Chapter 28
Regulation of Cyclic di-GMP Signaling in *Pseudomonas aeruginosa*

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Abstract *Pseudomonas aeruginosa* is found ubiquitously throughout the environment, and it readily adapts to survive in different environments. Genome analysis reveals *P. aeruginosa* has a proportionally high number of transcriptional regulators and two-component systems, and it has become a model for studying bacterial gene regulation. Furthermore, *P. aeruginosa* is an opportunistic pathogen, establishing infections that are often difficult to treat, due to its recalcitrance to antimicrobials and host immune factors. This tolerance can be attributed to its ability to form protective communities known as biofilms. The transition from a planktonic to biofilm lifestyle is complex, and many regulatory pathways are involved. The secondary messenger molecule, cyclic di-GMP, regulates many factors involved in this process, including type IV pili, flagella, and exopolysaccharides. Thus, understanding how *P. aeruginosa* modulates cyclic di-GMP levels has important implications for *P. aeruginosa* virulence and environmental lifestyle. *P. aeruginosa* encodes 38 proteins predicted to be involved in cyclic di-GMP metabolism, indicating intricate regulatory mechanisms are in place to control intracellular cyclic di-GMP levels in response to various stimuli. While the role and regulation of many of these proteins remains unknown, this chapter will review currently identified cyclic di-GMP regulatory mechanisms in *P. aeruginosa*, including the Wsp, Gac, and Roc networks.

Keywords Cyclic di-GMP · *Pseudomonas aeruginosa* · Biofilm · Motility · Exopolysaccharide · Secondary messengers

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28.1 Overview and Relevance of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that causes devastating diseases in immunocompromised individuals. *P. aeruginosa* is currently one of the most common causes of nosocomial infection, and is frequently isolated from chronic pulmonary, wound, and medical device-associated infections [1]. The incidence of multidrug resistance in *P. aeruginosa* has increased drastically in recent years, and *P. aeruginosa* is now ranked by the World Health Organization (WHO) as one of the most serious threats to human health [2].

Aside from its ability to establish infections, *P. aeruginosa* also thrives in marine and soil environments. Given the remarkable range of environments *P. aeruginosa* inhabits, it is no surprise that this bacterium has one of the largest bacterial genomes (up to 7.1 Mbp). Approximately 9% of its genes are predicted to function as transcriptional regulators or as part of two-component systems [3, 4]. These features emphasize the adaptability of *P. aeruginosa*, to respond to different environmental conditions. Perhaps the most prominent of these survival mechanisms is the formation of protective biofilm communities, which requires a complex coordination of regulatory mechanisms to transition from a planktonic to biofilm lifestyle. During biofilm formation, *P. aeruginosa* encases itself within a matrix composed of exopolysaccharides, extracellular DNA, and proteins. The biofilm enhances bacterial persistence, and *P. aeruginosa* becomes more tolerant toward environmental stress, antimicrobial treatment, and the host immune system [5, 6]. The exopolysaccharides alginate, Psl, and Pel are major structural components of the biofilm matrix and contribute to antimicrobial tolerance and defense against the host immune system [7–9]. Thus, biofilms have important clinical implications for chronic infection and antimicrobial tolerance, and it is apparent that understanding this bacterial process is essential for controlling infection.

It is now well-established that the transition from a motile to sessile biofilm lifestyle is primarily driven by modulating levels of the secondary messenger molecule cyclic dimeric (3′–5′) GMP (cyclic di-GMP) [10, 11]. High intracellular cyclic di-GMP concentrations generally activate production of various adhesins and biofilm matrix components while simultaneously downregulating motility [11]. Conversely, low levels of cyclic di-GMP promote biofilm dispersal and motility function [11]. The levels of cyclic di-GMP in the cell are controlled by the competing activity of two classes of enzymes known as diguanylate cyclases (DGCs) and phosphodiesterases (PDEs). DGCs are identified by a conserved GGDEF domain and synthesize cyclic di-GMP from two molecules of GTP. PDEs can be identified by an EAL or HD-GYP domain and degrade cyclic di-GMP into pGpG or two molecules of GMP, respectively [10]. Furthermore, many of these enzymes contain both DGC and PDE associated domains; however, typically only one of these domains exhibits catalytic activity. *P. aeruginosa* is predicted to produce up to 38 proteins (17 GGDEF, 5 EAL, and 16 GGDEF/EAL) involved in the metabolism of cyclic di-GMP depending on the strain [12]. Thus, a complex regulatory network must be in place to control cyclic di-GMP production in response to environmental signals.

Table 28.1 Identified *P. aeruginosa* DGCs and PDEs

Name	Function	Known regulators	Target/effect	Activating signal/domain[a]	References
WspR	DGC	WspA, WspC, WspF, WspR cyclic di-GMP	Promotes production of CdrA, Psl, and Pel	Surface sensing-WspA (**D**), NaCl (**D**)	[13–17]
SadC	DGC	Gac/RsmA, PilY1 (Pil-Chp)	Promotes surface attachment and inhibits swarming motility	Calcium-LadS (**I**)), Psl (**U**), Surface Contact (**I**)	[18–21]
RoeA	DGC	–	Promotes Pel production	–	[21]
SiaD	DGC	SiaA, Gac/RsmA	Promotes Cup fimbriae production	SDS-SiaA (**I**), Psl (**U**)	[18, 24]
GcbA	DGC	AmrZ, Gac/RsmA	Promotes attachment and suppresses Flagellar Reversal Rates	Calcium-LadS (**I**)	[23, 27]
YfiN	DGC	YfiB, YfiR	Promotes Pel and Psl production	Na-succinate (**I**), SDS (**I**), Membrane Stress-YifB (**I**)	[25]
PA3177	DGC	–	Promotes attachment and antimicrobial tolerance	ROS (**U**)	[26, 32]
NicD	DGC	–	Regulates DipA PDE activity via BdlA	Glutamate Sensing (**D**)	[28]
MorA	DGC/PDE	–	Enhances biofilm formation	–	[29, 30]
MucR	DGC/PDE	–	Biofilm dispersal	NO-(MHYT) (**D**), Glutamate (**U**)	[31]
BifA	PDE	Cyclic di-GMP	Inhibits Pel production and promotes swarming motility	GGDQF Allosteric Activation (**D**)	[81]
DipA	PDE	NicD, BdlA, ClpP	Biofilm dispersion	Glutamate (**I**), NO (**U**), NH_4Cl (**U**), $HgCl_2$ (**U**)	[33]
RocR/SadR	PDE	RocS1, RocA1	Represses Cup fimbriae production	PAS Domain (**I**)	[35, 36, 76, 77]
NbdA	PDE	–	Biofilm dispersal	NO-(MHYT) (**D**)	[31]
RbdA	PDE	GTP	Biofilm dispersal	PAS Domain (**D**)	[38, 39]
FimX	PDE	Cyclic di-GMP	Promotes T4P pole localization in high cyclic di-GMP conditions	GGDEF I-site (**D**)	[40–42]

[a]Indicates activating signal or domain is direct (**D**), indirect (**I**), or undetermined (**U**)

This chapter will discuss the regulation and function of the currently identified *P. aeruginosa* DGCs and PDEs, which are summarized in Table 28.1.

28.2 Principals of Cyclic di-GMP Regulation and Signaling Inputs

The transition between a motile and biofilm lifestyle requires the concerted regulation of many systems, and cyclic di-GMP serves as a master regulator of this process (Fig. 28.1). To exert its function, cyclic di-GMP binds to a protein or RNA, and the resulting conformational change alters activity. Depending on the target, cyclic di-GMP is capable of regulating at both a transcriptional or posttranslational level. Thus, cyclic di-GMP regulation is predominately controlled by modifying its concentration within the cell. One way this occurs is by activation or deactivation of DGCs and PDEs by a regulatory sensor, and in many *P. aeruginosa* cyclic di-GMP systems such sensors harbor an N-terminal domain that perceives signals. In these systems, signal transduction often occurs through a phosphotransfer event between the sensor histidine kinase and the response regulator, leading to its activation. Through this mechanism, it is possible for *P. aeruginosa* to modify DGC and PDE activity in response to environmental signals recognized by the sensor kinase. In Gram-negative bacteria, conserved signaling domains respond to a variety of factors including oxygen, phosphorylation, nutrients, antibiotics, protein/cyclic nucleotide binding, and light [43, 44]. In the context of cyclic di-GMP, multiple regulatory components with *P*er-*A*rnt-*S*im (PAS) and *rec*eiver (REC) sites have been identified which respond to oxygen, surface attachment, and nutrient conditions

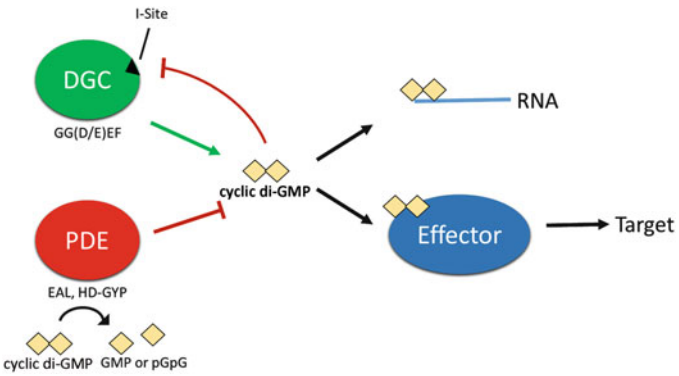

Fig. 28.1 Overview of cyclic di-GMP activity in *P. aeruginosa*. DGCs synthesize cyclic di-GMP from two molecules of GTP, while PDEs degrade cyclic di-GMP into pGpG or GMP. DGCs can be self-regulated at an allosteric I-site that binds to cyclic di-GMP to inhibit function. Once produced, cyclic di-GMP can regulate many bacterial processes by directly binding to RNA or proteins. Conformational changes in response to cyclic di-GMP binding regulate processes at the transcriptional, translational, and posttranslational level

(Table 28.1) [26, 28, 44]. This suggests environmental signals are crucial for controlling DGC and PDE activity depending on whether conditions are preferable for biofilm or planktonic growth.

Additionally, DGCs and PDEs frequently contain either a degenerate or nonfunctional GGDEF or EAL site, or an allosteric RxxD inhibitory site (I-site) [44]. These degenerate domains are enzymatically inactive but retain binding affinity. These serve as allosteric inhibitor or activator sites, where cyclic di-GMP or GTP can bind, leading to a conformational change that alters enzyme activity. *P. aeruginosa* encodes 16 proteins with predicted dual DGC and PDE domains, and often one of these sites is degenerate [12, 44]. These I-sites serve as an efficient self-regulatory mechanism, allowing these enzymes to control their own activity based on local concentrations of cyclic di-GMP and GTP.

28.3 *P. aeruginosa* Cyclic di-GMP Synthesis, Degradation, and Regulation

Currently, the function and regulation of only a few of the predicted *P. aeruginosa* DGC and PDE enzymes have been determined (Table 28.1). These proteins have a number of critical regulatory functions, and their identification has vastly improved our understanding of motility, biofilm formation, and dispersion. The following sections will summarize the function and regulation of the currently identified *P. aeruginosa* DGCs and PDEs.

28.3.1 Cell Motility

The regulation of motility is a crucial step in controlling the transition between a motile and biofilm lifestyle, and cyclic di-GMP has an important role in regulating flagella-mediated swarming motility and type IV pili-mediated twitching motility. One system involved in controlling swarming motility is the Gac/Rsm network. This system is a complex regulatory system involving multiple sensor kinases and more than 500 downstream targets [45, 46] (Fig. 28.2a). The activity of this system is controlled by two sensor kinase proteins, LadS and RetS [47, 48]. RetS recognition of an unknown carbohydrate signal represses the sensor kinase GacS [49]. Conversely, LadS calcium signaling activates GacS [50]. Once activated, GacS-mediated phosphorylation of GacA results in the production of two noncoding small RNAs, *rsmY* and *rsmZ*. These factors bind and sequester the translational repressor RsmA [51]. Inhibition of RsmA ultimately results in reduced motility and virulence factor production, but increased production of biofilm factors, including Psl and Pel [51, 52]. GacS-mediated inhibition of RsmA promotes translation of three DGCs, SiaD, GcbA (AdcA), and SadC [23, 53, 54]. SiaD activity promotes biofilm matrix

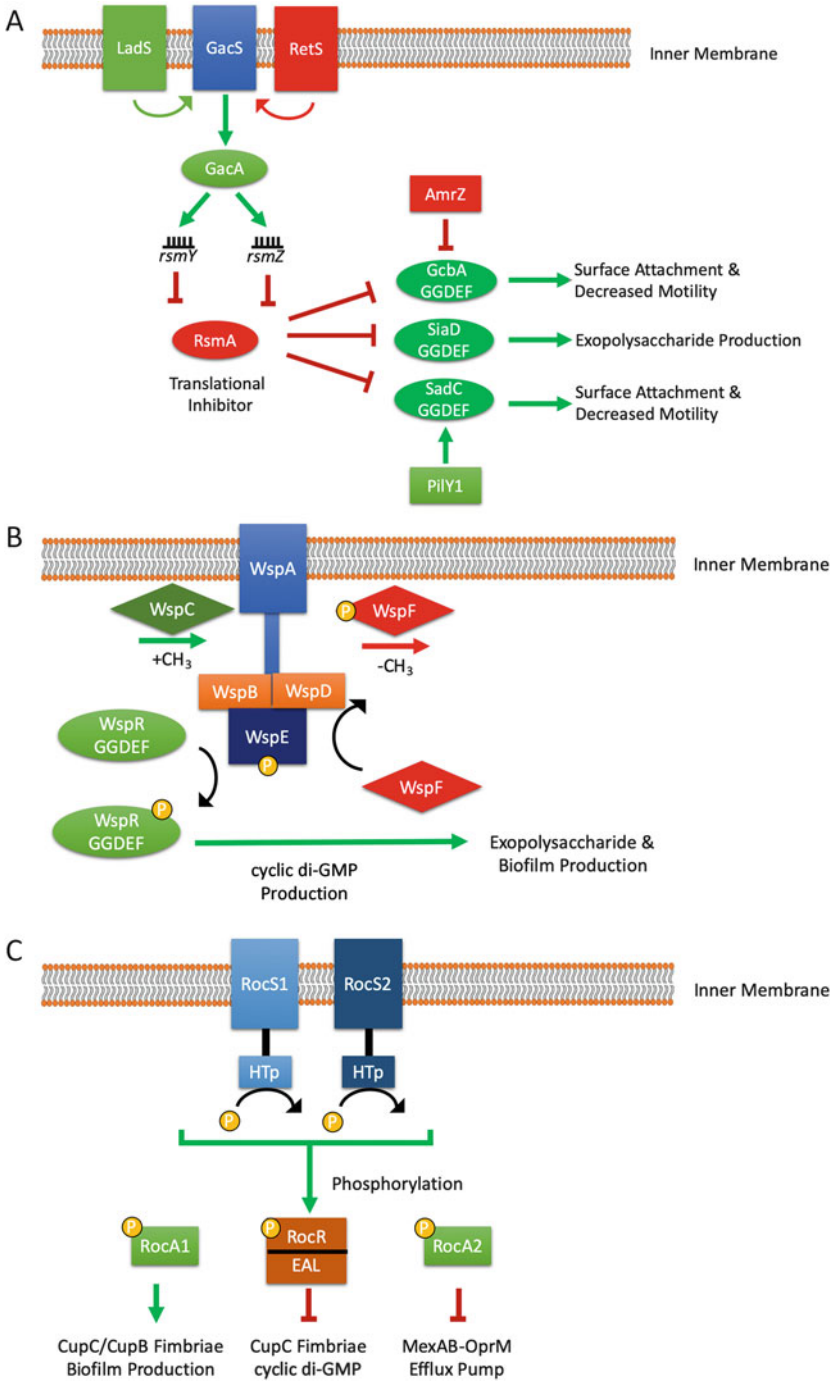

Fig. 28.2 *Pseudomonas aeruginosa* regulation of cyclic di-GMP. Schematic of three of the main cyclic di-GMP regulating pathways in *P. aeruginosa*. Green arrows indicate activation, while red bars indicate inhibition. (**a**) GacS activity is promoted by LadS and inhibited by RetS interaction.

production [18], while GcbA and SadC activity promotes attachment and downregulates swimming and swarming motility, respectively [19, 23]. Thus, these DGCs contribute toward biofilm formation.

Furthermore, GcbA and SadC are regulated by additional factors independent of the Gac/Rsm network. The transcriptional factor AmrZ regulates a number of virulence factors, and it directly inhibits transcription of GcbA, resulting in reduced cyclic di-GMP levels and increased motility [55]. SadC activity is further promoted by PilY1, which is part of the type IV pili (T4P) machinery [56]. T4P is necessary for twitching motility and biofilm maturation in *P. aeruginosa* [40, 57]. PilY1 is an important adhesion protein, containing a conserved von Willebrand A mechanosensory domain, which indicates it may have a signaling function in response to surface sensing [20]. Since PilY1 production is regulated by the FimS-AlgR two-component system and Pil-Chp chemotaxis complex, an intricate regulatory network involving interactions between all these systems is likely involved in modulating SadC activity [20]. While the details of this regulatory network remain to be elucidated, it is clear that SadC and PilY1 interaction enhances surface attachment, while inhibiting swarming motility during biofilm formation [20, 56].

Another role for cyclic di-GMP in T4P regulation has been reported. The PDE FimX promotes localization of T4P components to the cell pole, which is a critical step in pili formation. FimX contains both a GGDEF and EAL domain, but it appears to exhibit only PDE activity [40–42]. While PDE activity was required for T4P function, mutation of the nonfunctional GGDEF domain also inhibited T4P localization [58]. This could indicate the GGDEF site functions as an allosteric activation site that promotes PDE activity. Interestingly, the necessity of FimX for T4P production is limited to low cyclic di-GMP conditions, and *fimX* deletion mutants still produce T4P in high cyclic di-GMP conditions [40].

28.3.2 Biofilm Matrix Production

The Wsp (*w*rinkly *sp*reader) chemosensory system is one of the most well-defined cyclic di-GMP regulatory systems in *P. aeruginosa*. It has an active role in

Fig. 28.2 (continued) GacS activates GacA, which leads to the production of the sRNAs *rsmY* and *rsmZ*. These sRNAs bind and inhibit RsmA translational regulation, allowing for unimpeded translation of the DGCs, GcbA, SiaD, and SadC. DGC activity results in increased exopolysaccharide production and attachment, as indicated. AmrZ inhibits GcbA transcription, and PilY1 promotes GcbA activity. (**b**) WspC and WspF regulate WspA activity through transfer of a methyl group. In response methylation state and surface signals, WspA modulates WspE autophosphorylation activity, which in turn phosphorylates and activates the DGC, WspR. Activation of WspR leads to cyclic di-GMP production and increased production of the exopolysaccharides Pel and Psl [13–15, 17]. (**c**) RocS1 and RocS2 phosphorylate multiple response regulators in response to unknown environmental signals. Phosphorylation and activation of the PDE RocR results in reduced levels of cyclic di-GMP and CupC fimbriae production leading to reduced biofilm formation

regulating swimming motility and the production of biofilm matrix components [13, 59–61]. This system is encoded by the *wspABCDEFR* (PA3708-PA3702) operon, and its components closely resemble the enteric chemotaxis signal transduction system (Che) (Fig. 28.2b). WspA functions as a membrane-bound methyl-accepting chemotaxis protein (MCP), which responds to surface contact and environmental stresses that alter membrane fatty acid composition, such as high NaCl concentrations [14–16]. WspA is activated by the transfer of a methyl group via the methyltransferase WspC. Conversely, WspA is deactivated by the methylesterase WspF, which removes this methyl group [13]. WspB and WspD act as linkers to connect the sensor histidine kinase, WspE, to WspA. Activation of WspA results in a conformational change promoting WspE autophosphorylation. WspE is then able to phosphorylate and activate two response regulators, WspF and WspR [62]. Phosphorylation of WspF promotes methylesterase activity leading to WspA deactivation, while phosphorylation of the DGC WspR stimulates cyclic di-GMP production. WspR activity is further regulated through its oligomerization. At high concentrations of cyclic di-GMP, the molecule binds to the WspR RxxD allosteric I-site, which results in a conformational change that promotes the assembly of enzymatically inactive WspR tetramers, thus limiting its DGC activity [17].

Loss-of-function mutations inhibiting the regulatory activity of WspF result in constitutive WspR activity, providing insight into the downstream targets of WspR activity [13, 15, 60]. These studies suggest WspR activity promotes biofilm production and persistence by increasing Pel, Psl, and CdrA production [61, 63, 64]. The transcriptional regulator, FleQ, directly binds to both the *psl* and *pel* promoters [65, 66]. FleQ promoter binding represses *pel* transcription. In the presence of cyclic di-GMP, the molecule binds FleQ, leading to a conformational change, which elevates *pel* transcription due to derepression [65, 66]. While FleQ binding to the *psl* promoter was reported, a regulatory role for FleQ has not yet been directly demonstrated [65]. However, considering cyclic di-GMP is required to activate Psl production, it is likely FleQ regulates Psl similarly. Additionally, cyclic di-GMP promotes Pel production posttranslationally by directly binding to PelD, a component of the synthesis complex [67]. While it is unknown if WspR DGC activity is involved in alginate production, cyclic di-GMP similarly promotes alginate production by binding and activating the synthesis protein Alg44 [68].

Another DGC that promotes exopolysaccharide production is the DGC YfiN, which induces Pel and Psl production [69]. YfiN is embedded within the inner membrane, and is part of the YfiBNR three-component system. YfiN activity is regulated by interaction with YfiR, which binds to the PAS periplasmic domain of YfiN. Interaction with YfiR causes a conformational change of YfiN that disrupts its DGC activity. YfiB is located within the outer membrane and binds and sequesters YfiR, allowing for YfiN activity and cyclic di-GMP production [25]. This system is activated by the detergent SDS or high concentrations of Na-succinate, indicating that membrane stress promotes YfiN activity [25]. Combined with the Wsp system, cyclic di-GMP may be produced by multiple DGCs in response to membrane stress. However, whether YfiN activity is directly integrated with the Wsp system is unclear.

Lastly, two other DGCs are also involved in biofilm matrix production. RoeA activity promotes Pel production, likely through the interaction of cyclic di-GMP with FleQ and PelD [21]. MorA contains both active DGC and PDE domains and is capable of synthesizing and degrading cyclic di-GMP [29]. Upregulation of MorA was observed in a clinical *P. aeruginosa* isolate from a chronic lung infection, which indicates it may have an important role in bacterial persistence [30]. Deletion of *morA* in *Pseudomonas putida* enhanced motility and reduced biofilm formation. However, *morA* deletion in *P. aeruginosa* only resulted in decreased biofilm formation, with no effect on motility [70]. While this indicates MorA DGC activity may be important for biofilm formation in *P. aeruginosa*, the function of its PDE activity remains unclear.

28.3.3 Biofilm Dispersal

Biofilm dispersion is often an active bacterial process, triggered in mature biofilms by poor nutrient and anoxic conditions within the biofilm [71, 72]. Therefore, this process encourages the bacteria to seek nutrient-rich environments better suited for survival. The initiation of biofilm dispersal has been linked to low levels of cyclic di-GMP and the activity of a number of PDEs, including MucR, NbdA, RbdA, and DipA [31, 33, 38]. MucR and NbdA are anchored to the inner membrane and each contains a GGDEF and EAL domain. MucR exhibits both PDE and DGC activity, while NbdA only possesses PDE activity. However, deletion of either gene inhibits biofilm dispersion [31]. Nitric oxide (NO) accumulates in the biofilm during anaerobic growth, and it is a major stimulator of *P. aeruginosa* biofilm dispersal [72]. NbdA and MucR contain an MHYT domain, which is predicted to recognize diatomic gases including NO. NbdA-mediated biofilm dispersion was found to be specifically tied to NO signaling, and increased transcription of NbdA was observed following NO exposure [31]. Similarly, the PDE RbdA also promotes biofilm dispersal [73]. This protein contains both GGDEF and EAL domains; however, only exhibits PDE activity [73]. RbdA activity is regulated by the GGDEF domain, which acts as an allosteric activation site when bound to GTP [38, 73]. RbdA also contains a PAS domain and disruption of this domain inhibits biofilm dispersal, indicating that RbdA may be important for sensing anoxic conditions and promoting dispersal via PDE activity [73].

DipA (Pch) is another PDE that promotes biofilm dispersion, flagella-mediated motility, and chemotaxis in response to nutrient and environmental conditions, including glutamate, NO, ammonium chloride, and mercury chloride [33, 74]. DipA localizes to the flagellated cell pole leading to asymmetrical PDE distribution following cell division. This results in heterogeneous subpopulations that exhibit a range of motility phenotypes, which is predicted to promote bacterial survival in dynamic environments [41]. As an additional level of control, DipA activity is regulated by the inner membrane-bound DGC, NicD, and the chemotaxis protein, BdlA. In the presence of extracellular glutamate, NicD is dephosphorylated

leading to increased DGC activity [28]. BdlA is subsequently phosphorylated and activated by NicD-produced cyclic di-GMP and nonprocessive proteolysis via ClpP and GcbA [34, 39, 75]. Once activated, BdlA promotes DipA PDE activity resulting in biofilm dispersion. Thus, NicD and GcbA appears to temporarily increase cyclic di-GMP in response to extracellular nutrients, which leads to DipA PDE activation and biofilm dispersal through reducing cyclic di-GMP levels. As mentioned previously, GcbA DGC activity also promotes surface attachment by inhibiting flagella-mediated motility. These seemingly opposing dual functions for GcbA further highlight the intricate regulatory system required to transition between sessile and motile lifestyles.

The RocSAR system in PAO1 and SadARS in PA14 are homologous three-component systems with multiple response regulators (Fig. 28.2c). This system regulates the production of cup fimbriae adhesins and the Type III secretion system (T3SS) [35, 36]. One of the response regulators in each of these systems (RocR/SadR) contains an active EAL domain [76]. RocS1 functions as the sensor kinase, which has a domain associated with solute-binding proteins (Sbp3) and a PAS domain in the periplasm [35]. The presence of a PAS domain suggests oxygen or redox signaling activates the phosphorelay in this system [37]. A paralog of RocS1, named RocS2, has also been identified and functions similarly to RocS1 [77]. RocS1 and RocS2 activate RocR, RocA1, and RocA2 via phosphorylation at the histidine-containing phosphotransfer (HPt) domain [35, 76]. Activation of RocA1 results in increased Cup fimbriae formation and inhibited T3SS gene expression, while RocA2 phosphorylation leads to decreased mexAB-oprM-dependent efflux activity [36, 77]. RocR contains an active PDE domain and functions antagonistically on the Roc system [76, 77]. RocR activation by RocS1 and RocS2 inhibits *cupC* fimbriae expression and attachment, likely due to reduced cyclic di-GMP levels.

28.4 Conclusions and Moving Forward

Our understanding of the regulation and function of cyclic di-GMP in *P. aeruginosa* has improved considerably since its original discovery in 1987 [78]. As discussed here, a significant amount of work has gone into identifying cyclic di-GMP regulators, and the effect these systems have on the cell. However, considering that the *P. aeruginosa* genome encodes 38 proteins with conserved GGDEF and EAL domains, many proteins involved in the synthesis and degradation of cyclic di-GMP remain to be discovered. Moreover, there remains a large disparity in the number of DGC/PDEs compared to cyclic di-GMP effectors. To date, only four cyclic di-GMP effectors that bind cyclic di-GMP have been identified, Alg44, PelD, FimX, and FleQ [42, 65, 67, 68]. The role of these effectors is related to the transition between motile and sessile lifestyles. However, a recent study determined that the DGC PA3177 promotes antibiotic tolerance independent of biofilm formation [79]. These findings suggest that a broader role for cyclic di-GMP effectors may exist for *P. aeruginosa*, and given the quantity of DGCs and PDEs, it is likely that

many cyclic di-GMPs effectors are still unidentified. Two studies have attempted to address this, and using cyclic di-GMP binding affinity assays have identified a number of predicted possible effectors [22, 80]. Future studies that verify and identify the function of cyclic di-GMP effectors will be necessary to fully understand the extent of cyclic di-GMP regulation in *P. aeruginosa*.

Another major hurdle in understanding cyclic di-GMP regulation is related to signal specificity. Cyclic di-GMP controls a range of processes which effectively share the same signaling molecule, and it is unclear how a specific process is targeted by cyclic di-GMP produced by any given DGC. In the simplest model, cyclic di-GMP could be thought of as a global pool within the cell. Here, all active DGCs and PDEs would contribute to raising or lowering the total pool of cyclic di-GMP, which in turn alters cyclic di-GMP effector activity. The relative activity of a given cyclic di-GMP target could then be further controlled by differences in binding affinity among individual effectors. In this system, reaching various cyclic di-GMP concentrations would result in either motility or biofilm phenotypes. A global pool model could explain the observed redundancy among DGC effects, such as WspR and YfiN, which both upregulate Pel and Psl production [25, 64]. Further evidence supporting a global pool model is observed in regards to T4P formation. As described previously, T4P assembly at low cyclic di-GMP concentrations requires functional FimX PDE activity, but high levels of cyclic di-GMP bypass the requirement of FimX [40]. Jain and colleagues (2012) also report that FimX could be bypassed in *P. aeruginosa* by overexpression of an unrelated DGC isolated from *Caulobacter crescentus*. This suggests the source of cyclic di-GMP may be irrelevant in this system and high global levels of cyclic di-GMP are sufficient to stimulate T4P production. Contrary to this model, there is also evidence suggesting cyclic di-GMP signaling is highly specific to a particular target. In a local pool model, DGCs and PDEs are trafficked to the vicinity of their target effector. This would allow for the creation of local cyclic di-GMP pools that act only on their nearby effectors. Merritt and colleagues (2010) report that the activity of two DGCs, RoeA and SadC, have different and distinct effects on biofilm formation, despite producing similar total levels of cyclic di-GMP [21]. Furthermore, SadC and RoeA appear to be differentially distributed throughout the cell. While both proteins are localized to the inner membrane, SadC forms distinct foci around the cell periphery, and RoeA forms a diffuse patchy background. Differing localization patterns could explain why SadC controls swarming motility, and RoeA controls Pel production, despite producing similar levels of cyclic di-GMP [20]. It is possible that a combination of these models function in *P. aeruginosa*, depending on the effector, but future studies will be necessary to fully understand cyclic di-GMP signaling.

In conclusion, we have made great strides in understanding cyclic di-GMP regulation, but many of the details regarding its function and regulation remain elusive. Cyclic di-GMP controls a number of virulence factors associated with chronic infection, and the continued study of cyclic di-GMP in *P. aeruginosa* will provide important insights into biofilm formation, development, and dispersal.

References

1. Magill SS, Edwards JR, Bamberg W et al (2014) Multistate point-prevalence survey of health care-associated infections. N Engl J Med 370:1198–1208. https://doi.org/10.1056/NEJMoa1306801
2. European Centre for Disease Prevention and Control (2015) Antimicrobial resistance surveillance in Europe 2015. Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net)
3. Stover CK, Pham XQ, Erwin AL et al (2000) Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature 406:959–964
4. Galán-Vásquez E, Luna B, Martínez-Antonio A (2011) The regulatory network of *Pseudomonas aeruginosa*. Microb Inform Exp 1:3. https://doi.org/10.1186/2042-5783-1-3
5. Hall-Stoodley L, Stoodley P (2009) Evolving concepts in biofilm infections. Cell Microbiol 11:1034–1043. https://doi.org/10.1111/j.1462-5822.2009.01323.x
6. Costerton JW (1999) Bacterial biofilms: a common cause of persistent infections. Science 284:1318–1322. https://doi.org/10.1126/science.284.5418.1318
7. Ma L, Jackson KD, Landry RM et al (2006) Analysis of *Pseudomonas aeruginosa* conditional psl variants reveals roles for the *psl* polysaccharide in adhesion and maintaining biofilm structure postattachment. J Bacteriol 188:8213–8221. https://doi.org/10.1128/JB.01202-06
8. Billings N, Millan M, Caldara M et al (2013) The extracellular matrix component Psl provides fast-acting antibiotic defense in *Pseudomonas aeruginosa* biofilms. PLoS Pathog 9:e1003526. https://doi.org/10.1371/journal.ppat.1003526
9. Colvin KM, Gordon VD, Murakami K et al (2011) The pel polysaccharide can serve a structural and protective role in the biofilm matrix of *Pseudomonas aeruginosa*. PLoS Pathog 7. https://doi.org/10.1371/journal.ppat.1001264
10. Römling 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
11. Ha D-G, O'Toole GA (2015) c-di-GMP and its effects on biofilm formation and dispersion: a *Pseudomonas aeruginosa* review. Microbiol Spectr 3. https://doi.org/10.1128/microbiolspec.MB-0003-2014.c-di-GMP
12. Kulasakara H, Lee V, Brencic A et al (2006) Analysis of *Pseudomonas aeruginosa* diguanylate cyclases and phosphodiesterases reveals a role for bis-(3′-5′)-cyclic-GMP in virulence. Proc Natl Acad Sci USA 103:2839–2844. https://doi.org/10.1073/pnas.0511090103
13. Hickman JW, Tifrea DF, Harwood CS (2005) A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. Proc Natl Acad Sci USA 102:14422–14427. https://doi.org/10.1073/pnas.0507170102
14. Guvener Z, Harwood C (2007) Subcellular location characteristics of the *Pseudomonas aeruginosa* GGDEF protein, WspR, indicate that it produces cyclic-di-GMP in response to growth on surfaces. Mol Microbiol 66:1459–1473. https://doi.org/10.1016/j.biotechadv.2011.08.021.Secreted
15. Blanka A, Düvel J, Dötsch A et al (2015) Constitutive production of c-di-GMP is associated with mutations in a variant of *Pseudomonas aeruginosa* with altered membrane composition. Sci Signal 8:ra36. https://doi.org/10.1126/scisignal.2005943
16. O'Connor JR, Kuwada NJ, Huangyutitham V et al (2012) Surface sensing and lateral subcellular localization of WspA, the receptor in a chemosensory-like system leading to c-di-GMP production. Mol Microbiol 86:720–729. https://doi.org/10.1111/mmi.12013.Surface
17. De N, Pirruccello M, Krasteva PV et al (2008) Phosphorylation-independent regulation of the diguanylate cyclase WspR. PLoS Biol 6:0601–0617. https://doi.org/10.1371/journal.pbio.0060067
18. Irie Y, Borlee BR, O'Connor JR et al (2012) Self-produced exopolysaccharide is a signal that stimulates biofilm formation in *Pseudomonas aeruginosa*. Proc Natl Acad Sci USA 109:20632–20636. https://doi.org/10.1073/pnas.1217993109

19. Merritt JH, Brothers KM, Kuchma SL, O'Toole GA (2007) SadC reciprocally influences biofilm formation and swarming motility via modulation of exopolysaccharide production and flagellar function. J Bacteriol 189:8154–8164. https://doi.org/10.1128/JB.00585-07
20. Luo Y, Zhao K, Baker AE et al (2015) A hierarchical cascade of second messengers regulates *Pseudomonas aeruginosa* surface behaviors. MBio 6:1–11. https://doi.org/10.1128/mBio.02456-14
21. Merritt JH, Ha D-G, Cowles KN et al (2010) Specific control of *Pseudomonas aeruginosa* surface-associated behaviors by two c-di-GMP diguanylate cyclases. MBio 1:1–9. https://doi.org/10.1128/mBio.00183-10.Editor
22. Düvel J, Bertinetti D, Möller S et al (2012) A chemical proteomics approach to identify c-di-GMP binding proteins in *Pseudomonas aeruginosa*. J Microbiol Methods 88:229–236. https://doi.org/10.1016/j.mimet.2011.11.015
23. Petrova OE, Cherny KE, Sauer K (2014) The *Pseudomonas aeruginosa* diguanylate cyclase GcbA, a homolog of *P. fluorescens* GcbA, promotes initial attachment to surfaces, but not biofilm formation, via regulation of motility. J Bacteriol 196:2827–2841. https://doi.org/10.1128/JB.01628-14
24. Klebensberger J, Birkenmaier A, Geffers R et al (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
25. Malone JG, Jaeger T, Manfredi P et al (2012) The YfiBNR signal transduction mechanism reveals novel targets for the evolution of persistent *Pseudomonas aeruginosa* in cystic fibrosis airways. PLoS Pathog 8:e1002760. https://doi.org/10.1371/journal.ppat.1002760
26. Strempel N, Nusser M, Neidig A et al (2017) The oxidative stress agent hypochlorite stimulates c-di-GMP synthesis and biofilm formation in *Pseudomonas aeruginosa*. Front Microbiol 8:1–15. https://doi.org/10.3389/fmicb.2017.02311
27. 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
28. Roy A, Sauer K (2014) Diguanylate cyclase NicD-based signalling mechanism of nutrient-induced dispersion by *Pseudomonas aeruginosa*. Mol Microbiol 94:771–793. https://doi.org/10.1111/mmi.12802
29. Phippen CW, Mikolajek H, Schlaefli HG et al (2014) Formation and dimerization of the phosphodiesterase active site of the *Pseudomonas aeruginosa* MorA, a bi-functional c-di-GMP regulator. FEBS Lett 588:4631–4636. https://doi.org/10.1016/j.febslet.2014.11.002
30. Rau MH, Hansen SK, Johansen HK et al (2010) Early adaptive developments of *Pseudomonas aeruginosa* after the transition from life in the environment to persistent colonization in the airways of human cystic fibrosis hosts. Environ Microbiol 12:1643–1658. https://doi.org/10.1111/j.1462-2920.2010.02211.x
31. Li Y, Heine S, Entian M et al (2013) NO-induced biofilm dispersion in *Pseudomonas aeruginosa* is mediated by an MHYT domain-coupled phosphodiesterase. J Bacteriol 195:3531–3542. https://doi.org/10.1128/JB.01156-12
32. Poudyal B, Sauer K (2018) The PA3177 gene encodes an active diguanylate cyclase that formation by *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 62:1–17
33. Roy AB, Petrova OE, Sauer K (2012) The phosphodiesterase DipA (PA5017) is essential for *Pseudomonas aeruginosa* biofilm dispersion. J Bacteriol 194:2904–2915. https://doi.org/10.1128/JB.05346-11
34. Petrova OE, Cherny KE, Sauer K (2015) The diguanylate cyclase GcbA facilitates *Pseudomonas aeruginosa* biofilm dispersion by activating BdlA. J Bacteriol 197:174–187. https://doi.org/10.1128/JB.02244-14
35. Kulasekara HD, Ventre I, Kulasekara BR et al (2005) A novel two-component system controls the expression of *Pseudomonas aeruginosa* fimbrial *cup* genes. Mol Microbiol 55:368–380. https://doi.org/10.1111/j.1365-2958.2004.04402.x

36. Kuchma SL, Connolly JP, O'Toole GA (2005) A three-component regulatory system regulates biofilm maturation and type III secretion in *Pseudomonas aeruginosa*. J Bacteriol 187:1441–1454. https://doi.org/10.1128/JB.187.4.1441-1454.2005
37. Hengge R (2009) Principles of c-di-GMP signalling in bacteria. Nat Rev Microbiol 7:263–273. https://doi.org/10.1038/nrmicro2109
38. Liu C, Liew CW, Wong YH et al (2018) Insights into biofilm dispersal regulation from the crystal structure of the PAS-GGDEF-EAL region of RbdA from *Pseudomonas aeruginosa*. J Bacteriol 200:1–19. https://doi.org/10.1128/JB.00515-17
39. Petrova OE, Sauer K (2012) Dispersion by *Pseudomonas aeruginosa* requires an unusual posttranslational modification of BdlA. Proc Natl Acad Sci 109:16690–16695. https://doi.org/10.1073/pnas.1207832109
40. Jain R, Behrens AJ, Kaever V, Kazmierczak BI (2012) Type IV pilus assembly in *Pseudomonas aeruginosa* over a broad range of cyclic di-GMP concentrations. J Bacteriol 194:4285–4294. https://doi.org/10.1128/JB.00803-12
41. Kulasekara BR, Kamischke C, Kulasekara HD et al (2013) c-di-GMP heterogeneity is generated by the chemotaxis machinery to regulate flagellar motility. elife 2013:1–19. https://doi.org/10.7554/eLife.01402
42. Navarro M, De N, Bae N et al (2009) Structural analysis of the GGDEF-EAL domain-containing c-di-GMP receptor FimX. Structure 17:1104–1116. https://doi.org/10.1016/j.str.2009.06.010.Structural
43. Galperin MY, Nikolskaya AN, Koonin EV (2001) Novel domains of the prokaryotic two-component signal transduction systems. FEMS Microbiol Lett 203:11–21. https://doi.org/10.1016/S0378-1097(01)00326-3
44. Jenal U, Malone J (2006) Mechanisms of cyclic-di-GMP signaling in bacteria. Annu Rev Genet 40:385–407. https://doi.org/10.1146/annurev.genet.40.110405.090423
45. Francis VI, Stevenson EC, Porter SL (2017) Two-component systems required for virulence in *Pseudomonas aeruginosa*. FEMS Microbiol Lett 364:1–22. https://doi.org/10.1093/femsle/fnx104
46. 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
47. Ventre I, Goodman AL, Vallet-Gely I et al (2006) Multiple sensors control reciprocal expression of *Pseudomonas aeruginosa* regulatory RNA and virulence genes. Proc Natl Acad Sci USA 103:171–176. https://doi.org/10.1073/pnas.0507407103
48. Goodman AL, Merighi M, Hyodo M et al (2009) Direct interaction between sensor kinase proteins mediates acute and chronic disease phenotypes in a bacterial pathogen. Genes Dev 23:249–259. https://doi.org/10.1101/gad.1739009
49. Jing X, Jaw J, Robinson HH, Schubot FD (2010) Crystal structure and oligomeric state of the RetS signaling kinase sensory domain. Proteins 78:1631–1640. https://doi.org/10.1002/prot.22679.Crystal
50. Broder UN, Jaeger T, Jenal U (2016) LadS is a calcium-responsive kinase that induces acute-to-chronic virulence switch in *Pseudomonas aeruginosa*. Nat Microbiol 2:16184
51. Brencic A, Mcfarland KA, Mcmanus HR et al (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
52. Irie Y, Starkey M, Edwards AN et al (2010) *Pseudomonas aeruginosa* biofilm matrix polysaccharide Psl is regulated transcriptionally by RpoS and post-transcriptionally by RsmA. Mol Microbiol 78:158–172. https://doi.org/10.1111/j.1365-2958.2010.07320.x
53. Moscoso JA, Jaeger T, Valentini M et al (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

54. Colley B, Dederer V, Carnell M et al (2016) SiaA/D interconnects c-di-GMP and RsmA signaling to coordinate cellular aggregation of *Pseudomonas aeruginosa* in response to environmental conditions. Front Microbiol 7:1–13. https://doi.org/10.3389/fmicb.2016.00179
55. Jones CJ, Newsom D, Kelly B et al (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
56. Kuchma SL, Ballok AE, Merrit JH et al (2010) Cyclic-di-GMP-mediated repression of swarming motility by *Pseudomonas aeruginosa*: the pilY1 gene and its impact on surface-associated behaviors. J Bacteriol 192:2950–2964. https://doi.org/10.1128/JB.01642-09
57. Klausen M, Heydorn A, Ragas P et al (2003) Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. Mol Microbiol 48:1511–1524. https://doi.org/10.1046/j.1365-2958.2003.03525.x
58. Kazmierczak BI, Lebron MB, Murray TS (2006) Analysis of FimX, a phosphodiesterase that governs twitching motility in *Pseudomonas aeruginosa*. Mol Microbiol 60:1026–1043. https://doi.org/10.1111/j.1365-2958.2006.05156.x
59. D'Argenio DA, Calfee MW, Rainey PB, Pesci EC (2002) Autolysis and autoaggregation in *Pseudomonas aeruginosa* colony morphology mutants. J Bacteriol 184:6481–6489. https://doi.org/10.1128/JB.184.23.6481
60. Starkey M, Hickman JH, Ma L et al (2009) *Pseudomonas aeruginosa* rugose small-colony variants have adaptations that likely promote persistence in the cystic fibrosis lung. J Bacteriol 191:3492–3503. https://doi.org/10.1128/JB.00119-09
61. Borlee BR, Goldman AD, Murakami K et al (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
62. Bantinaki E, Kassen R, Knight CG et al (2007) Adaptive divergence in experimental populations of *Pseudomonas fluorescens*. III. Mutational origins of wrinkly spreader diversity. Genetics 176:441–453. https://doi.org/10.1534/genetics.106.069906
63. Pestrak MJ, Chaney SB, Eggleston HC et al (2018) *Pseudomonas aeruginosa* rugose small-colony variants evade host clearance, are hyper-inflammatory, and persist in multiple host environments. PLoS Pathog 14:1–22. https://doi.org/10.1371/journal.ppat.1006842
64. Colvin KM, Irie Y, Tart CS et al (2012) The Pel and Psl polysaccharides provide *Pseudomonas aeruginosa* structural redundancy within the biofilm matrix. Environ Microbiol 14:1913–1928. https://doi.org/10.1111/j.1462-2920.2011.02657.x.The
65. Baraquet C, Murakami K, Parsek MR, Harwood CS (2012) The FleQ protein from *Pseudomonas aeruginosa* functions as both a repressor and an activator to control gene expression from the Pel operon promoter in response to c-di-GMP. Nucleic Acids Res 40:7207–7218. https://doi.org/10.1093/nar/gks384
66. 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.Identification
67. Lee VT, Matewish JM, Kessler JL et al (2007) A cyclic-di-GMP receptor required for bacterial exopolysaccharide production. Mol Microbiol 65:1474–1484. https://doi.org/10.1111/j.1365-2958.2007.05879.x
68. Merighi M, Lee VT, Hyodo M et al (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
69. Malone JG, Jaeger T, Spangler C et al (2010) YfiBNR mediates cyclic di-GMP dependent small colony variant formation and persistence in *Pseudomonas aeruginosa*. PLoS Pathog 6: e1000804. https://doi.org/10.1371/journal.ppat.1000804
70. Choy WK, Zhou L, Syn CKC et al (2004) MorA defines a new class of regulators affecting flagellar development and biofilm formation in diverse *Pseudomonas* species. J Bacteriol 186:7221–7228. https://doi.org/10.1128/JB.186.21.7221-7228.2004

71. Sauer K, Cullen MC, Rickard AH et al (2004) Characterization of nutrient-induced dispersion in *Pseudomonas aeruginosa* PAO1 biofilm. J Bacteriol 186:7312–7326. https://doi.org/10.1128/JB.186.21.7312
72. Barraud N, Hassett DJ, Hwang SH et al (2006) Involvement of nitric oxide in biofilm dispersal of *Pseudomonas aeruginosa*. J Bacteriol 188:7344–7353. https://doi.org/10.1128/JB.00779-06
73. An S, Wu J, Zhang LH (2010) Modulation of *Pseudomonas aeruginosa* biofilm dispersal by a cyclic-di-gmp phosphodiesterase with a putative hypoxia-sensing domain. Appl Environ Microbiol 76:8160–8173. https://doi.org/10.1128/AEM.01233-10
74. Li Y, Xia H, Bai F et al (2007) Identification of a new gene PA5017 involved in flagella-mediated motility, chemotaxis and biofilm formation in *Pseudomonas aeruginosa*. FEMS Microbiol Lett 272:188–195. https://doi.org/10.1111/j.1574-6968.2007.00753.x
75. Petrova OE, Sauer K (2012) PAS domain residues and prosthetic group involved in BdlA dependent dispersion response by *Pseudomonas aeruginosa* biofilms. J Bacteriol 194:5817–5828. https://doi.org/10.1128/JB.00780-12
76. Rao F, Yang Y, Qi Y, Liang ZX (2008) Catalytic mechanism of cyclic di-GMP-specific phosphodiesterase: a study of the EAL domain-containing RocR from *Pseudomonas aeruginosa*. J Bacteriol 190:3622–3631. https://doi.org/10.1128/JB.00165-08
77. Sivaneson M, Mikkelsen H, Ventre I et al (2011) Two-component regulatory systems in *Pseudomonas aeruginosa*: an intricate network mediating fimbrial and efflux pump gene expression. Mol Microbiol 79:1353–1366. https://doi.org/10.1111/j.1365-2958.2010.07527.x
78. Ross P, Weinhouse H, Aloni Y et al (1987) Regulation of cellulose synthesis in *Acetobacter xylinum* by cyclic diguanylic acid. Nature 325:279–281
79. Poudyal B, Sauer K (2018) The PA3177 gene encodes an active diguanylate cyclase that contributes to biofilm antimicrobial tolerance but not biofilm formation by *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 62:e01049-18. https://doi.org/10.1128/AAC.01049-18
80. Nesper J, Reinders A, Glatter T et al (2012) A novel capture compound for the identification and analysis of cyclic di-GMP binding proteins. J Proteome 75:4874–4878. https://doi.org/10.1016/j.jprot.2012.05.033
81. Kuchma SL, Brothers KM, Merritt JH et al (2007) BifA, a cyclic-di-GMP phosphodiesterase, inversely regulates biofilm formation and swarming motility by *Pseudomonas aeruginosa* PA14. J Bacteriol 189:8165–8178. https://doi.org/10.1128/JB.00586-07