yang et al., 2008). More importantly, deletion of *rsmB* in $gcpA^{D418A}$ ($gcpA^{D418A}\Delta rsmB$) drastically reduced both its Pel production and *pelD* promoter activities to a level that is similar to that of the Δ*rsmB* mutant (Fig. 2C and 2D). This result suggested that RsmB plays a predominate 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 titrating the effect of RNA-binding protein RsmA, we speculated that 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 $gcpA^{D418A}$ mutant. As expected, over-

expression of *rsmA* reduced the Pel production and *pelD* promoter activity to near-wild-type levels in the *gcpA*^{D418A} mutant (Fig. S5). Therefore, we concluded that the RsmA/RsmB system plays an important role in the c-di-GMP signaling pathway mediated by GcpA to regulate Pel activity.

H-NS is involved in the GcpA-Rsm pathway

It has been previously shown that H-NS positively modulates Pel synthesis in *D. dadantii* (Nasser & Reverchon, 2002). Interestingly, we did not observe a significant reduction of Pel production or *pelD* promoter transcription when *hns* was deleted in the wild-type strain (Fig. 2E and 2F). However, when *hns* was deleted in the *gcpA*^{D418A} mutant, Pel production and *pelD* promoter activity were dramatically reduced (Fig. 2E and 2F). These results, together with the observation that expression of *hns* was elevated in the *gcpA*^{D418A} mutant (Fig. 2A), suggested that GcpA also represses Pel through H-NS.

As we showed that both H-NS and the Rsm system were involved in the 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*^{D418A}, Δ*hns*, and a *gcpA*^{D418A}Δ*hns* double mutant using qRT-PCR. In contrast to the approximate 4-fold increase of *rsmB* in the *gcpA*^{D418A} mutant, the relative level of *rsmB* was decreased by about 4-fold in the Δ*hns* mutant (Fig. 3). More importantly, *rsmB* expression was recovered to near-wild-type levels in the *gcpA*^{D418A}Δ*hns* double mutant. The difference of *rsmB* RNA levels in the different strains was further confirmed by northern blot analysis (data not shown). Together, our data supported that GcpA regulates Pel through the GcpA-H-NS-RsmB-RsmA-*pelD* pathway.

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 to 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 Δ*egcpB* and Δ*ecpC* mutants to 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 suggested that EGcpB might regulate Pel through the H-NS-RsmB-RsmA-*pelD* pathway while EcpC might regulate *rsmB* expression through a different mechanism. To further investigate their genetic interactions, the *egcpB* or *ecpC* gene was deleted in *gcpA*^{D418A} resulting in the double mutants, *gcpA*^{D418A}Δ*egcpB* and *gcpA*^{D418A}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 a near-wild-type level in the *gcpA*^{D418A}Δ*egcpB* double mutant, while it appeared to be even more reduced in the *gcpA*^{D418A}Δ*ecpC* double mutant than the Δ*ecpC* mutant. Moreover, the expression levels

of *pelD* in various mutants were detected by qRT-PCR assays. The results showed that 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 $gcpA^{D418A}$Δ*egcpB* double mutant, while it remained low in $gcpA^{D418A}$Δ*ecpC* (Fig. 4D). In summary, our results suggested that GcpA and EGcpB inversely regulate *pelD* gene expression through the same regulatory pathway while EcpC utilizes a different mechanism.

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

Since mutation of *gcpA* showed an opposite effect on Pel production as mutation of either *egcpB* or *ecpC*, and the virulence of Δ*egcpB* and Δ*ecpC* on host plants were reduced, we further determined whether the virulence of $gcpA^{D418A}$ was altered. As shown in Fig. 5A, $gcpA^{D418A}$ exhibited a two-fold increase in maceration areas relative to the wild-type strain in the leaves of 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 $gcpA^{D418A}$Δ*egcpB* and $gcpA^{D418A}$Δ*ecpC* partially restored the virulence phenotype, but not to the extent of the wild-type strain (Fig. 5A). This result was noteworthy for $gcpA^{D418A}$Δ*ecpC* since its *pelD* expression was not restored at all (Fig. 4D), indicating that GcpA might regulate other virulence factors besides Pel in *D. dadantii*.

Thus, we further tested the swimming motility and expression of the T3SS gene, *hrpA*, in the $gcpA^{D418A}$ mutant, both of which are virulence factors previously shown to be regulated by c-di-GMP signaling in *D. dadantii* (Yi et al., 2010). The results showed that the swimming motility and *hrpA* promoter activity were significantly enhanced in the $gcpA^{D418A}$ mutant compared with the wild-type strain (Fig. 5B and 5C). 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 $gcpA^{D418A}$Δ*ecpC* mutant exhibited low *hrpA* promoter activity that was equivalent to the Δ*ecpC* mutant (Fig. 5C), while its swimming motility was partially restored from Δ*ecpC* (Fig. 5B). These results could explain the partial restoration of virulence in the $gcpA^{D418A}$Δ*egcpB* and $gcpA^{D418A}$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 played different roles in GcpA-dependent regulation of swimming motility and T3SS

Since we 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 GcpA-dependent regulation of swimming motility and T3SS gene expression. As shown in Fig. 6A, Δ*hns* and Δ*rsmB* mutants exhibited wild-type levels of swimming motility, however, deletion of *hns* but not *rsmB* in the $gcpA^{D418A}$ mutant fully recovered its swimming motility to the wild-type level. This result, together with the observation that GcpA negatively regulated H-NS (Fig. 2A), suggested that H-NS is essential for GcpA to control swimming motility. Next, the *hrpA* promoter activity was determined, and the results indicated that H-NS is not involved in the regulation of T3SS in both the *D. dadantii* wild-

type and gcpA*D418A* strains (Fig. 6B). Deletion of *rsmB* drastically decreased the promoter activity of *hrpA* relative to the wild-type, and deletion of *rsmB* in gcpA^{D418A} reduced its *hrpA* promoter activity to the Δ*rsmB* level (Fig. 6B). To investigate how H-NS and RsmB contribute to virulence through various GcpA-regulated virulence factors, virulence assay was performed in the host plant, *B. campestris*. The maceration ability of Δ*hns* was reduced compared with that of the wild-type strain. In addition, deletion of *hns* in *gcpA*^{D418A} significantly reduced its maceration ability to nearly wild-type levels (Fig. 6C). On the other hand, despite a hyper swimming motility phenotype observed in the *gcpA*^{D418A}Δ*rsmB* double mutant (Fig. 6A), deletion of *rsmB* in either the wild-type strain or the *gcpA*^{D418A} mutant resulted in a non-pathogenic phenotype (Fig. 6C). Together, we concluded that the molecular mechanisms of GcpA to control various virulence factors are diverse; H-NS and RsmB are essential for the GcpA-dependent regulations of swimming motility and T3SS, respectively. However, the H-NS-RsmB-RsmA pathway was not a major component modulating these two virulence factors in a GcpA-dependent manner.

Discussion

D. dadantii produces Pels to degrade the plant cell wall, and 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 signaling network for the negative regulation of *pelD*.

We confirmed 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 and 1C). Moreover, the unsuccessful attempt to delete *gcpA* suggests that GcpA might be essential for bacterial viability and may play additional roles other than 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 signaling pathways.

PelD and PelE, which share high homology (89% similarity in amino acid sequence), are the most important Pels for the virulence of *D. dadantii* (Boccara *et al.*, 1988). Expressions of *pelD* and *pelE* are differentially regulated in both plant tissues and media (Tardy *et al.*, 1997, Hugouvieux-Cotte-Pattat *et al.*, 1992, Masclaux *et al.*, 1996). Here, we observed that the expression of *pelD* was much more significantly induced (22-fold) than *pelE* (4.5-fold) when PGA was supplied in minimal medium (MM), probably because the basal expression level of *pelE* is higher than *pelD* (Fig. S3). These results are in agreement with the previous studies, which indicate that the high basal level of *pelE* expression is essential to initiate rapid pectin degradation, while the highly induced expression of *pelD* is necessary for the maximum production of Pels during the infection in plant (Robert-Baudouy *et al.*, 2000, Ouafa et al., 2012). Furthermore, the transcription of *pelD* was monitored along with the growth in MM supplemented with PGA, and the results showed that the expression of *pelD* gene was only highly induced at the mid-log phase of growth, but not in the early log phase of growth (Fig. S6). This result suggested 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 $gcpA^{D418A}$ A-site mutant relative to the wild-type at 12 h, which corresponded to early log phase, while the expression levels of *pelE* were not altered (Fig. 1D, 1E and 1F). A recent study 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 implied that the GcpA-mediated c-di-GMP signaling pathway is involved in the mechanism of differentially expression of *pelD* and *pelE*.

The post-transcriptional regulatory system, RsmA/RsmB has been shown to regulate several virulence factors including Pel in soft-rot pathogens *D. dadantii* and *Pectobacterium carotovorum*, but how RsmA/RsmB regulates *pel* gene expression remains unclear (Yang et al., 2008, Chatterjee *et al.*, 1995, Mukherjee *et al.*, 1996). Deletion of *rsmA* was lethal to *D. dadantii* 3937, unlike previous results studying *P. carotovorum*. Therefore, we were not able to generate a *rsmA* mutant to examine its direct effect on Pel production. Instead, we observed that over-expression of *rsmA* significantly repressed the *pelD* expression and Pel production (data not shown). Nevertheless, our results demonstrated that GcpA regulates the expression of RsmB and RsmA (Fig. 2). This regulation was further determined to be essential for the GcpA-dependent Pel regulation since either deletion of *rsmB* or overexpression of *rsmA* in the $gcpA^{D418A}$ mutant drastically reduced its *pelD* promoter activity and Pel production (Fig. 2C, 2D and S5). In *P. aeruginosa*, the RsmY and RsmZ sRNAs sequester the mRNA-binding protein RsmA in a mechanism similar to the RsmA/RsmB system (Lapouge *et al.*, 2008). Several studies 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 signaling on 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 (Falconi *et al.*, 1998, Yu & DiRita, 2002, Castang *et al.*, 2008). In *D. dadantii*, it has been shown that H-NS positively regulates swimming motility and Pel production, but negatively regulates exopolysaccharides (EPS) synthesis (Nasser *et al.*, 2001). Our findings demonstrated that GcpA negatively regulates *hns* expression at the post-transcriptional level (Fig. 2A and S4). Strikingly, deletion of *hns* in the $gcpA^{D418A}$ mutant not only restored its *pelD* promoter activity and Pel production (Fig. 2E and 2F), 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. Besides directly functioning as a repressor of *pelD* gene expression, H-NS is also known to be a positive regulator for Pel synthesis due to its negative impacts on PecT production (Nasser & Reverchon, 2002). Since we did not observe significant changes of *pecT* expression in the $gcpA^{D418A}$ 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 behaviors in a sophisticated manner (Lindenberg *et al.*, 2013, Valentini et al., 2016). Our findings demonstrated that the Rsm system plays an essential

role in the c-di-GMP signaling and regulation of Pel production in *D. dadantii*. Although EGcpB, EcpC, and GcpA were shown to modulate the expression of *rsmB* at a post-transcriptional level (Fig. 2A and 4C), our results hinted 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 H-NS-RsmB-RsmA pathway (Fig. 7A). In *Acetobacter xylinum*, Qi and colleagues 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 (Qi *et al.*, 2009). Similarly, oxygen levels may play a role in modulating c-di-GMP metabolism in *D. dadantii*. EcpC, the sole-EAL domain protein, is very likely to modulate a different c-di-GMP pool that directly targets RsmB bypassing H-NS. This convergence and divergence in c-di-GMP signaling is also supported by the regulation of swimming motility and T3SS gene expression in *D. dadantii* (Fig. 7B and 7C). We showed here that GcpA and EGcpB inversely modulate swimming motility through H-NS, which is different from EcpC. It is worth noting that our data indicated that the regulatory mechanism of GcpA on T3SS gene expression might be different from EGcpB and EcpC, which previous reports had 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 $gcpA^{D418A}$ A-site mutant compared with the wild-type strain (Fig. S7). 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 the c-di-GMP-dependent regulation, the expression patterns and localizations of different DGCs and PDEs, and the c-di-GMP effectors that contribute to the signaling specificity on diverse cellular behaviors remain to be determined.

Overall, this multilevel regulation of c-di-GMP signaling on diverse virulence factors assures an accurate control of the virulence process for the infection of *D. dadantii*. It provides evidence on the complexity and specificity of c-di-GMP signaling 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 (see Supporting Information). *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% $MgSO_4$) or M9 minimal medium (MM) supplemented with 0.1% polygalacturonic acid (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 (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 *pecT* 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 polymerase chain reaction (PCR) with specific primers (Table S2). The kanamycin cassette was amplified from the pKD4 plasmid (Datsenko & Wanner, 2000) and was cloned between two flanking regions using three-way cross-over PCR. The PCR construct was inserted into the suicide plasmid pWM91, and the resulting plasmid was transformed into *D. dadantii* 3937 by conjugation using *E. coli* strain S17-1 λ-pir. Recombinants that grew on kanamycin medium, were plated on 10% sucrose plate to select strains with chromosomal deletions. Cells that were resistant to sucrose due to the loss of SacB-mediated toxicity were then plated on 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). Briefly, a primer set, *gcpA*-D418A-1 and *gcpA*-D418A-2 (Table S2), was used to generate $gcpA^{D418A}$, in which the SGDEF motif was changed to SGAEF. Substitution was confirmed by sequencing. The $gcpA^{D418A}$ fragment was then amplified using primer set, *gcpA*-A-SacI and *gcpA*-B (Table S2), and cloned upstream to the kanamycin cassette followed by the downstream fragment flanking *gcpA* using three-way cross-over PCR. The construct was inserted into pWM91, and the resulting plasmid was transferred into *D. dadantii* by conjugation using *E. coli* strain S17-1 λ-pir. The above described allelic exchange mutagenesis was conducted to replace wild-type *gcpA* with $gcpA^{D418A}$. Mutation was confirmed by sequencing using outside primers.

To construct double mutants, *rsmB*, *hns*, *egcpB*, and *ecpC*, were allelic exchanged in a $gcpA^{D418A}$ unmarked mutant strain, respectively. In brief, the pFLP2 plasmid encoding FLP (flipase) recombinase enzyme was transferred into the $gcpA^{D418A}$::Km strain by conjugation using *E. coli* S17-1 λ-pir. Two FLP recombinase target (*FRT*) sites flanking the kanamycin cassette allowed for flipase mediated excision of Km. Transconjugants that were sensitive to Km and high-concentration of sucrose, were then confirmed using outside primers and sequencing. To generate complemented strains, the promoter and 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 inoculating 10 μl of overnight bacterial cultures (OD_{600}=1.0) onto the center of MG plates containing 0.2% agar. The inoculated plates were incubated at 28°C for 16 h. The diameter of the radial growth was measured (Antúnez-Lamas *et al.*, 2009).

Pel activity assay

Extracellular Pel activity was measured by spectrometry as previously described (Matsumoto *et al.*, 2003). Bacterial cells were cultured in MM media supplemented with 0.1% PGA at 28°C for 16 h. 1 ml bacterial cultures were then centrifuged at 15,000 rpm for 2 min, and the supernatant was collected. 10 μl of supernatant was added to 990 μl of the reaction buffer (0.05% PGA, 0.1 M Tris-HCl [pH 8.5], and 0.1 mM $CaCl_2$, prewarmed to 30°C). Pel activity was monitored at A_{230} for 3 min and calculated based on one unit of Pel activity being equal to an increase of 1×10^{-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 (Miller *et al.*, 2000, Leveau & Lindow, 2001). The reporter plasmids pAT-*pelD*, pAT-*hrpA* and pAT-*rsmB* was constructed previously following the same procedure (Li *et al.*, 2015, Peng *et al.*, 2006, Yang et al., 2007). Promoter activity was monitored by measuring GFP intensity through flow cytometry (BD Biosciences, San Jose, CA) as previously described (Peng et al., 2006). Briefly, bacterial cells with the reporter plasmid were grown in LB media overnight and inoculated 1:100 into MM media with or without 0.1% PGA. Samples were collected at 6 h, 12 h and 24 h, respectively, and promoter activity was quantified by detecting GFP intensity using flow cytometry.

Determination of the intracellular c-di-GMP concentration

Intracellular c-di-GMP concentrations were determined by using ultra performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS-MS) using a method that has been previously described (Massie *et al.*, 2012). Briefly, overnight bacterial cultures were inoculated 1:1000 into 50 ml LB media in a flask. After the OD_{600} of the bacterial culture 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 4,000 rpm. The supernatant was then removed, and the pellet was re-suspended in 1.5 ml extraction buffer (40% acetonitrile-40% methanol in 0.1 N formic acid). To lyse the cells and release intracellular c-di-GMP, cells re-suspended in extraction buffer were dried by speed-vac, resuspended in 100 μL 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 (PVDF, 0.45 μm, 4 mm), and analyzed by UPLC-MS-MS.

Western blot analysis

D. 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) buffer and lysed by sonication. The protein in crude lysates was quantified using the Bradford protein assay (Bio-Rad). Samples were boiled before loading onto 12% sodium dodecyl sulfate polyacrylamide gels. Proteins were then transferred onto a polyvinylidene fluoride membrane (Millipore). Blots were washed with PBS containing 0.05% Tween-20 and probed with an anti-RsmA antibody (Proteintech,

Rosemont, IL). Anti-RNA polymerase monoclonal antibody (Neoclone) was used as a control. The resulting blots were incubated for 1 min in enhanced chemiluminescence reagent (GE Healthcare) and detected using O-MAT X-ray film.

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). The residual DNA was removed with a Turbo DNA-free DNase kit (Invitrogen, Ausin, TX). Northern blot analysis was performed using biotin-labelled probe and a biotin detection system (BrightStar Psolaren-Biotin and Bright Star BioDetect, Ambion). 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 PureLink RNA Mini Kit (Ambion, Carlsbad, CA) according to the manufacturer's instruction. On-column DNase treatment (Invitrogen, Carlsbad, CA) 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). The relative levels of gene expression were determined by using the $2^{-\Delta\Delta CT}$ method (Livak & 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 (Yuan et al., 2015). In brief, 10 μl of bacterial suspension at 10^7 CFU ml^{-1} 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 growth chamber at 28°C with 100% relative humidity for 16 h before pictures were taken. To evaluate disease symptoms, APS 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) or Fisher's Lease Significant Difference (LSD) test using DPS data processing system (http://www.dpsw.cn/dps_eng).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Xiaochen Yuan was supported by the Postdoctoral Workstation of Jiangsu Academy of Agricultural Sciences. This work was funded by United States Department of Agriculture-National Institute of Food and Agriculture-Agriculture and Food Research Initiative-Exploratory Research program (2016-67030-24856) and Research Growth Initiative of the University of Wisconsin-Milwaukee awarded to Ching-Hong Yang; National Key Research and Development program of China (2017YFC200604) awarded to Fengquan Liu; China Scholarship Council (201503250007) awarded to Fang Tian; National Science Foundation of China (31370160; 31671990) awarded to Chenyang He; National Institutes of Health grants GM109259 awarded to Christopher M. Waters.

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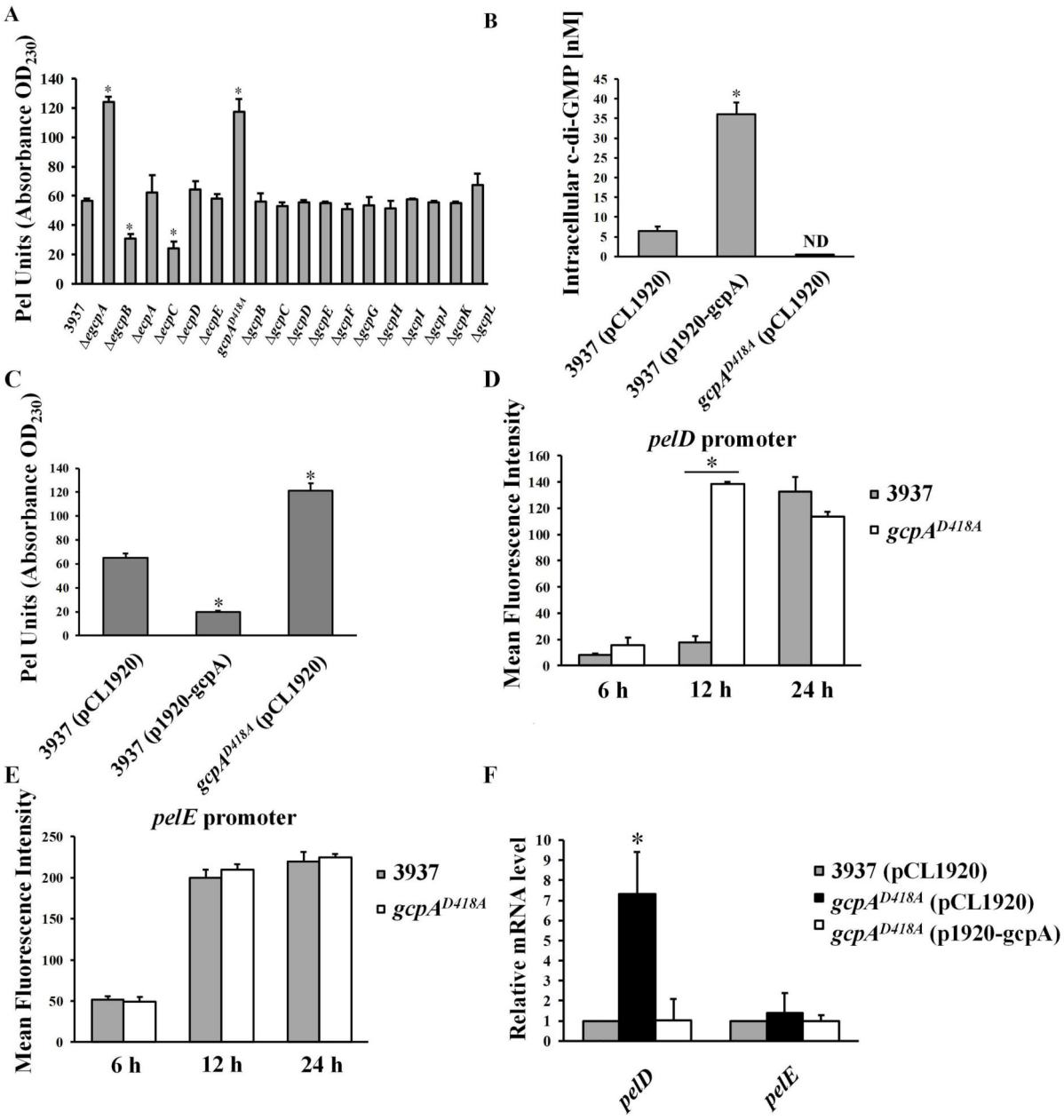

Fig. 1.

GcpA synthesizes c-di-GMP to negatively regulate Pel production and *pelD* gene expression in *D. dadantii*. (A) Pel production of wild-type *D. dadantii* and GGDEF and/or EAL deletion mutant strains cultured in MM+0.1% PGA for 12 h at 28°C. (B) Measurement of intracellular c-di-GMP and (C) Pel production in wild-type *D. dadantii* harboring empty vector pCL1920, wild type harboring pCL1920-*gcpA* and $gcpA^{D418A}$ harboring pCL1920-*gcpA* strains. The *pelD* (D) and *pelE* (E) promoter activities were measured in the parental strain *D. dadantii* and $gcpA^{D418A}$. Cells cultured in MM+0.1% PGA were harvested at 6, 12 and 24 h respectively to measure the mean fluorescence intensity (MFI) by flow cytometry.

(F) Quantitative RT-PCR (qRT-PCR) analysis of mRNA levels of *pelD* and *pelE* in *D. dadantii* strains. The data represent expression levels of each gene relative to that in the wild type, which was mathematically designated as 1. *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).

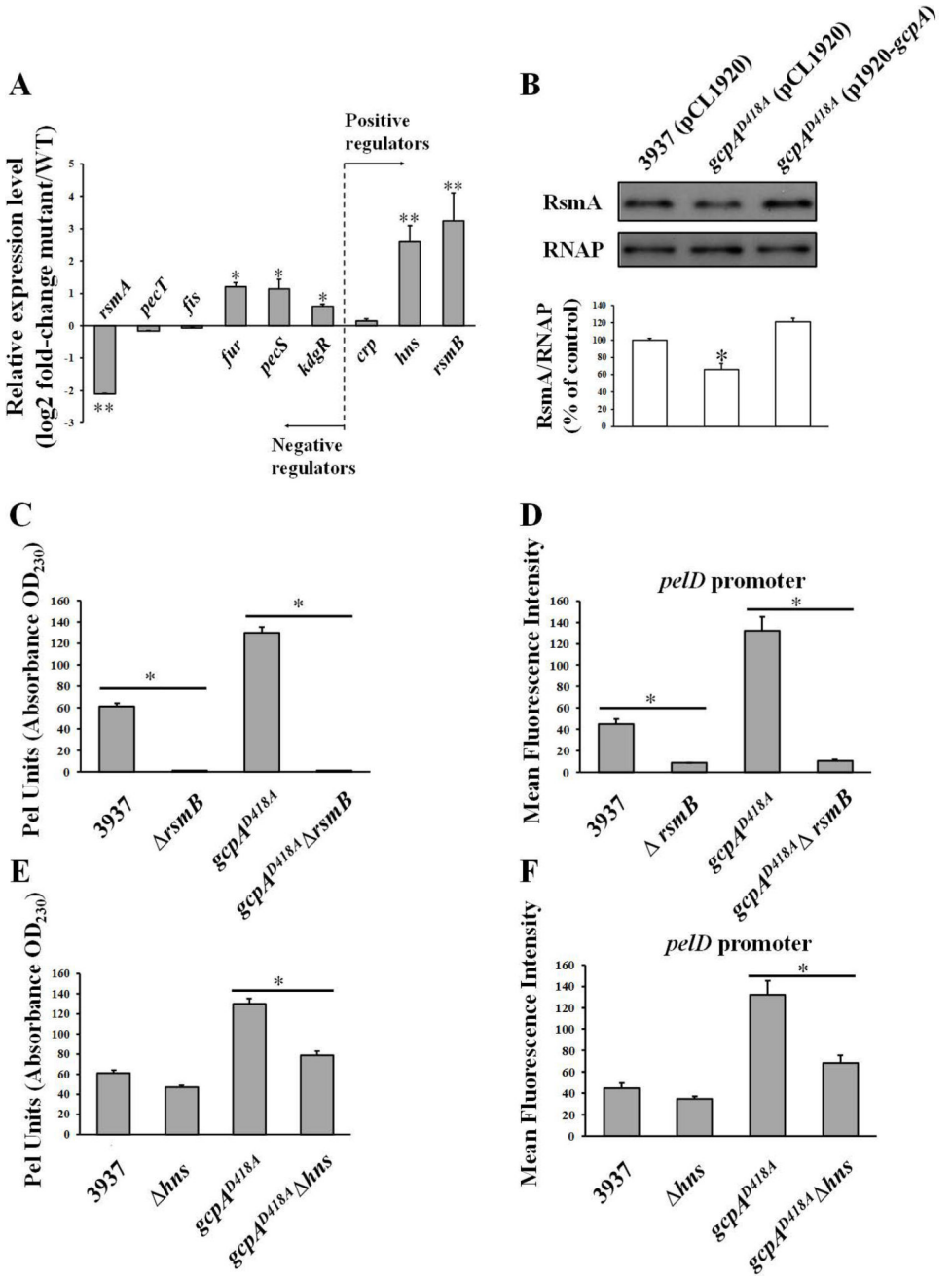

Fig. 2.

RsmB and H-NS play important roles in the GcpA-dependent Pel regulation. (A) Quantitative RT-PCR analysis of RNA levels of *rsmA*, *pecT*, *fis*, *fur*, *pecS*, *kdgR*, *crp*, *hns* and *rsmB* in *D. dadantii* wild type and $gcpA^{D418A}$. The mutant/WT ratio for each gene expression was calculated as described in the Experimental Procedures. (B) Western blot analysis of RsmA protein in *D. dadantii* strains. Pel production (C and E) and *pelD* promoter activities (D and F) were tested in *D. dadantii* strains. Values are a 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).

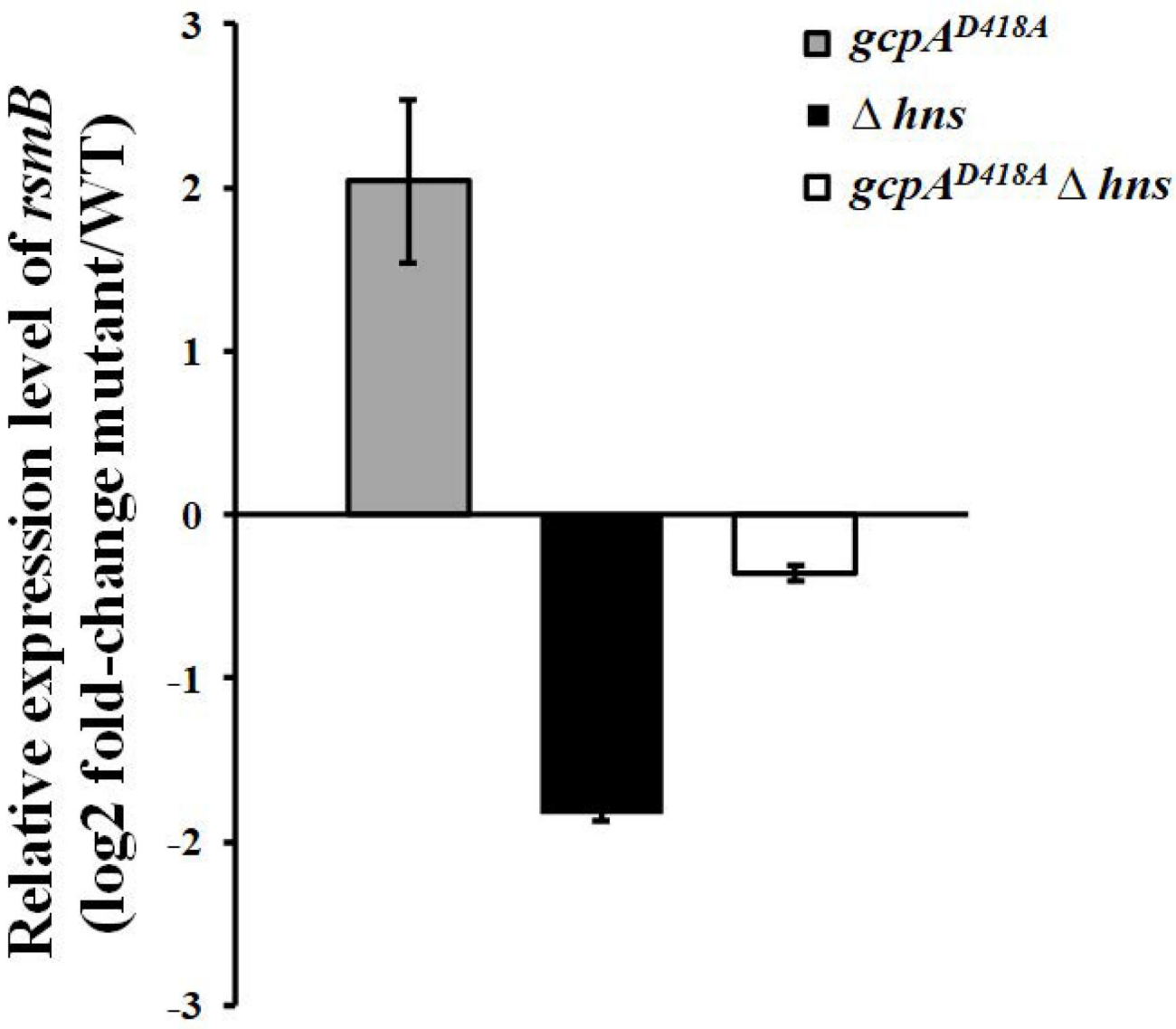

Fig. 3.

H-NS is involved in the GcpA-dependent regulation on *rsmB*. *rsmB* RNA levels were examined in *D. dadantii* using qRT-PCR. The mutant/wild-type ratio for *rsmB* gene expression was calculated as described in the 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).

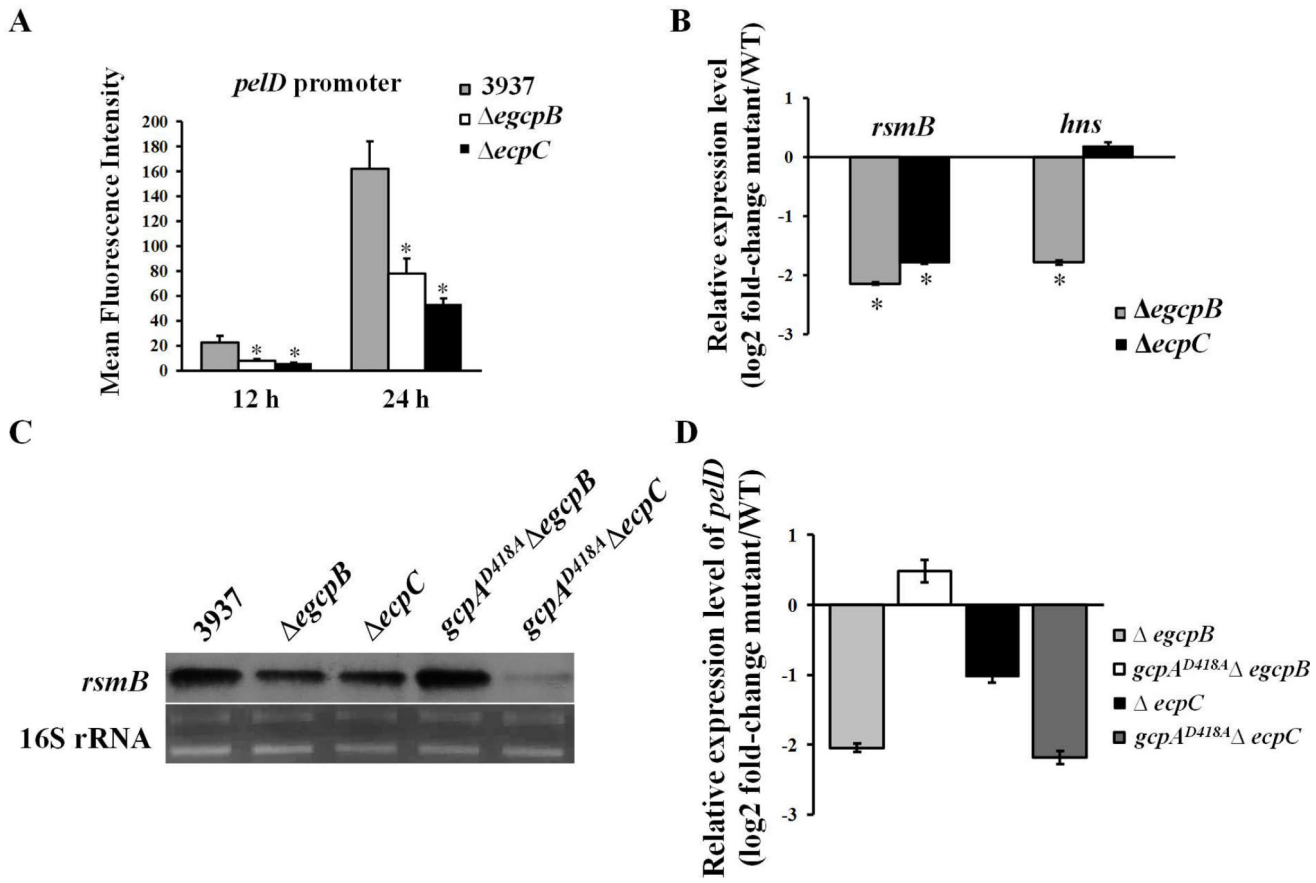

Fig. 4.

EGcpB and EcpC positively regulate *pelD* gene expression via different pathways. (A) The promoter activity of *pelD* was examined in *D. dadantii*. (B) RNA levels of *rsmB* and *hns* were examined using qRT-PCR. The mutant/WT ratio of each gene was calculated as described in the Experimental Procedures. (C) Northern blot analysis of *rsmB* mRNA in *D. dadantii* strains. (D) Relative mRNA levels of *pelD* in mutant strains to that in the wild-type strain. Values are a 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).

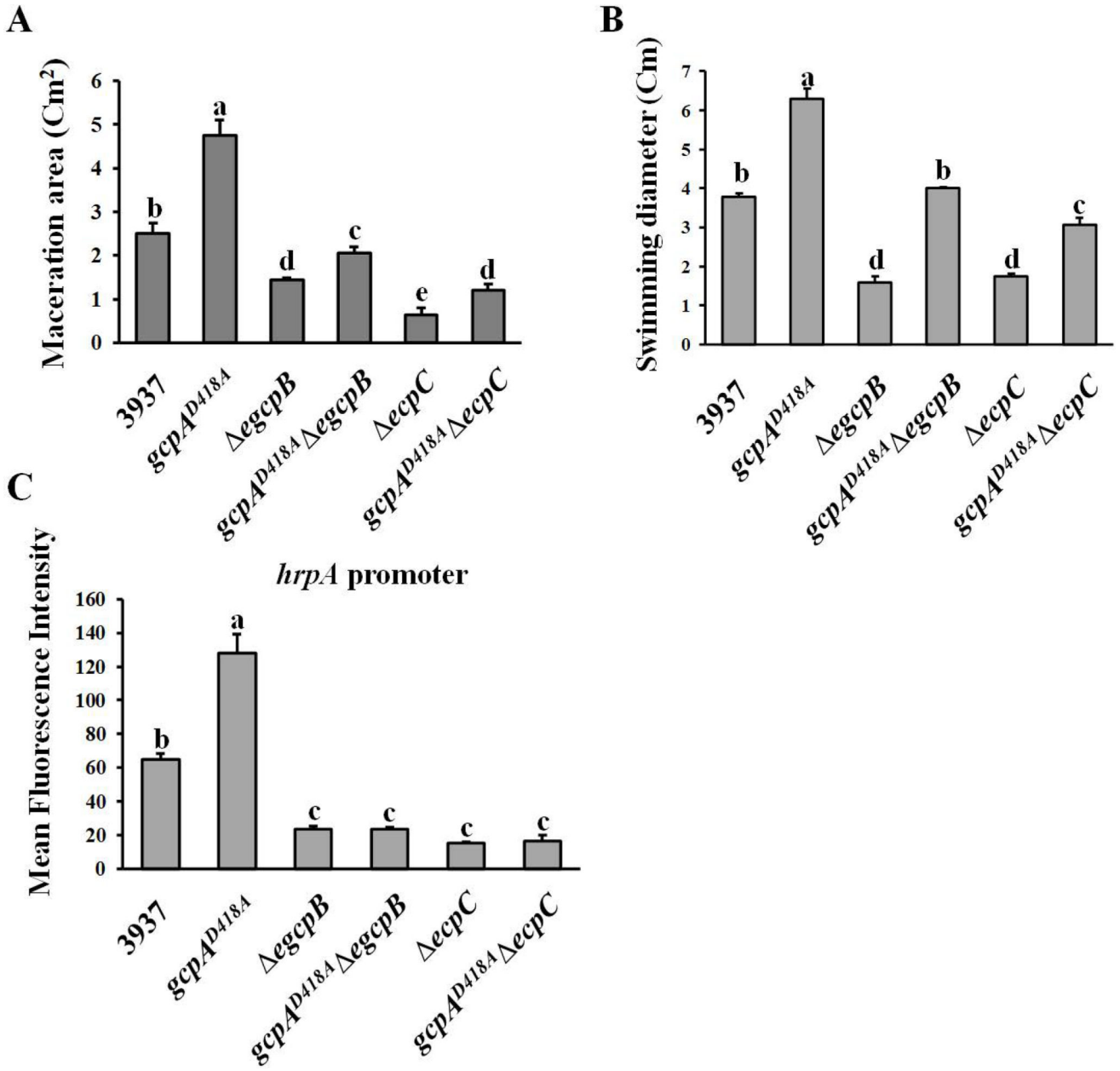

Fig. 5.

Effects of GcpA on swimming motility, T3SS gene expression and virulence. (A) Bacterial cells of *D. dadantii* were inoculated in the leaves of Chinese cabbage (*Brassica campestris*). The maceration areas were measured 16 h post-inoculation. The swimming motility (B), T3SS gene *hrpA* promoter activity (C) were examined. Values are a 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 bar indicate statistically significant differences between treatments (Fisher's LSD, $P<0.05$).

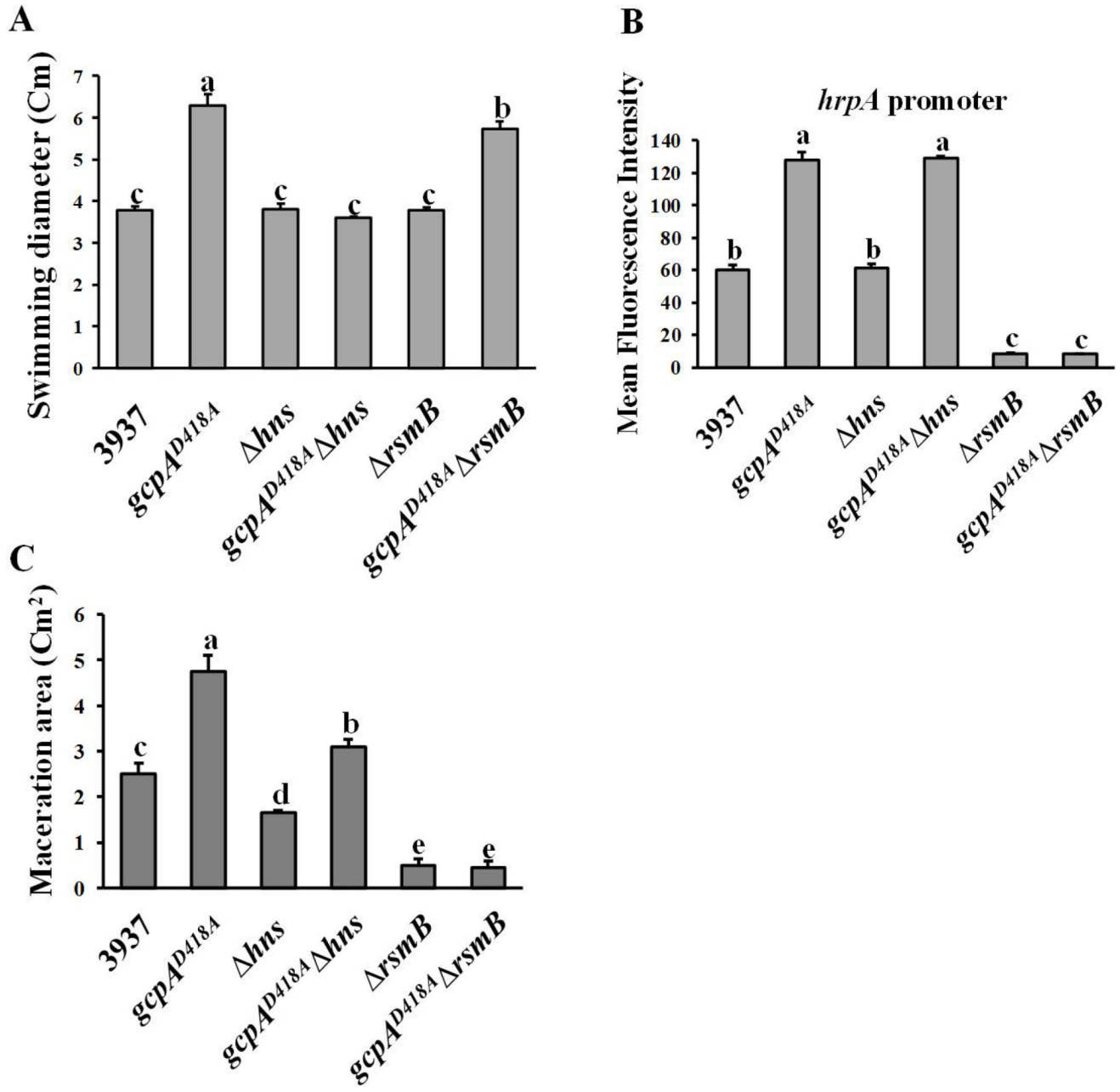

Fig. 6.

Effects of H-NS and RsmB on swimming motility, T3SS gene expression, and virulence. The swimming motility (A), T3SS gene *hrpA* promoter activity (B) and maceration on the leaves of Chinese cabbage (C) were examined. Values are a 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 bar indicate statistically significant differences between treatments (Fisher's LSD, $P<0.05$).

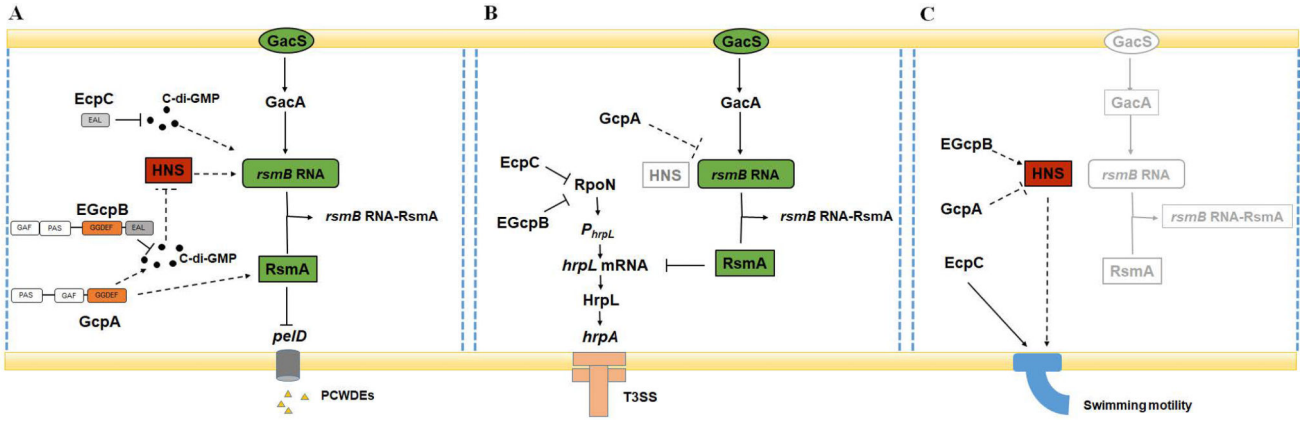

Fig. 7.

Working model for the c-di-GMP signaling pathway in *D. dadantii*.

(A) The regulation of c-di-GMP signaling on Pel production in *D. dadantii* is complex, involves several Gcp and Ecp proteins and takes place at both transcriptional and post-transcriptional levels. Rsm system is a central component in the c-di-GMP-related Pel regulation. GcpA and EGcpB modulate the same c-di-GMP pool to control *pelD* gene expression through 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 post-transcriptional level. (B) The regulation of GcpA on T3SS gene expression is dependent on its impact on RsmB, which controls the expression of *hrpL* at post-transcriptional level. This regulation is different from EGcpB and EcpC that regulate *hrpL* at transcriptional level via RpoN-*hrpL* pathway. (C) GcpA and EGcpB regulate swimming motility through H-NS, while EcpC is different from them. ⊥represents negative control; →represents positive control. The dotted lines indicate regulatory mechanisms identified in this study.