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The Pyocin Regulator PrtR Regulates Virulence Expression of *Pseudomonas aeruginosa* by Modulation of Gac/Rsm System and c-di-GMP Signaling Pathway

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ABSTRACT In *Pseudomonas aeruginosa*, the second messenger cyclic-di-GMP and Gac/Rsm signaling pathways are associated with the transition from acute to chronic infection. Therefore, identification of the molecular mechanisms that govern lifestyle choice in bacteria is very important. Here, we identified a novel cyclic-di-GMP modulator, PrtR, which was shown to repress pyocin production by inhibition of PrtN and activate the type III secretion system (T3SS) through PtrB. Compared to a wild-type strain or a *prtN* mutant, the *prtR prtN* double mutant exhibited a wrinkly colony and hyperbiofilm phenotype, as well as an increase in intracellular c-di-GMP levels. Interestingly, a diguanylate cyclase (DGC) gene, *siaD*, was repressed by PrtR. Further experiments revealed that PrtR directly interacts with SiaD and facilitates the accumulation of c-di-GMP in cells. We also demonstrated that PrtR regulates the activity of the Gac/Rsm system, thus affecting expression of the T3SS and type VI secretion system (T6SS) and the formation of biofilm. Taken together, the present findings indicate that PrtR, as a c-di-GMP modulator, plays key roles in the adaptation to opportunistic infection of *P. aeruginosa*. Additionally, this study revealed a novel mechanism for PrtR-mediated regulation of the lifestyle transition via the Gac/Rsm and c-di-GMP signaling networks.

KEYWORDS cyclic di-GMP, pyocin regulator PrtR, motile-sessile switch, Gac/Rsm system, *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a ubiquitous and opportunistic pathogen responsible for human infections in hospitalized and cystic fibrosis (CF) patients. In general, *P. aeruginosa* can form matrix-encased and surface-associated communities known as biofilms (1). In a biofilm, bacteria are surrounded by an extracellular matrix composed of exopolysaccharides, extracellular DNA, and proteins. Several exopolysaccharides have been described in *P. aeruginosa*, including Pel, which is a glucose-rich polymer (2–4), and Psl, which is a polymer with a repeating pentamer that contains D-mannose, L-rhamnose, and D-glucose (5).

The development of biofilms and the switch from acute to chronic infections involve multicellular processes that are associated with environmental signals and regulated by complex regulatory networks (6). In many bacterial species, the intracellular signaling molecule c-di-GMP stimulates the synthesis of biofilm matrix components, and generally, cells with elevated levels of intracellular c-di-GMP form thicker and more robust biofilms (7–9). In *P. aeruginosa*, c-di-GMP is required for the synthesis of two different extracellular polysaccharides, Psl and Pel (8, 10). c-di-GMP also influences

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CdrA, which is the extracellular matrix adhesin protein (10) and modulates the production of alginate (11). These phenotypes are also regulated by the Gac/Rsm signaling pathway (12, 13). The Gac/Rsm system includes two histidine kinases, RetS and LadS, which exert opposing influences on GacS histidine kinase (12–14). On the other hand, this system controls the transcription of two small regulatory RNAs, RsmY and RsmZ, that antagonize the activities of RsmA to control the expression of multiple genes, including *pel* and *psl* (13, 15, 16). Recently, it was shown that a mutation in *retS* leads to an increase in intracellular c-di-GMP levels, which indicates that the Gac/Rsm and c-di-GMP signaling networks interact (17). It has also been shown that a diguanylate cyclase (DGC) protein, SadC, is a central player in Gac/Rsm-mediated biofilm formation in *P. aeruginosa* (18). Thus, these two networks play critical roles in modulating the transition from a planktonic to biofilm state (19–21).

More than 90% of *P. aeruginosa* strains can produce bacteriocins called pyocins (22). The production of pyocins is tightly regulated by PrtR, which is a homologue of λ CI that represses the expression of *prtN* by directly binding to its promoter region (23). Upon activation of the bacterial SOS response, pyocin production is induced by DNA-damaging agents such as UV light and mitomycin C. Furthermore, RecA promotes the cleavage of PrtR, which leads to the upregulation of PrtN and the subsequent activation of pyocin genes (23). It has also been reported that hydrogen peroxide (H₂O₂) and ciprofloxacin stimulate the expression of pyocin genes (24, 25), presumably by causing DNA damage and repressing the activity of DNA topoisomerase, which would lead to the SOS response and the cleavage of the PrtR protein, respectively (26).

In addition to regulation of pyocin production, PrtR regulates the expression of the type III secretion system (T3SS) and plays an important role in *P. aeruginosa* infections (26, 27). However, the biological roles and regulatory mechanisms underlying PrtR activity remain elusive. The present study demonstrates that mutation of *prtR* and *prtN* resulted in wrinkly colony and hyperbiofilm phenotypes compared to the Δ *prtN* strain. It was also revealed that PrtR targeted SiaD to modulate c-di-GMP concentrations. Moreover, our data show that PrtR regulated the Gac/Rsm system. Taken together, these results indicate that Gac/Rsm and c-di-GMP signaling are required for PrtR-mediated inverse regulation and reveal one of the molecular mechanisms underlying the role of PrtR in the motile-sessile switch in *P. aeruginosa*.

RESULTS

PrtR controlled biofilm formation and bacterial motility in *P. aeruginosa*. PrtR is involved in regulation of pyocin production and T3SS expression (27), which led us to investigate the effect of PrtR on other virulence factors, such as biofilm formation and bacterial motility. It has been shown that the growth rate of the Δ *prtR* mutant is much lower than that of the wild-type PAO1 strain (27), and thus, Δ *prtN* Δ *prtR* and Δ *prtN* mutant strains were generated because their growth rates were similar to that of the wild-type parent strain (see Fig. S1A in the supplemental material). To investigate whether PrtR would affect biofilm formation and structure, the biofilms of the Δ *prtN* Δ *prtR* double mutant were compared with those of the wild-type parent and Δ *prtN* single mutant. Consistent with previous reports (28), wild-type PAO1 produced typical mushroom-shaped microcolonies (Fig. 1A). However, the Δ *prtN* Δ *prtR* mutant expressed large amounts of Psl and produced taller tower-like structures than those produced by the wild-type strain and Δ *prtN* mutant, which indicates that lack of *prtR* contributed to tower formation. Additionally, the induction of *prtN* and *prtR* in the Δ *prtN* Δ *prtR* strain appeared to complement the biofilm phenotype (Fig. 1A). A COMSTAT analysis revealed that the Δ *prtN* Δ *prtR* strain significantly increased biofilm biomass and microcolony height compared to the Δ *prtN* mutant and wild-type parent (Fig. S1B). These results suggest that PrtR is involved in the regulation of biofilm structure and production.

In *P. aeruginosa*, the regulation of biofilm formation and swarming motility is inversely regulated (29). Because there was enhanced biofilm production in the Δ *prtN* Δ *prtR*

FIG 1 PrtR controls biofilm formation and bacterial motility of *P. aeruginosa*. (A) Confocal scanning laser microscopy of 72-h biofilms grown in flow cell chambers. White scale bars represent 50 μ m. Green fluorescence by SYTO 9 stain indicates biomass, while red staining represents antibody detection of Psl. Error bars indicate the mean $\pm$ SD from three biological replicates. (B) Deletion of *prtN* and *prtR* reduces bacterial swimming and swarming motility. (C) Swimming diameters of the indicated strains. Error bars indicate the mean $\pm$ SD from three independent experiments: **, $P < 0.01$ based on Student's *t* test.

mutant, whether PrtR would influence bacterial motility was investigated. The deletion of *prtR* and *prtN* resulted in reduced swimming and swarming motility (Fig. 1B and C). Taken together, these results indicate that PrtR is an important regulator of *P. aeruginosa* virulence-associated phenotype.

Psl, which is a neutral polysaccharide, contributes to biofilm formation and the maintenance of chronic infections, and its elimination in mucoid strains leads to abolishment of microcolony structure (30, 31). Next, the effects of PrtR on *PpsIA* promoter activity were measured *in vitro* using reporter-*lux* construction. Thus, a genetic cassette carrying the *psIA-lux* fusion was introduced into the wild-type PAO1 strain and $\Delta prtN$ and $\Delta prtN \Delta prtR$ mutant strains, and promoter activity was measured along the growth curve. As shown in Fig. S1C, *psIA* expression was elevated in the $\Delta prtN \Delta prtR$ mutant, whereas complementation with a *prtR* gene restored *psIA* promoter activity. These results suggest that deletion of *prtN* and *prtR* activates *psl* expression.

Deletion of *prtR* and *prtN* resulted in a wrinkly colony morphology and elevated intracellular c-di-GMP levels. Since PrtR controls biofilm production and represses *psIA* activity, it was hypothesized that the role of PrtR in virulence would be more expansive than has been previously described. Observation of growth on Congo red agar plates revealed that the $\Delta prtN \Delta prtR$ mutant formed wrinkled colonies that contrasted with the smooth appearances of colonies of the wild-type PAO1 and $\Delta prtN$ mutant strains (Fig. 2A); chromosomal complementation of the $\Delta prtN \Delta prtR$ mutant returned the colony morphology. This phenotype is different than those of $\Delta wspF$ or $\Delta amrZ$ mutants, which form an aggregated rugose small colony variant (RSCV) morphology (10, 28). Indeed, unlike the $\Delta wspF$ strain, which exhibits clumps in liquid culture, both the $\Delta prtN$ and $\Delta prtN \Delta prtR$ mutants displayed a similar growth phenotype to the wild-type parent strain (Fig. 2B).

It is well known that this colony phenotype is associated with c-di-GMP production.

FIG 2 Mutation of *prtN* and *prtR* exhibits increased intracellular c-di-GMP levels. (A) Congo red binding of the indicated strains after 2 days of incubation and growth on Congo red plates. The wrinkly phenotype was observed in the $\Delta prtN \Delta prtR$ mutant but not in the parental PAO1 strain or $\Delta prtN$ mutant. (B) The $\Delta prtN$ and $\Delta prtN \Delta prtR$ mutants displayed similar growth to the wild-type parent but were different from the $\Delta wspF$ mutant. The photos represent growth of these strains in LB for 12 h. (C) The expression of *cdrA-lux* was increased in the $\Delta prtN \Delta prtR$ mutant compared to that in the $\Delta prtN$ mutant and wild-type parent. (D) Measurement of c-di-GMP by LC-MS/MS indicated that the $\Delta prtN \Delta prtR$ double mutant produced more intracellular c-di-GMP than the wild-type PAO1 strain or $\Delta prtN$ mutant. Error bars indicate the mean $\pm$ SD from three biological replicates, and statistical significance was calculated by one-way analysis of variance (ANOVA) with Dunnett's multiple-comparison test: ***, $P < 0.001$.

For example, the wrinkled phenotype of the $\Delta wspF$ strain has been attributed to the loss of repression of the diguanylate cyclase WspR, which leads to elevated levels of intracellular c-di-GMP (10). Therefore, it was hypothesized that the $\Delta prtN \Delta prtR$ mutant would display a wrinkled phenotype due to elevated intracellular c-di-GMP levels. To verify this hypothesis, c-di-GMP levels were monitored in these strains using the *PcdrA-lux* reporter fusion, which is responsive to intracellular levels of c-di-GMP in *P. aeruginosa* (32). The $\Delta prtN \Delta prtR$ mutant displayed elevated levels of c-di-GMP compared to the wild-type and $\Delta prtN$ strains and could be complemented by introducing the *prtR* and *prtN* genes in *trans* (Fig. 2C). Additionally, c-di-GMP concentrations were measured in these strains using liquid chromatography with tandem mass spectrometry (LC-MS/MS); the $\Delta prtN \Delta prtR$ mutant accumulated c-di-GMP levels that were nearly 2-fold those of parental wild-type PAO1 (Fig. 2D). These data are consistent with the present observation that the $\Delta prtN \Delta prtR$ mutant has a wrinkled phenotype.

PrtR directly interacted with SiaD. Given that the intracellular c-di-GMP is synthesized by the diguanylate cyclases (DGCs), we next determined whether the expression of DGCs was regulated by PrtR. To this end, mRNA levels of all DGCs were determined by quantitative real-time PCR (qRT-PCR), and the data showed that PrtR strongly repressed the expression of *siaD* (Fig. 3A), which encodes a DGC that is involved in SDS-induced aggregation and biofilm formation in *P. aeruginosa* (33, 34). To determine whether PrtR would directly control *siaD* promoter activity, electrophoretic mobility shift assays

FIG 3 Interaction between PrtR and SiaD. (A) Relative RNA levels of *siaD* in the wild-type PAO1 strain, the $\Delta prtN$ and $\Delta prtN \Delta prtR$ mutants, and the complemented strains were tested. Bacteria were grown to an OD_{600} of 1.0, and RNA samples were collected and analyzed by real-time PCR. Error bars indicate the mean $\pm$ SD from three biological replicates, and statistical significance was calculated by one-way ANOVA with Dunnett's multiple-comparison test: ***, $P < 0.001$. (B) The *E. coli* two-hybrid assay reveals an interaction between PrtR and SiaD. The recombinant strains harboring the indicated proteins were separately streaked on nonselective medium and dual-selective medium (3-amino-1,2,4-triazole plus streptomycin). The strain expressing LGF2 and Gal11p or RetS and PA1611 was used as a positive control. (C) *In vitro* pull-down of PrtR by GST and GST-SiaD.

(EMSAs) were conducted with an *siaD* promoter and purified PrtR. No band shift was observed in the EMSA experiments, and as a positive control, PrtR efficiently bound to the promoter region of *prtN* (see Fig. S2A in the supplemental material).

Given that PrtR did not directly bind to *siaD*, we hypothesized that PrtR might directly interact with the SiaD protein. To validate this hypothesis, the bacterial adenylate cyclase-based two-hybrid (BACTH) system (35), which has been used to detect the protein-protein interactions in bacteria, was employed as described previously (36, 37). To perform this experiment, *prtR* and *siaD* genes were fused to plasmids pBT and pTRG, respectively. Then, the resulting plasmids were cotransformed into the reporter strain *Escherichia coli* BTH101. Like the positive strains (bearing pTRG-Gal11p plus pBT-LGF2 or pBT-RetS plus pTRG-PA1611), the strain bearing pBT-PrtR and pTRG-SiaD grew on a dual-selective medium (Fig. 3B). A pull-down assay was conducted to confirm the interaction between PrtR and SiaD *in vitro*. Glutathione *S*-transferase (GST)-SiaD was able to retain PrtR but not GST alone (Fig. 3C), which is indicative of a direct interaction between PrtR and SiaD.

The PrtR-controlling hyperbiofilm phenotype was c-di-GMP-dependent. The present data clearly demonstrated that the *prtR*- and *prtN*-deficient strain formed larger biofilms than those formed by the parental wild-type stain (Fig. 1A). Furthermore, PrtR

FIG 4 Deletion of *siaD* abrogates the hyperbiofilm phenotype and reduces high c-di-GMP levels of a $\Delta prtN \Delta prtR$ mutant. (A) Confocal scanning laser microscopy of 72-h biofilms of the indicated strains grown in flow cell chambers. Green fluorescence by SYTO 9 stain indicates biomass. Experiments were performed in triplicate, and a representative image is shown. Scale bars, 50 μm . (B) *SiaD* contributes to increase of c-di-GMP in the $\Delta prtN \Delta prtR$ mutant. Direct LC-MS/MS measurement of c-di-GMP showed that deletion of *siaD* in the $\Delta prtN \Delta prtR$ mutant produced smaller amounts of c-di-GMP. (C) Overexpression of PA2133 or deletion of *sadC* abrogates the hyperbiofilm of the $\Delta prtN \Delta prtR$ mutant. Biofilm formation by the indicated strains was displayed with crystal violet staining (top) and quantified by optical density measurement (bottom). Each data point represents the average from three biological replicates performed in triplicate. Statistical significance was calculated by one-way ANOVA with Dunnett's multiple-comparison test: **, $P < 0.01$; ***, $P < 0.001$. "EV" represents the empty vector.

regulated intracellular c-di-GMP levels (Fig. 2C and D) and thus provided an additional control of biofilm formation. Therefore, it was hypothesized that the hyperbiofilm phenotype in the $\Delta prtN \Delta prtR$ mutant was due to derepression of *siaD* and the c-di-GMP accumulation in this strain. Because *siaD* was upregulated, it was tested whether a mutation in *siaD* would result in the suppression of the $\Delta prtN \Delta prtR$ mutant hyperbiofilm phenotype. To accomplish this, a $\Delta prtN \Delta prtR \Delta siaD$ mutant was engineered and cultured in a flow chamber system for 72 h to induce biofilm formation. The $\Delta prtN \Delta prtR \Delta siaD$ triple mutant produced biofilms with less biomass and a lower microcolony height than those of the $\Delta prtN \Delta prtR$ double mutant (Fig. 4A). Additionally, the $\Delta prtN \Delta prtR \Delta siaD$ mutant produced significantly lower levels of c-di-GMP than the $\Delta prtN \Delta prtR$ strain (Fig. 4B). These observations were consistent with the COMSTAT analysis (Fig. S2B). Taken together, these results suggest that the hyperbiofilm phenotype of the $\Delta prtN \Delta prtR$ mutant was due to *siaD* derepression and elevated intracellular c-di-GMP concentrations.

We also reasoned that if the hyperbiofilm phenotype of the $\Delta prtN \Delta prtR$ mutants was due to elevated c-di-GMP levels, then reducing intracellular c-di-GMP in these strains via overexpression of a phosphodiesterase (PDE) or the deletion of a DGC should return biofilm formation to $\Delta prtN$ levels. To address this, a plasmid containing PDE PA2133 (p-PA2133) was transformed into the $\Delta prtN \Delta prtR$ mutant, and the biofilm phenotype was examined using crystal violet (CV) staining. The overexpression of a

FIG 5 PrtR regulates the expression of *hcp1* and the Gac/Rsm system. (A and B) The promoter activities of *hcp1*, *gacA* and *gacS*, and *rsmY* and *rsmZ* were measured in the wild-type PAO1 strain, Δ prtN and Δ prtN Δ prtR mutants, and complemented strains. (C) The protein levels of GacA and GacS are repressed by PrtR but not influenced by SiaD. Protein fractions from the indicated strains were prepared, and proteins were detected by Western blot analysis using anti-Flag. Data are representative of three independent replications. Error bars indicate the mean $\pm$ SD from three independent experiments. Statistical significance was calculated by one-way ANOVA with Dunnett's multiple-comparison test: **, $P < 0.01$; ***, $P < 0.001$. "EV" represents the empty vector.

PDE or the mutation of *sadC* significantly reduces biofilm formation (Fig. 4C), which further supports the hypothesis that the altered biofilm phenotype of the Δ prtN Δ prtR mutant was due to elevated c-di-GMP contents.

Deletion of *prtN* and *prtR* activated the expression of T6SS. The activation of T3SS and T6SS can be regulated by intracellular c-di-GMP contents (18). Given that mutation of *prtN* and *prtR* resulted in increased levels of c-di-GMP (Fig. 2D) and the repression of T3SS (27), it was hypothesized that PrtR might regulate T6SS activity. *hcp1*, a core structure gene of the H1-T6SS, forms a tail tube for effectors to deliver the H1-T6SS (38). Therefore, the expression of *hcp1* was monitored in the wild-type PAO1 strain, the Δ prtN and Δ prtN Δ prtR mutants, and their complemented strains using the *hcp1-lux* reporter fusion. As shown in Fig. 5A, the Δ prtN Δ prtR mutant displayed elevated transcriptional levels of *hcp1*, but the introduction of either plasmid p-*prtR* or p-*prtR*/*prtN* into the Δ prtN Δ prtR mutant restored *hcp1* activity to wild-type levels. These data indicate that PrtR appeared to be involved in the control of T6SS activity.

PrtR repressed the Gac/Rsm system. Since the Gac/Rsm pathway mainly regulates T3SS and T6SS genes, we next examined the effect of PrtR on Gac/Rsm activity. As expected, the promoter activities of *GacS* and *GacA* were increased in the Δ prtN Δ prtR mutant (Fig. 5B). Similarly, a Western blot assay showed that the protein levels of *GacS* and *GacA* were more abundant in the Δ prtN Δ prtR mutant than in the Δ prtN strain (Fig. 5C). Additionally, the promoter activity of two small RNA (sRNA) genes, *rsmY* and *rsmZ*, was also upregulated in the Δ prtN Δ prtR strain. Complementation of *prtR* or *prtN* *prtR* restored the expression of *gacS* *gacA* and *rsmY* *rsmZ* to the wild-type levels (Fig. 5B). These data indicate that PrtR is involved in regulation of the Gac/Rsm system.

PrtR-mediated modulation of T3SS, T6SS, and biofilm formation via Gac/Rsm pathway. Given that PrtR directly regulates the expression of the Gac/Rsm system, we speculated that this activity might be involved in the regulation of T3SS, T6SS, and

FIG 6 The Gac/Rsm system is involved in PrtR-mediated virulence phenotypes. (A) Effects of GacA and RsmY and -Z on PrtR-regulated T3SS expression. The indicated strains were grown to an OD₆₀₀ of 1.0 in LB supplemented with EGTA. Intracellular ExoS and secreted ExoS were separated by centrifugation. Samples were subjected to SDS-PAGE gels and probed with an anti-ExoS antibody. (B) Immunoblots with antibodies against Flag showing the levels of Hcp1-Flag in whole-cell lysates of the indicated strains. (C) Biofilm formation of the indicated strains was displayed with crystal violet staining (top) and quantified with optical density measurement (bottom). Error bars indicate the mean $\pm$ SD, and data are representative of three independent experiments. Statistical significance was calculated by one-way ANOVA with Dunnett's multiple-comparison test: **, $P < 0.01$; ***, $P < 0.001$. "EV" represents the empty vector.

biofilm formation in the *prtN prtR* mutant. To address this hypothesis, the protein levels of ExoS and Hcp1 were examined in the wild-type PAO1 strain and $\Delta prtN$, $\Delta prtN \Delta prtR$, and $\Delta prtN \Delta prtR \Delta gacA$ mutants, as well as the complemented strains. The deletion of *gacA* in the $\Delta prtN \Delta prtR$ strain restored the expression levels of ExoS to those in the $\Delta prtN$ mutant and wild-type parent, but expression levels were lower for Hcp1 (Fig. 6A). The biofilm phenotypes in these strains were also assessed using crystal violet staining and revealed that the deletion of *gacA* in the $\Delta prtN \Delta prtR$ background abolished the hyperbiofilm phenotype (Fig. 6B). Similar to GacA, the deletion of *rsmY* and *rsmZ* increased ExoS levels and reduced Hcp1 levels in the $\Delta prtN \Delta prtR$ strain and abolished the hyperbiofilm phenotype (Fig. 6A and B).

The major roles of RsmY and RsmZ involve the antagonism of RsmA (13). Thus, we next investigated the effects of RsmA on PrtR-mediated T3SS expression and biofilm formation. It was shown that overexpression of *rsmA* increased the expression of ExoS and reduced biofilm formation in the $\Delta prtN \Delta prtR$ mutant (see Fig. S3 in the supplemental material). Taken together, these results clearly indicate that the PrtR-mediated regulation of T3SS and biofilm formation occurred via the LadS/Gac/Rsm pathway.

DISCUSSION

An important objective of the current study is to understand the molecular mechanisms supporting the bacterial transition from acute to chronic infections. Although significant progress has been achieved in this area in recent years, many unknown interconnected pathways remain to be elucidated. Here, we demonstrated that PrtR directly interacted with SiaD, which is a DGC, and potentially affected biofilm formation via the regulation of intracellular c-di-GMP levels. Additionally, it was shown that PrtR was interconnected with the Gac/Rsm system and the c-di-GMP signaling pathway, both of which play important roles in modulating the transition from acute to chronic infection (19, 20).

In *P. aeruginosa*, PrtR is required for T3SS gene expression via its repressive role on PtrB (27) and has been shown to control pathogenesis in a mouse acute pneumonia model (26). However, the exact physiological roles played by PrtR remain unclear. We demonstrated that PrtR influenced the formations of a wrinkly colony and biofilm and activated bacterial motility but repressed the intracellular c-di-GMP production (Fig. 1 and 2). These data indicate that PrtR may serve as a molecular switch that triggers biofilm maturation in *P. aeruginosa*. Similar phenotypes have been observed in the well-

FIG 7 A proposed model of PrtR involved in transitions between acute and chronic infections. PrtR-mediated T3SS and pyocin regulation was reported in a previous study (27). In response to environmental signals, the expression of PrtR was repressed, and thus levels of LadS, GacA and GacS, and RsmY and RsmZ are elevated, which in turn antagonize the activity of RsmA, resulting in a shift toward the biofilm-associated phenotype. We also find that PrtR interacts with SiaD and thus regulates intracellular c-di-GMP levels. PrtR involved in biofilm formation is dependent on SiaD and c-di-GMP concentrations. The regulatory pathways identified in the present study are represented by red lines.

characterized AmrZ and FleQ proteins of *P. aeruginosa* (10, 39). The present findings indicated that low PrtR levels activated the expression of the DGC *siaD* (Fig. 3A), which elevated c-di-GMP levels in the cells (Fig. 3D). This signaling molecule can influence the production of all the above-mentioned pathways as well as being regulated directly by PrtR. Deletion of *siaD* in a $\Delta prtN \Delta prtR$ mutant abrogated the hyperbiofilm phenotype and reduced c-di-GMP levels (Fig. 4). Importantly, the present data were the first to demonstrate that PrtR directly interacted with SiaD to potentiate biofilm formation (Fig. 4A).

The second messenger c-di-GMP is thought to form an intricate intracellular network that specifically regulates biological processes crucial for bacterial adaptation and virulence (20, 40). The switch from acute to chronic disease is governed by the global Gac/Rsm pathway (13). Additionally, it has been reported that the *retS* mutant elevated c-di-GMP levels, and this molecule governed the T3SS-T6SS switch that is dependent on the two sRNAs, *rsmY* and *rsmZ*, providing direct evidence that the Gac/Rsm system and c-di-GMP signaling are interconnected (17, 21). The present data showed that, in addition to regulation of T3SS, PrtR also controlled H1-T6SS activity (Fig. 5A). We also demonstrated that PrtR regulated the expression of GacA/GacS and two small RNAs, *rsmY* and *rsmZ* (Fig. 5B). Therefore, the deletion of *gacA* or *rsmY* and *rsmZ* in the $\Delta prtN \Delta prtR$ mutant diminished the expression of Hcp1 and biofilm formation (Fig. 6). Taken together, the present findings suggest that PrtR affected intracellular c-di-GMP concentrations and mediated the Gac/Rsm regulatory cascade, which establishes a link between these two signaling pathways and provides a clue as to how *P. aeruginosa* may switch from acute to chronic in an infection.

The DGC GGDEF domains are typically fused to sensor domains such as PAS and GAF, which are known to serve as universal signal sensors or interaction modules that modulate the activity of its vicinal GGDEF domain (41–43). SiaD is a stand-alone GGDEF domain and is not predicted to contain any other domain; thus, the obvious question is whether and how the activity of the GGDEF domain is regulated. The present data showed that PrtR directly interacted with SiaD (Fig. 3C and D) to regulate biofilm formation (Fig. 4A), which may be a novel clue that will aid the current understanding of SiaD function.

Based on the present results and those of previous studies, we propose a model for the regulation and function of PrtR in *P. aeruginosa* (Fig. 7). PrtR regulates the

expression of *prtN* and *ptrB*, PrtN activates the expression of pyocin synthesis genes, and PtrB represses T3SS genes (27). In addition to its pleiotropic effects, PrtR plays determinant roles in bacterial motility and the regulation of biofilm formation. It is possible that PrtR coordinates the activities of the Gac/Rsm cascade and c-di-GMP signaling, to reciprocally influence bacterial motility and biofilm formation as *P. aeruginosa* transitions between the planktonic and the sessile/biofilm lifestyles. Additionally, it appears that these two major signaling pathways are interconnected, which reflects the importance of timely, fine-tuning of cellular behaviors. This capability is likely to be crucial to bacterial survival because these organisms must quickly adapt to changing environments.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material. Primers used in this study were summarized in Table S2 in the supplemental material. *P. aeruginosa* PAO1 (37) and its derivatives were grown at 37°C on LB agar plates or in LB broth with shaking at 220 rpm. For quantification of intracellular c-di-GMP concentrations, *P. aeruginosa* was grown in ABTG medium [1.51 mM (NH₄)₂SO₄, 3.37 mM Na₂HPO₄, 2.2 mM KH₂PO₄, 5 mM NaCl, 1 mM MgCl₂, 6H₂O, 100 μM CaCl₂·2H₂O, and 1 μM FeCl₃·6H₂O, supplemented with 0.4 g/liter glucose] (44). Antibiotics were used at the following concentrations: for *Escherichia coli*, gentamicin at 15 μg/ml, ampicillin at 100 μg/ml, and tetracycline at 10 μg/ml; for *P. aeruginosa*, gentamicin at 50 μg/ml in LB or 150 μg/ml in PIA (*Pseudomonas* isolate agar), tetracycline at 150 μg/ml in LB or 300 μg/ml in PIA, and carbenicillin at 500 μg/ml in LB.

Construction of plasmids. Plasmids p-*prtR*, p-*prtN*&*prtR*, p-*sadC*, p-PA2133, and p-*rsmA* were constructed as previously described (36, 37). Briefly, the fragments were amplified with the corresponding primers (Table S2) p-*prtN*-A/S, p-*prtN*/*prtR*-A/S, p-*sadC*-A/S, p-PA2133-A/S, and p-*rsmA*-A/S by PCR. The PCR products were digested with the indicated enzymes and cloned into pAK1900 plasmid (45).

The *hcp1*-wF-up/*hcp1*-wR-down primer pairs were used to amplify the DNA fragment, including the *hcp1* promoter that was intended to fuse with a C-terminal Flag tag by PCR. The indicated enzyme-digested PCR products were cloned into the corresponding enzyme sites of Mini-CTX-*lacZ* to generate Mini-CTX-*hcp1*-Flag.

The CTX-*prtN*/*prtR* plasmid was generated by amplifying fragments with the corresponding primers *prtN*/*prtR*-C-up and *prtN*/*prtR*-C-down by PCR. The resultant PCR products were digested with the indicated enzymes and cloned into Mini-CTX-*lacZ*, yielding Mini-CTX-*prtN*/*prtR*.

The plasmid pMS402, carrying a promoterless *luxCDABE* reporter gene cluster, was used to construct promoter-*luxCDABE* reporter vector as previously described (36, 46). For instance, *prtR* promoter regions were amplified by PCR using primer pair pro-*prtR*-A/S. The PCR products were cloned into the pMS402 plasmid, yielding pKD-*prtR*. Other promoter-*luxCDABE* reporter fusions were constructed accordingly. Besides the plasmid base reporter system, an integration plasmid CTX6.1 originating from plasmid mini-CTX-*lux* was used to construct chromosomal fusion reporters. The pMS402 fragments containing the kanamycin resistance marker, the multiple-cloning site (MCS), and the promoter-*luxCDABE* reporter cassette were then isolated and ligated to CTX6.1, yielding the CTX-promoter-*lux* plasmid. The plasmid generated was first transferred into *E. coli* SM10-λpir, and the *P. aeruginosa* reporter integration strain was obtained by biparental mating. All resultant mutants and plasmid constructs were verified by PCR and DNA sequencing.

Construction of *P. aeruginosa* mutants. In order to obtain the *P. aeruginosa* *prtN* insertion mutant, a SacB-based strategy was employed as previously described (36, 47). Briefly, two DNA regions including upstream (972-bp) and downstream (984-bp) fragments of the *prtN* were amplified by PCR using specific primer pairs (Table S2). The two PCR products were digested with appropriate restriction enzymes and directionally cloned into pEX18Ap (47), yielding pEX18Ap-*prtN*. A gentamicin resistance cassette was digested from pPS858 with XbaI. The fragment was cloned into XbaI-digested pEX18Ap-*prtN*. The resultant plasmid was electroporated into PAO1 with selection for gentamicin resistance. Colonies showing gentamicin resistance, carbenicillin susceptibility, and sucrose (5%) resistance were selected on LB agar plates, which indicates a double-crossover event and thus gene replacement occurring (47). Deletion events were verified by PCR and DNA sequencing, and the resulting mutant was Δ*prtN*::Gm. The *wspF* insertion mutant was generated by a similar strategy. To generate the *gacA* deletion mutant, two fragments were amplified using corresponding primer pairs (Table S2). The PCR products were digested and cloned into pEX18Ap, yielding pEX18Ap-*gacA*. This plasmid was electroporated into PAO1 with selection for carbenicillin resistance. Colonies were screened for carbenicillin sensitivity and sucrose (5%) resistance. The deletion mutant was further confirmed by PCR and DNA sequencing. Other deletion mutants were generated by a similar strategy.

Quantification of c-di-GMP by LC-MS. Extraction of c-di-GMP from *P. aeruginosa* cells as previously described (48). Briefly, *P. aeruginosa* was grown overnight in ABTG medium and subcultured the next day to an optical density at 600 nm (OD₆₀₀) of 0.02 in 50-ml tubes containing 10 ml of ABTG. When cells reached an OD₆₀₀ of ~0.1, 5-ml cell cultures were centrifuged for 5 min at 14,000 × *g*, and the supernatant was removed. The cell pellet was washed once with 1 mM ice-cold ammonium acetate and resuspended in 1 ml of ice-cold acetonitrile-methanol-water (2:2:1) solution. Samples were sonicated for 30 min in an ice-filled water sonicator, and cell debris was removed by centrifugation at 4°C for 5 min.

Supernatants (150 μ l) were removed, and samples were neutralized by addition of 30 μ l 2.5 M KHCO_3 . The resulting precipitate was removed by centrifugation at 4°C. These neutralized supernatants were stored at -80°C until analyzed by LC-MS. For protein determinations, three 1.5-ml samples of each culture were removed and centrifuged to pellet cells. These cell pellets were resuspended in 150 μ l 1 M NaOH and boiled in a water bath for 10 min. Samples were then stored on ice. The total protein level was measured by the Bio-Rad protein assay kit, which was then used for normalization of the c-di-GMP value.

Protocols for c-di-GMP separations and analysis by mass spectrometry were based on previous studies (28, 48). Compounds were separated on an Acuity UPLC equipped with a C_{18} guard cartridge (Phenomenex) and using a 2.0- by 50-mm Synergi Hydro RP column (Phenomenex). A gradient system was used starting with a 98% aqueous phase (10 mM ammonium formate, pH 4.0) and 2% organic phase (acetonitrile). The aqueous concentration was adjusted to 70% at 2 min, 20% at 2.5 min, and 100% at 3 min and finally held at 98% from 5 to 7.5 min. The m/z 691 > 152 transition was used for the identification of c-di-GMP. For a standard curve, 50, 100, 250, 500, and 1,000 fmol pure c-di-GMP (Invivogen, San Diego, CA) were analyzed by the method described above. The c-di-GMP levels are normalized to total protein per milliliter of culture. Data represent averages from two independent cultures.

Development of biofilm in flow chamber. The experiments were set up as previously described (49–51). *P. aeruginosa* wild-type and mutant strains were cultured overnight in 2 ml of LB medium in a 37°C incubator with shaking (200 rpm). The overnight cultures were diluted to 1:100 $\times$ with ABTG medium, and 500 μ l of the diluted cultures was injected into the flow chambers (52) for 1 h of incubation in an inverted position without medium flow. Then, the flow chambers were placed in the upright position before the bacteria were fed with ABTG medium via a Masterflex peristaltic pump (Cole-Parmer, Vernon Hills, IL) at a rate of 4 ml h^{-1} at 37°C for 72 h for biofilm development. After this, 300 μ l of 3.34 μ M SYTO9 (LIVE/DEAD BacLight bacterial viability kit; Thermo Fisher Scientific) and 100 μ g ml^{-1} -HHA-tetramethyl rhodamine isothiocyanate (HHA-TRITC; EY Labs) were used for 30 min for staining of total biofilm and Psl exopolysaccharide, respectively. After staining, the medium flow was resumed to remove the unbound stains for 30 min before the biofilms were subjected to confocal microscopy imaging.

The biofilms were observed by confocal laser scanning microscopy (CLSM) (LSM 780, Carl Zeiss, Germany), using a $\times 20$ magnification lens objective. The argon laser (488 nm) and HeNe laser (561 nm) were used to observe the green (SYTO9) and red (HHA-TRITC) fluorescence, respectively. The confocal images were acquired and processed using IMARIS version 9 (Bitplane AG, Zurich, Switzerland) to generate the orthogonal view of the biofilms as well as the biovolumes of the total biofilms and Psl exopolysaccharide. The statistical analysis was calculated using GraphPad Prism 7 (GraphPad Software, La Jolla, CA). Experiments were performed in triplicate, and results are shown as the mean $\pm$ standard deviation (SD).

Quantification of biofilm formation. Static biofilm was measured as previously described (53) with minor modifications. Visualization of biofilm formation was carried out in 10-ml borosilicate tubes. Briefly, bacteria from overnight cultures were inoculated at 1:100 dilutions into LB medium supplemented with appropriate antibiotics and grown at 30°C for 10 h. Biofilms were stained with 0.1% crystal violet (CV) and tubes were washed with water to remove unbound dye. Quantification of biofilm formation was performed in 24-well polystyrene microtiter plates. LB and appropriate antibiotics were inoculated to a final OD_{600} of 0.01. The plates were incubated for 20 h at 30°C. Crystal violet was added to each tube and stained for 15 min prior to removal by aspiration. Wells were rinsed three times by submerging the tubes in distilled water, and the remaining crystal violet was dissolved in 1 ml of 95% ethanol. A 1-ml portion of this solution was transferred to a new polystyrene tube, and the absorbance was measured at 600 nm.

PrtR protein expression and purification. The plasmid encoding the full-length PrtR was constructed and transferred into *E. coli* strain BL21(DE3). The plasmid encoding the *prtR* was constructed as following. Genomic DNA from *P. aeruginosa* was utilized as the template for PCR with two primers—*prtR*-P-F and *prtR*-P-R—the PCR products were resolved on agarose gel and extracted according to the Miniprep protocol. The samples were then digested with BamHI and XhoI, and the target fragment was recovered and recombined into the pET28a vector, which was also treated with BamHI and XhoI. The recombinant vector was then transferred into the *E. coli* BL21(DE3) competent cell.

The PrtR recombinant strains were cultured in LB medium supplemented with 50 μ g/ml kanamycin at 37°C overnight. Twenty milliliters of revived bacterium suspension was inoculated into 1 liter LB medium and cultured at 37°C with continuous 200-rpm shaking. Overexpression of the proteins was induced at $\text{OD}_{600} \approx 0.6$ by addition of 1.0 mM isopropyl β -D-1-thiogalactopyranoside (IPTG). The induced cultures were then grown at 16°C for an additional 16 h. The overnight culture was harvested at 4°C by centrifugation at $6,000 \times g$ for 8 min.

Pellets of PrtR were resuspended and lysed in buffer A (10 mM Tris-HCl [pH 7.5], 500 mM NaCl, 1 mM dithiothreitol [DTT], 10 mM phenylmethylsulfonyl fluoride [PMSF]). The cells were lysed by sonication and then centrifuged at 12,000 rpm for 25 min. The supernatant was filtered through a 0.45- μ m-pore filter and applied to a Ni-nitrilotriacetic acid (NTA) column (Qiagen). Then, the proteins were further purified by the HiLoad 16/60 Superdex 200 column (GE Healthcare) with buffer B (10 mM Tris-HCl [pH 7.5], 200 mM NaCl, 1.0 mM DTT). The samples were concentrated to 2 mg/ml in the gel filtration buffer and stored at -80°C until use.

Electrophoretic mobility shift assay. PrtR proteins were mixed with DNA probes (Table S2) in 20 μ l of the gel shift loading buffer (20 mM Tris-HCl, pH 7.4, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, and 15 μ g/ml sheared salmon sperm DNA). After incubation at 25°C for 20 min, aliquots of binding reaction mixtures were then electrophoresed on 6% nondenaturing acrylamide gels in 0.5 $\times$ Tris-borate-

EDTA (TBE) buffer. The gels were stained with SYBR gold dye and subjected to a screen on a phosphor screen (LAS 600; GE Healthcare).

Bacterial two-hybrid assays. Bacterial two-hybrid experiments were accomplished using the BacterioMatch II Two-Hybrid System Vector kit (Agilent Technologies). The fragments of PrtR were cloned into the bait vector pBT in order to create a fusion protein with λ repressor protein (λ cl). The DNA fragment of SiaD was inserted into the vector pTRG in frame with the α -subunit of RNA polymerase. The pBT-derived plasmid and pTRG-derived plasmid were then cotransformed into the validation reporter strain. Then the resulting strains, grown at 37°C in SOC medium for 90 min, were harvested by centrifugation ($5,000 \times g$, 2 min) and washed twice with M9⁺ His-dropout broth (TaKaRa, catalog no: 630415). The cells were diluted to different gradients, the nonselective screening medium and selective screening medium were spotted with the cells, and finally the cells were incubated at 30°C for 2 days. The colonies grown on the selective screening medium were selected and streaked on the dual screening medium for further verification. The cotransformants containing pBT-LGF2 and pTRG-Gal11p plasmids were used as the positive control.

GST pulldown assay. For GST pulldown experiments, 100 μ g purified GST or an equal amount of GST-SiaD was mixed with 50 μ l of prewashed glutathione bead slurry (Promega) for 2 h at 4°C; the unbound proteins were washed with GST binding buffer. After addition of 20 μ g PrtR-Flag protein, binding was allowed for 6 h at 4°C. The beads were then washed five times with GST binding buffer containing 500 mM NaCl. Retained proteins and the input samples were separated by SDS-PAGE, and then a Western blot was carried out using a standard protocol with the anti-GST (TransGen Biotech) and anti-Flag (Sigma) antibodies. The signal was detected by the use of an ECL Plus enhanced chemiluminescence kit (Amersham Biosciences).

Luminescence screening assays. Expression of *lux*-based reporters from cells grown in liquid culture was measured as counts per second (cps) of light production in a Synergy 2 plate reader (Biotek) as previously described (36, 46). Overnight cultures were diluted to an OD₆₀₀ of 0.2 with fresh LB medium and then cultured for another 2 h. The cultures were added to a black 96-well plate with a transparent bottom. Sixty microliters of sterilized mineral oil was added to the culture. Promoter activities and bacterial growth (OD₅₉₅) were continuously measured every 30 min for 24 h in a Synergy 2 plate reader (BioTek).

Western blot analysis. Overnight cultures of bacteria were subcultured with 100-fold dilution in fresh LB or 50-fold dilution in LB containing 5 mM EGTA and 20 mM MgCl₂ for 3 h. The supernatant and pellet were separated by centrifugation. Samples from equivalent numbers of bacterial cells were loaded and separated on 12% SDS-PAGE gels. The proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane and hybridized with a rabbit polyclonal ExoS antibody (generated in Shouguang Jin's laboratory) or a mouse monoclonal Flag antibody (Sigma). The signal was detected with an ECL Plus kit (Amersham Biosciences).

Colony morphology. Tryptone (10 g/liter) agar (1%) plates supplemented with Congo red (40 μ g/ml) and Coomassie blue dyes (20 μ g/ml) were used. The surfaces of the plates were spotted with bacteria at 5 μ l, and the bacteria were grown at 25°C. The colony morphology and staining were recorded after 3 days.

Swarming and swimming motility assays. The motility assay was carried out as described previously (54). The swarming medium was based on M9 minimal medium, supplemented with MgSO₄ (1 mM), glucose (0.2%), and Casamino Acids (CAA; 0.5%) and solidified with agar (0.5%). Swarm agar plates were spot inoculated with bacteria as 2.5- μ l aliquots taken directly from overnight LB cultures. Swarming agar plates were incubated for 16 h at 37°C. The swimming motility medium was 10 g/liter tryptone, 5 g/liter NaCl, and 0.3% agar (54). Plates were carefully spotted with 2 μ l of overnight cultures and incubated at room temperature for 16 h.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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REFERENCES

- Costerton JW, Stewart PS, Greenberg EP. 1999. Bacterial biofilms: a common cause of persistent infections. *Science* 284:1318–1322. <https://doi.org/10.1126/science.284.5418.1318>.
- Friedman L, Kolter R. 2004. Two genetic loci produce distinct carbohydrate-rich structural components of the *Pseudomonas aeruginosa* biofilm matrix. *J Bacteriol* 186:4457–4465. <https://doi.org/10.1128/JB.186.14.4457-4465.2004>.
- Vasseur P, Vallet-Gely I, Soscia C, Genin S, Filloux A. 2005. The pel genes of the *Pseudomonas aeruginosa* PAK strain are involved at early and late stages of biofilm formation. *Microbiology (Reading)* 151:985–997. <https://doi.org/10.1099/mic.0.27410-0>.
- Jennings LK, Storek KM, Ledvina HE, Coulon C, Marmont LS, Sadovskaya I, Secor PR, Tseng BS, Scian M, Filloux A, Wozniak DJ, Howell PL, Parsek MR. 2015. Pel is a cationic exopolysaccharide that cross-links extracellular

- DNA in the *Pseudomonas aeruginosa* biofilm matrix. *Proc Natl Acad Sci U S A* 112:11353–11358. <https://doi.org/10.1073/pnas.1503058112>.
5. Byrd MS, Sadovskaya I, Vinogradov E, Lu H, Sprinkle AB, Richardson SH, Ma L, Ralston B, Parsek MR, Anderson EM, Lam JS, Wozniak DJ. 2009. Genetic and biochemical analyses of the *Pseudomonas aeruginosa* Psl exopolysaccharide reveal overlapping roles for polysaccharide synthesis enzymes in Psl and LPS production. *Mol Microbiol* 73:622–638. <https://doi.org/10.1111/j.1365-2958.2009.06795.x>.
 6. Karatan E, Watnick P. 2009. Signals, regulatory networks, and materials that build and break bacterial biofilms. *Microbiol Mol Biol Rev* 73:310–347. <https://doi.org/10.1128/MMBR.00041-08>.
 7. Boyd CD, O'Toole GA. 2012. Second messenger regulation of biofilm formation: breakthroughs in understanding c-di-GMP effector systems. *Annu Rev Cell Dev Biol* 28:439–462. <https://doi.org/10.1146/annurev-cellbio-101011-155705>.
 8. Borlee BR, Goldman AD, Murakami K, Samudrala R, Wozniak DJ, Parsek MR. 2010. *Pseudomonas aeruginosa* uses a cyclic-di-GMP-regulated adhesin to reinforce the biofilm extracellular matrix. *Mol Microbiol* 75:827–842. <https://doi.org/10.1111/j.1365-2958.2009.06991.x>.
 9. Irie Y, Borlee BR, O'Connor JR, Hill PJ, Harwood CS, Wozniak DJ, Parsek MR. 2012. Self-produced exopolysaccharide is a signal that stimulates biofilm formation in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* 109:20632–20636. <https://doi.org/10.1073/pnas.1217993109>.
 10. Hickman JW, Tifrea DF, Harwood CS. 2005. A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. *Proc Natl Acad Sci U S A* 102:14422–14427. <https://doi.org/10.1073/pnas.0507170102>.
 11. Merighi M, Lee VT, Hyodo M, Hayakawa Y, Lory S. 2007. The second messenger bis-(3'-5')-cyclic-GMP and its PilZ domain-containing receptor Alg44 are required for alginate biosynthesis in *Pseudomonas aeruginosa*. *Mol Microbiol* 65:876–895. <https://doi.org/10.1111/j.1365-2958.2007.05817.x>.
 12. Ventre I, Goodman AL, Vallet-Gely I, Vasseur P, Soscia C, Molin S, Bleves S, Lazdunski A, Lory S, Filloux A. 2006. Multiple sensors control reciprocal expression of *Pseudomonas aeruginosa* regulatory RNA and virulence genes. *Proc Natl Acad Sci U S A* 103:171–176. <https://doi.org/10.1073/pnas.0507407103>.
 13. Goodman AL, Kulasekara B, Rietsch A, Boyd D, Smith RS, Lory S. 2004. A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in *Pseudomonas aeruginosa*. *Dev Cell* 7:745–754. <https://doi.org/10.1016/j.devcel.2004.08.020>.
 14. Vincent F, Round A, Reynaud A, Bordini C, Filloux A, Bourne Y. 2010. Distinct oligomeric forms of the *Pseudomonas aeruginosa* RetS sensor domain modulate accessibility to the ligand binding site. *Environ Microbiol* 12:1775–1786. <https://doi.org/10.1111/j.1462-2920.2010.02264.x>.
 15. Brencic A, Lory S. 2009. Determination of the regulon and identification of novel mRNA targets of *Pseudomonas aeruginosa* RsmA. *Mol Microbiol* 72:612–632. <https://doi.org/10.1111/j.1365-2958.2009.06670.x>.
 16. Brencic A, McFarland KA, McManus HR, Castang S, Mogno I, Dove SL, Lory S. 2009. The GacS/GacA signal transduction system of *Pseudomonas aeruginosa* acts exclusively through its control over the transcription of the RsmY and RsmZ regulatory small RNAs. *Mol Microbiol* 73:434–445. <https://doi.org/10.1111/j.1365-2958.2009.06782.x>.
 17. Moscoso JA, Mikkelsen H, Heeb S, Williams P, Filloux A. 2011. The *Pseudomonas aeruginosa* sensor RetS switches type III and type VI secretion via c-di-GMP signalling. *Environ Microbiol* 13:3128–3138. <https://doi.org/10.1111/j.1462-2920.2011.02595.x>.
 18. Moscoso JA, Jaeger T, Valentini M, Hui K, Jenal U, Filloux A. 2014. The diguanylate cyclase SadC is a central player in Gac/Rsm-mediated biofilm formation in *Pseudomonas aeruginosa*. *J Bacteriol* 196:4081–4088. <https://doi.org/10.1128/JB.01850-14>.
 19. Mikkelsen H, Sivaneson M, Filloux A. 2011. Key two-component regulatory systems that control biofilm formation in *Pseudomonas aeruginosa*. *Environ Microbiol* 13:1666–1681. <https://doi.org/10.1111/j.1462-2920.2011.02495.x>.
 20. Romling U, Galperin MY, Gomelsky M. 2013. Cyclic di-GMP: the first 25 years of a universal bacterial second messenger. *Microbiol Mol Biol Rev* 77:1–52. <https://doi.org/10.1128/MMBR.00043-12>.
 21. Grenga L, Little RH, Malone JG. 2017. Quick change: post-transcriptional regulation in *Pseudomonas*. *FEMS Microbiol Lett* 364:fnx125. <https://doi.org/10.1093/femsle/fnx125>.
 22. Michel-Briand Y, Bayse C. 2002. The pyocins of *Pseudomonas aeruginosa*. *Biochimie* 84:499–510. [https://doi.org/10.1016/S0300-9084\(02\)01422-0](https://doi.org/10.1016/S0300-9084(02)01422-0).
 23. Matsui H, Sano Y, Ishihara H, Shinomiya T. 1993. Regulation of pyocin genes in *Pseudomonas aeruginosa* by positive (*prfN*) and negative (*prfR*) regulatory genes. *J Bacteriol* 175:1257–1263. <https://doi.org/10.1128/jb.175.5.1257-1263.1993>.
 24. Brazas MD, Hancock RE. 2005. Ciprofloxacin induction of a susceptibility determinant in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 49:3222–3227. <https://doi.org/10.1128/AAC.49.8.3222-3227.2005>.
 25. Chang W, Small DA, Toghiani F, Bentley WE. 2005. Microarray analysis of *Pseudomonas aeruginosa* reveals induction of pyocin genes in response to hydrogen peroxide. *BMC Genomics* 6:115. <https://doi.org/10.1186/1471-2164-6-115>.
 26. Sun Z, Shi J, Liu C, Jin Y, Li K, Chen R, Jin S, Wu W. 2014. PrfR homeostasis contributes to *Pseudomonas aeruginosa* pathogenesis and resistance against ciprofloxacin. *Infect Immun* 82:1638–1647. <https://doi.org/10.1128/IAI.01388-13>.
 27. Wu W, Jin S. 2005. PrfR of *Pseudomonas aeruginosa* suppresses the type III secretion system under the stress of DNA damage. *J Bacteriol* 187:6058–6068. <https://doi.org/10.1128/JB.187.17.6058-6068.2005>.
 28. Jones CJ, Newsom D, Kelly B, Irie Y, Jennings LK, Xu B, Limoli DH, Harrison JJ, Parsek MR, White P, Wozniak DJ. 2014. ChIP-Seq and RNA-Seq reveal an AmrZ-mediated mechanism for cyclic di-GMP synthesis and biofilm development by *Pseudomonas aeruginosa*. *PLoS Pathog* 10:e1003984. <https://doi.org/10.1371/journal.ppat.1003984>.
 29. Caiazza NC, Merritt JH, Brothers KM, O'Toole GA. 2007. Inverse regulation of biofilm formation and swarming motility by *Pseudomonas aeruginosa* PA14. *J Bacteriol* 189:3603–3612. <https://doi.org/10.1128/JB.01685-06>.
 30. Ghafoor A, Hay ID, Rehm BH. 2011. Role of exopolysaccharides in *Pseudomonas aeruginosa* biofilm formation and architecture. *Appl Environ Microbiol* 77:5238–5246. <https://doi.org/10.1128/AEM.00637-11>.
 31. Jones CJ, Wozniak DJ. 2017. Psl produced by mucoid *Pseudomonas aeruginosa* contributes to the establishment of biofilms and immune evasion. *mBio* 8:e00864-17. <https://doi.org/10.1128/mBio.00864-17>.
 32. Rybtke MT, Borlee BR, Murakami K, Irie Y, Hentzer M, Nielsen TE, Givskov M, Parsek MR, Tolker-Nielsen T. 2012. Fluorescence-based reporter for gauging cyclic di-GMP levels in *Pseudomonas aeruginosa*. *Appl Environ Microbiol* 78:5060–5069. <https://doi.org/10.1128/AEM.00414-12>.
 33. Klebensberger J, Birkenmaier A, Geffers R, Kjelleberg S, Philipp B. 2009. SiaA and SiaD are essential for inducing autoaggregation as a specific response to detergent stress in *Pseudomonas aeruginosa*. *Environ Microbiol* 11:3073–3086. <https://doi.org/10.1111/j.1462-2920.2009.02012.x>.
 34. Kulasakara H, Lee V, Brencic A, Liberati N, Urbach J, Miyata S, Lee DG, Neely AN, Hyodo M, Hayakawa Y, Ausubel FM, Lory S. 2006. Analysis of *Pseudomonas aeruginosa* diguanylate cyclases and phosphodiesterases reveals a role for bis-(3'-5')-cyclic-GMP in virulence. *Proc Natl Acad Sci U S A* 103:2839–2844. <https://doi.org/10.1073/pnas.0511090103>.
 35. Fields S, Song O. 1989. A novel genetic system to detect protein-protein interactions. *Nature* 340:245–246. <https://doi.org/10.1038/340245a0>.
 36. Zhao J, Yu X, Zhu M, Kang H, Ma J, Wu M, Gan J, Deng X, Liang H. 2016. Structural and molecular mechanism of CdpR involved in quorum-sensing and bacterial virulence in *Pseudomonas aeruginosa*. *PLoS Biol* 14:e1002449. <https://doi.org/10.1371/journal.pbio.1002449>.
 37. Kong W, Chen L, Zhao J, Shen T, Surette MG, Shen L, Duan K. 2013. Hybrid sensor kinase PA1611 in *Pseudomonas aeruginosa* regulates transitions between acute and chronic infection through direct interaction with RetS. *Mol Microbiol* 88:784–797. <https://doi.org/10.1111/mmi.12223>.
 38. Mougous JD, Cuff ME, Raunser S, Shen A, Zhou M, Gifford CA, Goodman AL, Joachimiak G, Ordóñez CL, Lory S, Walz T, Joachimiak A, Mekalanos JJ. 2006. A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus. *Science* 312:1526–1530. <https://doi.org/10.1126/science.1128393>.
 39. Jones CJ, Ryder CR, Mann EE, Wozniak DJ. 2013. AmrZ modulates *Pseudomonas aeruginosa* biofilm architecture by directly repressing transcription of the psl operon. *J Bacteriol* 195:1637–1644. <https://doi.org/10.1128/JB.02190-12>.
 40. Hengge R. 2009. Principles of c-di-GMP signalling in bacteria. *Nat Rev Microbiol* 7:263–273. <https://doi.org/10.1038/nrmicro2109>.
 41. Moglich A, Ayers RA, Moffat K. 2009. Structure and signaling mechanism of Per-ARNT-Sim domains. *Structure* 17:1282–1294. <https://doi.org/10.1016/j.str.2009.08.011>.
 42. Pandit J, Forman MD, Fennell KF, Dillman KS, Menniti FS. 2009. Mechanism for the allosteric regulation of phosphodiesterase 2A deduced from the X-ray structure of a near full-length construct. *Proc Natl Acad Sci U S A* 106:18225–18230. <https://doi.org/10.1073/pnas.0907635106>.
 43. Schirmer T. 2016. C-di-GMP synthesis: structural aspects of evolution,

- catalysis and regulation. *J Mol Biol* 428:3683–3701. <https://doi.org/10.1016/j.jmb.2016.07.023>.
44. Chen Y, Yuan M, Mohanty A, Yam JK, Liu Y, Chua SL, Nielsen TE, Tolker-Nielsen T, Givskov M, Cao B, Yang L. 2015. Multiple diguanylate cyclase-coordinated regulation of pyoverdine synthesis in *Pseudomonas aeruginosa*. *Environ Microbiol Rep* 7:498–507. <https://doi.org/10.1111/1758-2229.12278>.
 45. Jansons I, Touchie G, Sharp R, Almquist K, Farinha MA, Lam JS, Kropinski AM. 1994. Deletion and transposon mutagenesis and sequence analysis of the pRO1600 OriR region found in the broad-host-range plasmids of the pQF series. *Plasmid* 31:265–274. <https://doi.org/10.1006/plas.1994.1028>.
 46. Kang H, Gan J, Zhao J, Kong W, Zhang J, Zhu M, Li F, Song Y, Qin J, Liang H. 2017. Crystal structure of *Pseudomonas aeruginosa* RsaL bound to promoter DNA reaffirms its role as a global regulator involved in quorum-sensing. *Nucleic Acids Res* 45:699–710. <https://doi.org/10.1093/nar/gkw954>.
 47. Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP. 1998. A broad-host-range FLP-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* 212:77–86. [https://doi.org/10.1016/S0378-1119\(98\)00130-9](https://doi.org/10.1016/S0378-1119(98)00130-9).
 48. Hickman JW, Harwood CS. 2008. Identification of FleQ from *Pseudomonas aeruginosa* as a c-di-GMP-responsive transcription factor. *Mol Microbiol* 69:376–389. <https://doi.org/10.1111/j.1365-2958.2008.06281.x>.
 49. Chua SL, Yam JKH, Hao P, Adav SS, Salido MM, Liu Y, Givskov M, Sze SK, Tolker-Nielsen T, Yang L. 2016. Selective labelling and eradication of antibiotic-tolerant bacterial populations in *Pseudomonas aeruginosa* biofilms. *Nat Commun* 7:10750. <https://doi.org/10.1038/ncomms10750>.
 50. Sternberg C, Tolker-Nielsen T. 2006. Growing and analyzing biofilms in flow cells. *Curr Protoc Microbiol* Chapter 1:Unit 1B.2. <https://doi.org/10.1002/9780471729259.mc01b02s21>.
 51. Chua SL, Liu Y, Yam JK, Chen Y, Vejborg RM, Tan BG, Kjelleberg S, Tolker-Nielsen T, Givskov M, Yang L. 2014. Dispersed cells represent a distinct stage in the transition from bacterial biofilm to planktonic lifestyles. *Nat Commun* 5:4462. <https://doi.org/10.1038/ncomms5462>.
 52. Sternberg C, Tolker-Nielsen T. 2011. Growing and analyzing biofilms in flow cells. *Curr Protoc Microbiol* 21:1B.2.1–1B.2.15. <https://doi.org/10.1002/9780471729259.mc01b02s21>.
 53. O'Toole GA, Kolter R. 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol Microbiol* 30:295–304. <https://doi.org/10.1046/j.1365-2958.1998.01062.x>.
 54. Rashid MH, Kornberg A. 2000. Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* 97:4885–4890. <https://doi.org/10.1073/pnas.060030097>.