Received: 7 August 2019 | Revised: 7 November 2019 | Accepted: 11 November 2019

DOI: 10.1002/mbo3.975

ORIGINAL ARTICLE

MicrobiologyOpen Open Access WILEY

Evaluation and characterization of the predicted diguanylate cyclase-encoding genes in *Pseudomonas aeruginosa*

Pramod Bhasme[1,2] | **Qing Wei**[1] | **Anming Xu**[1,2] | **Syed Tatheer Alam Naqvi**[1] | **Di Wang**[1] | **Luyan Z. Ma**[1,2]

[1]State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China

[2]University of Chinese Academy of Sciences, Beijing, China

Correspondence
Luyan Z. Ma, State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China.
Email: Luyanma27@im.ac.cn

Present address
Syed Tatheer Alam Naqvi, Department of Biotechnology, COMSATS University Islamabad, Islamabad, Pakistan

Funding information
National Natural Science Foundation of China, Grant/Award Number: 31570126 and 31300066; Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry

Abstract

Opportunistic pathogen *Pseudomonas aeruginosa* can cause acute and chronic infections in humans. It is notorious for its resistance to antibiotics due to the formation of biofilms. Cyclic-di-GMP is a bacterial second messenger that plays important roles during biofilm development. There are 40 genes in *P. aeruginosa* predicted to participate in c-di-GMP biosynthesis or degradation. It is time-consuming for the functional characterization of these genes. Here, we cloned 16 genes from *P. aeruginosa* PAO1 that are predicted to encode diguanylate cyclases (DGCs, responsible for c-di-GMP biosynthesis) and constructed their corresponding in-frame deletion mutants. We evaluated the methods to measure the intracellular c-di-GMP concentration by using deletion mutants and PAO1 strains containing a plasmid expressing one of the 16 genes, respectively. Functional outputs of all PAO1-derived stains were also detected and evaluated, including biofilm formation, production of exopolysaccharide, swimming and swarming motilities. Our data showed that measuring the c-di-GMP level only characterized a few DGC by using either pCdrA::*gfp* as a reporter or LC/MS/MS. Functional output results indicated that overexpression of a DGC gave more pronounced phenotypes than the corresponding deletion mutant and suggested that the swimming motility assay could be a quick way to briefly estimate a predicted DGC for further studies. The overall evaluation suggested 15 out of 16 predicted DGCs were functional DGCs, wherein six were characterized to encode DGCs previously. Altogether, we have provided not only a cloning library of 16 DGC-encoding genes and their corresponding in-frame deletion mutants but also paved ways to briefly characterize a predicted DGC.

KEYWORDS
Cyclic-di-GMP, diguanylate cyclases, motility, Pseudomonas aeruginosa

1 | INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen that can infect humans, animals, and plants (Plotnikova, Rahme, & Ausubel, 2000; Stover et al., 2000). It can cause nosocomial infections such as pneumonia and cystic fibrosis (CF) that are refractory to eradicate due to its capability to form biofilms (Lebeaux, Ghigo, & Beloin, 2014; Lyczak, Cannon, & Pier, 2000; Mulcahy, Isabella, & Lewis, 2014). The surface-associated microbial communities called biofilms are surrounded by a matrix of

extracellular polymeric substances including exopolysaccharides, extracellular DNA, and proteins (Decho & Gutierrez, 2017; Fong & Yildiz, 2015; Koo, Allan, Howlin, Stoodley, & Hall-Stoodley, 2017; Wei & Ma, 2013). The *bis*-(3′-5′)-cyclic guanosine monophosphate (c-di-GMP) is an important second messenger that controls the switch from a planktonic to biofilm lifestyle (Boyd & O'Toole, 2012; Hengge, 2009; Romling, Galperin, & Gomelsky, 2013) and regulates a wide range of biological activities (Fu et al., 2018; Ryan, Fouhy, Lucey, & Dow, 2006). For example, c-di-GMP regulates the expression of biofilm matrix polysaccharide Psl and Pel in *P. aeruginosa* (Ha & O'Toole, 2015; Klausen, Aaes-Jorgensen, Molin, & Tolker-Nielsen, 2003; Liang, 2015) and regulates the virulence in a murine model of acute infection (Kulasakara et al., 2006). The c-di-GMP has also been involved in the regulation of bacterial motility such as flagella-mediated swimming and swarming motilities. It was demonstrated that c-di-GMP could fine-tune swimming speed by binding to a molecular brake, YcgR (Boehm et al., 2010).

The c-di-GMP is synthesized from two molecules of GTP by diguanylate cyclases (DGCs) and degraded into 5′-phosphoguanylyl-(3′-5′)-guanosine (pGpG) by specific phosphodiesterases (PDEs); later, pGpG was split into two GMP molecules by ribonuclease Orn (Orr et al., 2015; Stelitano et al., 2013). Bioinformatic analysis showed that DGC activity is associated with the presence of conserved GGDEF domain while PDE activity is associated with conserved EAL or HD-GYP domains (Chan et al., 2004; Paul et al., 2004; Schmidt, Ryjenkov, & Gomelsky, 2005). Therefore, the intracellular c-di-GMP is depended on the active DGCs and PDEs. In *P. aeruginosa*, there are at least 40 predicted c-di-GMP metabolizing proteins (Ha, Richman, & O'Toole, 2014; Kulasakara et al., 2006). Comprehensive analyses of those c-di-GMP metabolizing genes were performed on strain PA14, which is a native Psl-negative strain (Liberati et al., 2006). Afterward, the deletion library of c-di-GMP metabolizing genes was constructed and characterized briefly for their ability to form biofilms and motilities (Ha et al., 2014). Those data have provided means to demonstrate the potential use of this library. However, the phenotype of mutants was very complex and difficult to figure out a gene functioned as DGC or PDE. Thus, it is necessary to evaluate the current methods to find an effective way to characterize a DGC or PDE.

In this study, we selected 16 predicted DGC-encoding genes from *P. aeruginosa* strain PAO1 that only contain conserved GGDEF domain to evaluate the methods for DGC characterization, which includes the measurement of intracellular c-di-GMP concentration and the functional outputs of all PAO1-derived stains, including biofilm formation, the production of exopolysaccharide Psl, swimming and swarming motilities. Our work would help the researcher to go depth into their interested DGCs in bacteria.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains and growth conditions

Strains, plasmids, and primers used in this study are listed in Tables A1 and A2. *Pseudomonas aeruginosa* strain PAO1 and *Escherichia coli* were used in this study. PAO1 and *E. coli* strains were routinely grown at 37°C in Luria-Bertani (LB) medium. *Pseudomonas aeruginosa* strains were grown in LB medium without sodium chloride or in Jensen's, a chemically defined medium. Antibiotics were added at the following final concentrations when required: ampicillin (Ap), 100 μg/ml; carbenicillin (Carb), 300 μg/ml; gentamicin (Gm), 10 μg/ml; Irgasan (Irg) 25 μg/ml.

2.2 | Molecular techniques

The pUCP20 vector used to overexpress the GGDEF genes by standard transformation technique and pEX18Gm vector was used to construct single and double mutants by in-frame deletion technique.

2.3 | Biofilm assays

Biofilm assay was performed as previously described (O'Toole & Kolter, 1998). Briefly, bacterial cultures grown overnight in LB were diluted 1:100 in Jensen's medium and grown in triplicate in a polyvinylchloride plate (Costar) overnight at 30°C without agitation, followed by staining with 0.1% crystal violet for 30 min at room temperature and then washed twice by dipping into standing water bath. The adherent stain was solubilized in 30% acetic acid and quantified by measuring its optical density at 560 nm.

2.4 | Motility assays

Motility assay was performed as previously described (Caiazza, Shanks, & O'Toole, 2005). Briefly, bacterial cultures were grown overnight in LB and inoculated culture on swarm agar (nutrient broth-8 g/L, D-glucose-5 g/L, bacto agar-5 g/L) and swim agar (tryptone-10 g/L, yeast extract-5 g/L agar-1.5 g/L) plates. Plates were incubated upright at 37°C overnight. Swarming and swimming zones were measured accordingly.

2.5 | Psl immunoblotting

The immunoblotting assay was performed as previously described (Byrd et al., 2009). Briefly, bacterial strains were incubated in Jensen's liquid culture for 24 hr and bacterial surface-bound polysaccharide were extracted from cultures with OD600nm of 10. Cells were centrifuged, and the pellet was resuspended in 100 μl of 0.5 M EDTA. Cells were boiled at 100°C for 10 min and centrifuged. The supernatant was collected and treated with proteinase K for 60 min at 60°C (final concentration 0.5 mg/ml), followed by proteinase K inactivation for 30 min at 80°C. 3 μl of polysaccharide preparations was spotted onto a nitrocellulose membrane. The blocking step was performed using 10% milk solution in TBST (20 mM Tris, 137 mM NaCl, 0.1% Tween 20,

pH 7.6) for 1 hr at room temperature and washed with TBST, and then probed with antibody α-Psl (1:25,000 in TBST) for 1 hr with agitation then a second antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG used at 1:10,000 in TBST) for 1 hr. About 5 ml of Western blue (Promega) was added to stain the dots (Zhu et al., 2016). The analysis was done using Image laboratory 5.1 software.

2.6 | Quantification of c-di-GMP using pCdrA::*gfp*

Indirect c-di-GMP quantified by pCdrA::*gfp* was performed with some modifications as previously described (Rybtke et al., 2012). Briefly, pCdrA::*gfp* plasmid was transformed into bacteria by using a standard transformation technique. The strain containing pCdrA::*gfp* plasmid was grown overnight and was diluted 1:100 in Jensen's medium containing carbenicillin and grown in triplicate in 96-well plate (NEST) at 37°C for 24 hr. OD_{600} and fluorescence (excitation 485 nm, emission 520 nm) were read accordingly.

2.7 | Quantification of c-di-GMP using LC-MS/MS

Quantification of c-di-GMP was performed as previously described (Spangler, Bohm, Jenal, Seifert, & Kaever, 2010). Samples of interest were compared to a standard curve derived from measurements of known concentrations of pure c-di-GMP to determine the concentration (nM) of c-di-GMP in the samples. The data were then normalized to the total protein content of the sample determined by BCA assay (Thermo); for each strain, the experiment was done in biological triplicate, and LC-MS/MS measurements were repeated in duplicate. Data are presented as pmol of c-di-GMP/mg of total protein.

2.8 | Statistical analysis

The data of this study were analyzed by one-way ANOVA. Student's *t* test was used when one-way ANOVA revealed significant differences ($p < .05$). All statistical analyses were performed with GraphPad Prism statistical software (GraphPad Software) with the help of Excel (Microsoft).

3 | RESULTS

3.1 | Quantification of c-di-GMP generation potential of predicted DGC-encoding genes in PAO1

In bacteria, DGC is implicated in the synthesis of intracellular c-di-GMP. Imperatively, overexpression of DGC-encoding genes would produce a higher amount of intracellular c-di-GMP and deletion of DGC-encoding genes would generate less c-di-GMP in bacterial cells. We chose 16 predicted DGC-encoding genes that containing only GGDEF domain to evaluate the methods to measure the intracellular c-di-GMP level for a DGC characterization. We generated in-frame deletion mutants of the 16 genes in *P. aeruginosa* PAO1. Meanwhile, we cloned each of them into the expression vector pUCP20 and then transferred each plasmid into PAO1 to get overexpression variants. The LC/MS/MS method was used to quantify the intracellular c-di-GMP level of strains overexpressed DGC-encoding genes. As can be seen from Figure 1, among 16 genes, only six genes (PA4332, PA0169, PA4843, PA4929, PA2870, and PA3702) showed a significantly higher amount of c-di-GMP than that of wild type (Figure 1a). Among those six genes, four genes (PA4332, PA0169, PA3702, and PA4843) are well-studied DGC-encoding genes and confirmed in our study (Jones et al., 2015; Zhu et al., 2016). We then used the pCdrA::*gfp* as a reporter to monitor the relative c-di-GMP level in the 16 deletion mutants. The six mutants out of 16 (ΔPA0169, ΔPA0847, ΔPA1120, ΔPA4332, ΔPA3177, and ΔPA3702) exhibited significantly lower c-di-GMP level than that of wild type (Figure 1b). PA4332, PA0169, and PA3702 exhibited significant changes in either in-frame deletion mutants or DGC overexpression strains, while the other genes could not detect a change on the c-di-GMP level by one of the methods or both even if the genes have been characterized as DGCs previously, such as PA1107 and PA1120. This revealed that the quantification of c-di-GMP is not sufficient for the identification of DGC activities in *P. aeruginosa* under the tested conditions.

3.2 | C-di-GMP-related phenotypes by overexpression analysis

We then performed four major c-di-GMP associated phenotypes (functional output of DGC) of DGC-overexpressed strains in order to identify a DGC. Firstly, biofilm formation of all overexpression mutants was determined. Quantification of biofilm formation was performed in 96-well polyvinylchloride plate. There were 10 predicted DGC-encoding genes that showed significant higher biofilm formation ability than the control strain PAO1/pUCP20 (Figure 2a). Among those genes, PA0169, PA0290, PA0847, and PA4929 produced more than 200% of biofilms as compared to wild-type PAO1, while another six genes (PA0338, PA1107, PA1120, PA2870, PA3702, and PA4332) produced more than 150% of biofilm compared to control.

Afterward, we examined the production of Psl exopolysaccharide. Out of 16 genes, six genes (PA0169/*siaD*, PA0847, PA2870, PA3702, PA4332, PA4929, and PA5487) produced 150% of Psl polysaccharide as compared to wild-type PAO1, while other overexpression of other genes gave rise to no differential results (Figure 2b). Finally, we detected the swarming and swimming motilities. Strikingly, all 16 genes significantly reduced the swarming and swimming motilities (Figure 2c,d). In particular, PA0847, PA3702, and PA4332 completely lost swimming motility (Figure 2d). Taken together, for overexpression analysis, the motility assay showed the most distinct phenotype and could be a quick way to estimate a predicted DGC.

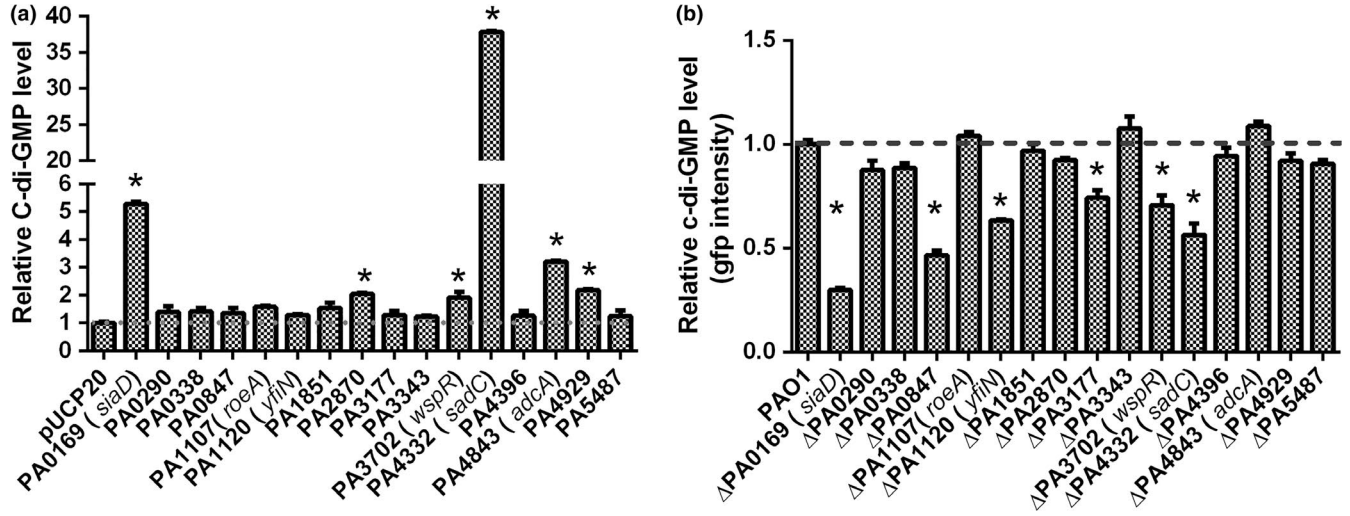

FIGURE 1 Quantification of c-di-GMP level in PAO1-derived strains with overexpression of a DGC-encoding gene or a gene deletion. (a) The total intracellular c-di-GMP concentration of overexpressed GGDEF domain genes measured by LC/MS/MS. Strains were incubated in Jensen's medium. The total intracellular c-di-GMP was extracted from cultures post 24 hr of growth. Shown are the value related to PAO1/pUCP20 (the concentration of c-di-GMP was 5.14 pmol/mg total protein); (b) The c-di-GMP level of GGDEF domain mutants was indicated by green fluorescent intensity of plasmid pCdrA::*gfp* measured by OD 560 post 24h of growth. Dotted lines indicate the wild-type level. Results were normalized to the level of wild type (the green fluorescent intensity of PAO1 is 5,842). *$p < .05$

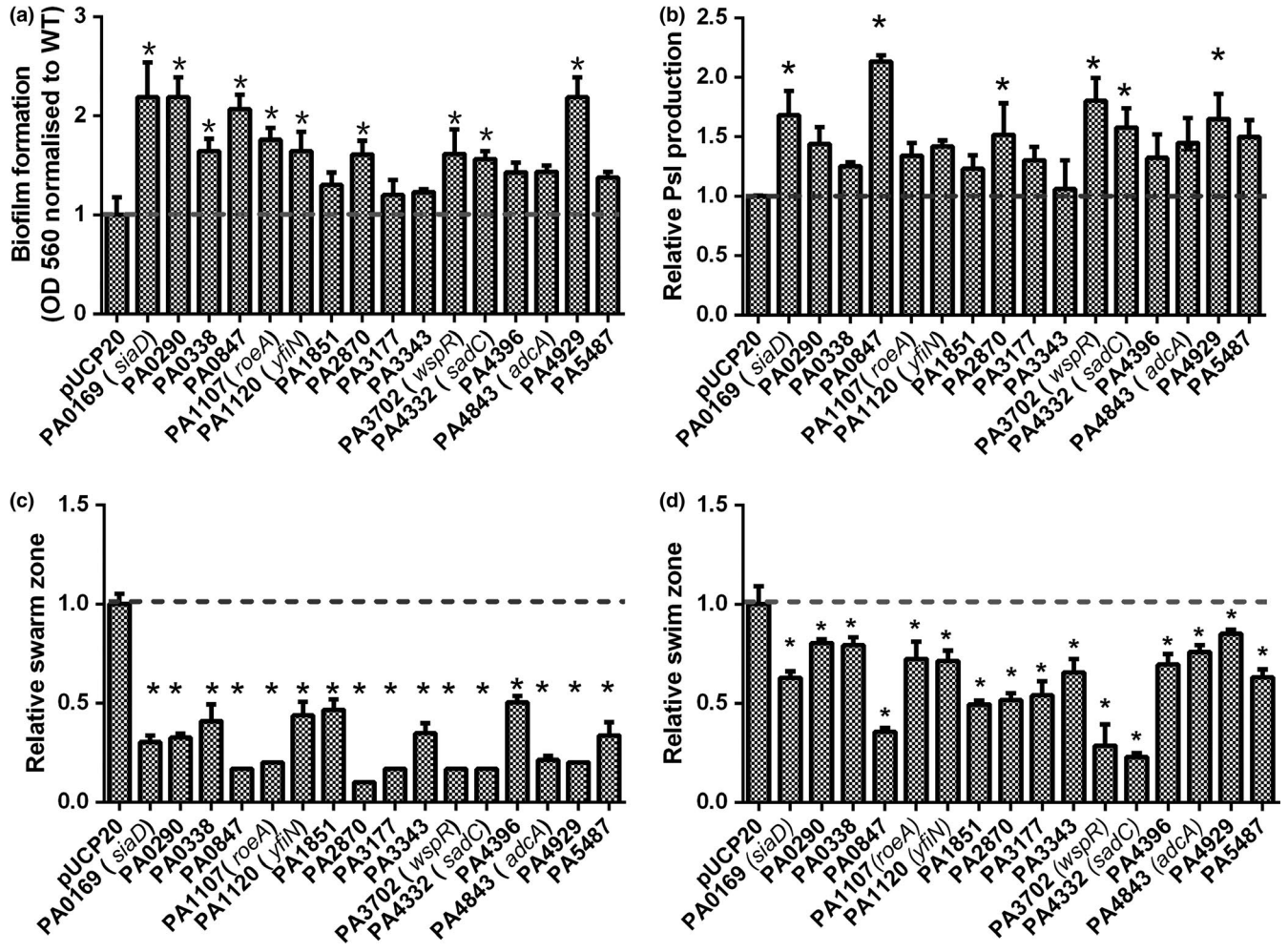

FIGURE 2 C-di-GMP-related phenotypic assays for overexpression of DGC-encoding genes in PAO1. (a) Biofilm assay; (b) Psl production; (c) swarming motility; and (d) swimming motility. Dotted lines indicate the wild-type level. Results were normalized to the value of wild-type control (PAO1/pUC20) that is the biofilm biomass (OD_{560}), 5.82; Psl, 29 μg/OD_{600}; swarm zone, 29 mm; and swim zone, 32 mm. *$p < .05$

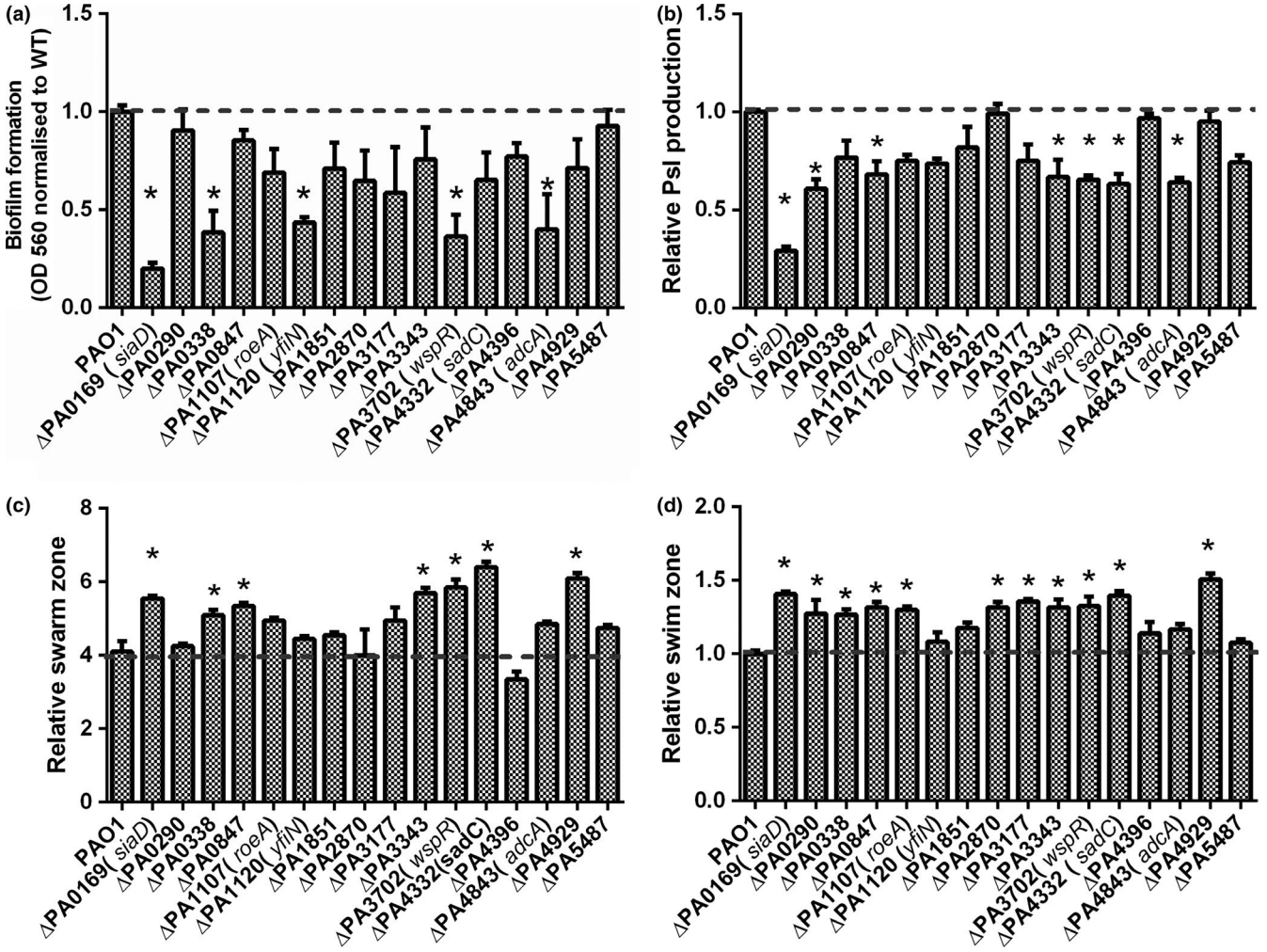

FIGURE 3 C-di-GMP-related phenotypic assays for DGC-encoding gene mutants in PAO1. (a) Biofilm assay; (b) Psl production; (c) swarming motility ; (d) swimming motility. Dotted lines indicate the wild-type level. Results were normalized to the value of PAO1 (OD_{560}, 6.42; swarm zone, 48 mm; Psl, 39 μg/OD_{600}; swim zone, 38 mm). *$p < .05$

3.3 | C-di-GMP-related phenotypes by in-frame deletion analysis

We then evaluated the phenotypic output of the in-frame deletion of 16 DGC-encoding genes. As can be seen from Figure 3a, most of the mutants produced less biofilm; noticeably, five mutants (ΔPA0169, ΔPA0338, ΔPA1120, ΔPA3702, and ΔPA4843) produced significantly less biofilm compared to other DGC-encoding genes and the wild-type PAO1 control. Psl production was also tested for all mutants. As compared to the wild-type strain PAO1, seven mutants (ΔPA0169, ΔPA0338, ΔPA0847, ΔPA3343, ΔPA3702, ΔPA4332, and ΔPA4929) produced significantly less Psl (Figure 3b). Regard to motilities, seven mutants (ΔPA0169, ΔPA0338, ΔPA0847, ΔPA3343, ΔPA3702, ΔPA4332, and ΔPA4929) showed significantly increased in swarming motility (Figure 3c), while 11 mutants (ΔPA0169, ΔPA0290, ΔPA0338, ΔPA0847, ΔPA2870, ΔPA3177, ΔPA3343, ΔPA3702, ΔPA4332, and ΔPA4929) showed significant increased swimming motility compared to wild type (Figure 3d), suggesting that the swimming motility assay was also a good way to briefly evaluate a predicted DGC-encoding gene by deletion analysis.

3.4 | Correlation analysis of c-di-GMP-related phenotypes of DGC-encoding genes

In order to further evaluate the function of those DGC-encoding genes, we introduced a quantitative way to classify them into different groups. We first assigned to each phenotype a score based on the relative results as compared to that of wild-type PAO1 and then calculated the overall contribution of each overexpression or mutation of DGC-encoding genes to those phenotypes. As can be seen from Table 1, the mutation of 16 DGC-encoding genes resulted in the overall index above 4, which is the score of wild-type control. The most pronounced effects were caused by the deletion of PA0169, PA0338, PA3702, and PA4843, which have an overall contribution index higher than 7.5. PA0169 showed the highest score (12.35) among the 16 genes. PA1107 gave the lowest score (6.41 in Table 1) among previously characterized DGC. As for the overexpression of those DGC-encoding genes (Table 2), there have even higher scores than that of corresponding deletion mutants. Among them, five genes (PA0847, PA2870, PA3177, PA3702, and PA4332) showed a score higher than 10. PA3702/*wspR* gave the highest value (12.83) among 16 genes.

TABLE 1 Contribution index for deletion mutants of predicted DGC-encoding genes

	Contribution index				
	Swarming	Swimming	Biofilm	Psl	Overall
ΔPA0169 (*siaD*)	1.79	1.91	4.98	3.66	12.34
ΔPA4843 (*adcA*)	1.80	1.71	2.50	1.61	7.62
ΔPA0338	1.78	1.85	2.60	1.35	7.58
ΔPA3702 (*wspR*)	1.13	1.94	2.75	1.37	7.19
ΔPA1120 (*yfiN*)	1.70	1.59	2.29	1.40	6.98
ΔPA2870	1.67	1.93	1.54	1.65	6.78
ΔPA4332 (*sadC*)	1.55	2.00	1.53	1.63	6.71
ΔPA3177	1.54	1.99	1.70	1.38	6.61
ΔPA3343	1.75	1.93	1.37	1.55	6.60
ΔPA0290	1.81	1.87	1.10	1.70	6.48
ΔPA1107 (*roeA*)	1.68	1.90	1.45	1.38	6.40
ΔPA4929	1.41	2.21	1.40	1.30	6.33
ΔPA0847	1.73	1.93	1.08	1.45	6.18
ΔPA5487	1.67	2.00	1.03	1.39	6.09
ΔPA1851	1.53	1.78	1.41	1.37	6.09
ΔPA4396	1.37	1.90	1.30	1.08	5.65
PAO1	1.00	1.00	1.00	1.00	4.00

TABLE 2 Contribution index for overexpression of predicted DGC-encoding genes

	Contribution index				
	Swarming	Swimming	Biofilm	Psl	Overall
PA3702 (*wspR*)	5.93	3.48	1.61	1.80	12.83
PA0847	5.93	2.23	2.07	2.13	12.37
PA4332 (*sadC*)	5.93	1.85	1.56	1.58	10.93
PA2870	5.84	1.93	1.61	1.51	10.90
PA3177	5.93	1.85	1.20	1.30	10.29
PA4929	4.94	1.18	2.19	1.65	9.95
PA1107 (*roeA*)	4.94	1.24	1.76	1.34	9.29
PA4843 (*adcA*)	4.68	1.32	1.43	1.45	8.89
PA0169 (*siaD*)	3.30	1.21	2.19	1.68	8.38
PA0290	3.07	1.24	2.19	1.44	7.94
PA5487	2.97	1.58	1.38	1.50	7.42
PA1120 (*yfiN*)	2.28	1.40	1.64	1.42	6.75
PA3343	2.87	1.53	1.23	1.06	6.69
PA1851	1.93	2.02	1.30	1.23	6.49
PA0338	2.23	1.26	1.64	1.25	6.38
PA4396	1.75	1.24	1.43	1.32	5.74
pUCP20	1.00	1.00	1.00	1.00	4.00

PA1120/*yfiN* showed a relatively low score (6.74) among previously characterized DGC. Our results also suggested that nine predicted DGC-encoding genes (PA0290, PA0338, PA0847, PA1851, PA2870, PA3177, PA3343, PA4396, PA4929, and PA5487) were very likely functional DGCs. PA4929 gave the lowest value in both analyses and required further analysis to confirm its function. The overall evaluation also showed that overexpression analysis gave a higher score in general than in-frame deletion analysis.

4 | DISCUSSION

Opportunistic pathogen *P. aeruginosa* is making lots of obstacles in human's life by producing diseases such as urinary tract infections, pneumonia, and lung infection, and it is getting resistant to a wide range of antibiotics because of its nature of producing biofilm (Cornforth et al., 2018; Prat & Lacoma, 2016). C-di-GMP is an

important second messenger involved in the regulation of biofilm formation. Understanding the mechanisms of c-di-GMP signaling in bacteria will provide a strategy to solve biofilm-related problems. In many bacteria, there are multiple genes involved in the biosynthesis and degradation of c-di-GMP. In this study, we have used *P. aeruginosa* PAO1 as a model microorganism to evaluate the methods and functional outputs of 16 putative DGC-encoding genes in this bacterium. The results will be beneficial for the research on *Pseudomona* spp. and bacteria using c-di-GMP as a signal.

In *P. aeruginosa* PAO1, 40 predicted genes (16 GGDEF-only, 16 GGDEF + EAL, five EAL only and three HD-GYP only) are involved in c-di-GMP synthesis and degradation. We have focused on the 16 predicted DGC-encoding genes that contain only GGDEF domain. We have cloned all 16 genes into shuttle vector pUCP20 and transformed into *P. aeruginosa* and also have used in-frame deletion technique to mutate the 16 genes, respectively. In our overexpression study, PA4332 (*sadC*) and PA0169 (*siaD*), the two well-studied DGC in *P. aeruginosa*, produced remarkably higher c-di-GMP than other genes (Valentini & Filloux, 2016; Zhu et al., 2016). Among DGC mutants, ΔPA0169, ΔPA0847, ΔPA1120 (*yfiN*), ΔPA3702 (*wspR*), and ΔPA4332 produced significantly less c-di-GMP compared to wild type. These are consistent with the previous reports (Malone et al., 2012; Valentini & Filloux, 2016; Zhu et al., 2016). It is interesting to note that the results from our detection of c-di-GMP in PAO1 derivatives were comparable to what has been published previously and even can be compared with the PA14, a native Psl-negative strain (Ha et al., 2014; Ueda & Wood, 2009; Zhu et al., 2016).

Our results in this study have revealed that the intracellular c-di-GMP measurement is not enough for evaluation or identification of a functional DGC and several methods have to be used. Overall evaluation is critical because the regulation of c-di-GMP level in bacteria is far more complex than what we have imaged. We have also noted that DGC genes still have distinct c-di-GMP relevant phenotypes, with some having more of an effect in one assay versus another even though they are overexpressed to the same level in PAO1. This could be due to the localization of DGC that changes the subcellular c-di-GMP level, leading the phenotypic difference as reported previously (Zhu et al., 2016). This would also suggest that the DGCs' activity is still regulated at some level even when overexpressed. Another possible explanation is due to the complex protein architecture of those DGCs that would involve more signaling pathways to mediate the downstream physiology (Kulasakara et al., 2006). For example, YfiN (PA1120) has previously been reported to cause a significant increase in the c-di-GMP level when overexpressed or mutated, possibly due to a change in the stoichiometry to its regulatory components (YfiB and YfiR) (Malone et al., 2012). In addition, a regulator of swimming motility, FleQ, can function as both a repressor and an activator in response to c-di-GMP to control the transcription of *pel* genes in *P. aeruginosa* (Baraquet, Murakami, Parsek, & Harwood, 2012).

By comparing the phenotypes of overexpression with deletion of DGC-encoding genes, our results have shown that the overexpression technique overall has given more distinctive phenotype. Most strikingly, the motility assay has shown relevant phenotype for all 16 predicted DGC-encoding genes by overexpression. This has suggested that the motility assay could be a quick way to briefly evaluate a predicted DGC for further studies. However, our data have also revealed that it is not easy to evaluate whether putative DGC genes are truly functional DGCs. It is very important to use multiple methods to give an overall evaluation for the characterization of a predicted DGC. By overall evaluation, we have revealed nine functional DGCs in *P. aeruginosa*, which have not been identified previously. In summary, we have provided a cloning library of 16 DGC-encoding genes from *P. aeruginosa* and their corresponding in-frame deletion mutants. Our work paves ways to briefly evaluate a predicted DGC and would help the researcher to go depth into their interested DGCs in bacteria.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (31570126 to L.M, 31300066 to Q.W.) and by the Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry (Q.W.). P.B. is an awardee of the Chinese Academy of Sciences (CAS) and The World Academy of Sciences (TWAS).

CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

Pramod Bhasme: Investigation, writing-original draft, writing-review & editing; Qing Wei: Investigation, writing-original draft, writing-review & editing; Anming Xu: Formal analysis, methodology, writing-review & editing; Syed Tatheer Alam Naqvi: Formal analysis, methodology, writing-review & editing; Di Wang: Formal analysis, methodology, writing-review & editing; and Luyan Zulie Ma: Data curation, funding acquisition, project administration, resources, supervision, writing-original draft, writing-review & editing.

ETHICS STATEMENT

None required.

ORCID

Luyan Z. Ma https://orcid.org/0000-0002-3837-6682

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article.

REFERENCES

Baraquet, C., Murakami, K., Parsek, M. R., & Harwood, C. S. (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 Research*, *40*(15), 7207–7218. https://doi.org/10.1093/nar/gks384

Boehm, A., Kaiser, M., Li, H., Spangler, C., Kasper, C. A., Ackermann, M., … Jenal, U. (2010). Second messenger-mediated adjustment of bacterial swimming velocity. *Cell*, *141*(1), 107–116. https://doi.org/10.1016/j.cell.2010.01.018

Boyd, C. D., & O'Toole, G. A. (2012). Second messenger regulation of biofilm formation: Breakthroughs in understanding c-di-GMP effector systems. *Annual Review of Cell and Developmental Biology*, *28*, 439–462. https://doi.org/10.1146/annurev-cellbio-101011-155705

Byrd, M. S., Sadovskaya, I., Vinogradov, E., Lu, H., Sprinkle, A. B., Richardson, S. H., … Wozniak, D. J. (2009). Genetic and biochemical analyses of the *Pseudomonas aeruginosa* Psl exopolysaccharide reveal overlapping roles for polysaccharide synthesis enzymes in Psl and LPS production. *Molecular Microbiology*, *73*(4), 622–638. https://doi.org/10.1111/j.1365-2958.2009.06795.x

Caiazza, N. C., Shanks, R. M., & O'Toole, G. A. (2005). Rhamnolipids modulate swarming motility patterns of *Pseudomonas aeruginosa*. *Journal of Bacteriology*, *187*(21), 7351–7361. https://doi.org/10.1128/jb.187.21.7351-7361.2005

Chan, C., Paul, R., Samoray, D., Amiot, N. C., Giese, B., Jenal, U., & Schirmer, T. (2004). Structural basis of activity and allosteric control of diguanylate cyclase. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(49), 17084–17089. https://doi.org/10.1073/pnas.0406134101

Cornforth, D. M., Dees, J. L., Ibberson, C. B., Huse, H. K., Mathiesen, I. H., Kirketerp-Moller, K., … Whiteley, M. (2018). *Pseudomonas aeruginosa* transcriptome during human infection. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(22), E5125–e5134. https://doi.org/10.1073/pnas.1717525115

Decho, A. W., & Gutierrez, T. (2017). Microbial extracellular polymeric substances (EPSs) in ocean systems. *Frontiers in Microbiology*, *8*, 922. https://doi.org/10.3389/fmicb.2017.00922

Fong, J. N. C., & Yildiz, F. H. (2015). Biofilm matrix proteins. *Microbiology Spectrum*, *3*(2). https://doi.org/10.1128/microbiolspec.MB-0004-2014

Fu, Y., Yu, Z., Liu, S., Chen, B. O., Zhu, L. I., Li, Z., … He, J. (2018). c-di-GMP regulates various phenotypes and insecticidal activity of gram-positive *Bacillus thuringiensis*. *Frontiers in Microbiology*, *9*, 45. https://doi.org/10.3389/fmicb.2018.00045

Ha, D. G., & O'Toole, G. A. (2015). c-di-GMP and its effects on biofilm formation and dispersion: A *Pseudomonas aeruginosa* Review. *Microbiology Spectrum*, *3*(2), Mb-0003-2014. https://doi.org/10.1128/microbiolspec.MB-0003-2014

Ha, D. G., Richman, M. E., & O'Toole, G. A. (2014). Deletion mutant library for investigation of functional outputs of cyclic diguanylate metabolism in *Pseudomonas aeruginosa* PA14. *Applied and Environment Microbiology*, *80*(11), 3384–3393. https://doi.org/10.1128/aem.00299-14

Hengge, R. (2009). Principles of c-di-GMP signalling in bacteria. *Nature Reviews Microbiology*, *7*(4), 263–273. https://doi.org/10.1038/nrmicro2109

Jones, C. J., Utada, A., Davis, K. R., Thongsomboon, W., Zamorano Sanchez, D., Banakar, V., … Yildiz, F. H. (2015). C-di-GMP regulates motile to sessile transition by modulating MshA pili biogenesis and near-surface motility Behavior in *Vibrio cholerae*. *PLoS Path*, *11*(10), e1005068. https://doi.org/10.1371/journal.ppat.1005068

Klausen, M., Aaes-Jorgensen, A., Molin, S., & Tolker-Nielsen, T. (2003). Involvement of bacterial migration in the development of complex multicellular structures in *Pseudomonas aeruginosa* biofilms. *Molecular Microbiology*, *50*(1), 61–68. https://doi.org/10.1046/j.1365-2958.2003.03677.x

Koo, H., Allan, R. N., Howlin, R. P., Stoodley, P., & Hall-Stoodley, L. (2017). Targeting microbial biofilms: Current and prospective therapeutic strategies. *Nature Reviews Microbiology*, *15*(12), 740–755. https://doi.org/10.1038/nrmicro.2017.99

Kulasakara, H., Lee, V., Brencic, A., Liberati, N., Urbach, J., Miyata, S., … Lory, S. (2006). Analysis of *Pseudomonas aeruginosa* diguanylate cyclases and phosphodiesterases reveals a role for bis-(3′-5′)-cyclic-GMP in virulence. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(8), 2839–2844. https://doi.org/10.1073/pnas.0511090103

Lebeaux, D., Ghigo, J. M., & Beloin, C. (2014). Biofilm-related infections: Bridging the gap between clinical management and fundamental aspects of recalcitrance toward antibiotics. *Microbiology and Molecular Biology Reviews*, *78*(3), 510–543. https://doi.org/10.1128/mmbr.00013-14

Liang, Z. X. (2015). The expanding roles of c-di-GMP in the biosynthesis of exopolysaccharides and secondary metabolites. *Natural Products Reports*, *32*(5), 663–683. https://doi.org/10.1039/c4np00086b

Liberati, N. T., Urbach, J. M., Miyata, S., Lee, D. G., Drenkard, E., Wu, G., … Ausubel, F. M. (2006). An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(8), 2833–2838. https://doi.org/10.1073/pnas.0511100103

Lyczak, J. B., Cannon, C. L., & Pier, G. B. (2000). Establishment of *Pseudomonas aeruginosa* infection: Lessons from a versatile opportunist. *Microbes and Infection*, *2*(9), 1051–1060.

Malone, J. G., Jaeger, T., Manfredi, P., Dötsch, A., Blanka, A., Bos, R., … Jenal, U. (2012). The YfiBNR signal transduction mechanism reveals novel targets for the evolution of persistent *Pseudomonas aeruginosa* in cystic fibrosis airways. *PLoS Path*, *8*(6), e1002760. https://doi.org/10.1371/journal.ppat.1002760

Mulcahy, L. R., Isabella, V. M., & Lewis, K. (2014). *Pseudomonas aeruginosa* biofilms in disease. *Microbial Ecology*, *68*(1), 1–12. https://doi.org/10.1007/s00248-013-0297-x

Orr, M. W., Donaldson, G. P., Severin, G. B., Wang, J., Sintim, H. O., Waters, C. M., & Lee, V. T. (2015). Oligoribonuclease is the primary degradative enzyme for pGpG in *Pseudomonas aeruginosa* that is required for cyclic-di-GMP turnover. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(36), E5048–5057. https://doi.org/10.1073/pnas.1507245112

O'Toole, G. A., & Kolter, R. (1998). Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: A genetic analysis. *Molecular Microbiology*, *28*(3), 449–461.

Paul, R., Weiser, S., Amiot, N. C., Chan, C., Schirmer, T., Giese, B., & Jenal, U. (2004). Cell cycle-dependent dynamic localization of a bacterial response regulator with a novel di-guanylate cyclase output domain. *Genes & Development*, *18*(6), 715–727. https://doi.org/10.1101/gad.289504

Plotnikova, J. M., Rahme, L. G., & Ausubel, F. M. (2000). Pathogenesis of the human opportunistic pathogen *Pseudomonas aeruginosa* PA14 in Arabidopsis. *Plant Physiology*, *124*(4), 1766–1774.

Prat, C., & Lacoma, A. (2016). Bacteria in the respiratory tract-how to treat? Or do not treat? *International Journal of Infectious Diseases*, *51*, 113–122. https://doi.org/10.1016/j.ijid.2016.09.005

Romling, U., Galperin, M. Y., & Gomelsky, M. (2013). Cyclic di-GMP: The first 25 years of a universal bacterial second messenger. *Microbiology and Molecular Biology Reviews*, *77*(1), 1–52. https://doi.org/10.1128/mmbr.00043-12

Ryan, R. P., Fouhy, Y., Lucey, J. F., & Dow, J. M. (2006). Cyclic di-GMP signaling in bacteria: Recent advances and new puzzles. *Journal of Bacteriology*, *188*(24), 8327–8334. https://doi.org/10.1128/jb.01079-06

Rybtke, M. T., Borlee, B. R., Murakami, K., Irie, Y., Hentzer, M., Nielsen, T. E., … Tolker-Nielsen, T. (2012). Fluorescence-based reporter for gauging cyclic di-GMP levels in *Pseudomonas aeruginosa*. *Applied and Environment Microbiology*, *78*(15), 5060–5069. https://doi.org/10.1128/aem.00414-12

Schmidt, A. J., Ryjenkov, D. A., & Gomelsky, M. (2005). The ubiquitous protein domain EAL is a cyclic diguanylate-specific phosphodiesterase: Enzymatically active and inactive EAL domains. *Journal of Bacteriology*, *187*(14), 4774–4781. https://doi.org/10.1128/jb.187.14.4774-4781.2005

Spangler, C., Bohm, A., Jenal, U., Seifert, R., & Kaever, V. (2010). A liquid chromatography-coupled tandem mass spectrometry method

for quantitation of cyclic di-guanosine monophosphate. *Journal of Microbiol Methods*, *81*(3), 226–231. https://doi.org/10.1016/j.mimet.2010.03.020

Stelitano, V., Giardina, G., Paiardini, A., Castiglione, N., Cutruzzola, F., & Rinaldo, S. (2013). C-di-GMP hydrolysis by *Pseudomonas aeruginosa* HD-GYP phosphodiesterases: Analysis of the reaction mechanism and novel roles for pGpG. *PLoS ONE*, *8*(9), e74920. https://doi.org/10.1371/journal.pone.0074920

Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., … Olson, M. V. (2000). Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature*, *406*(6799), 959–964. https://doi.org/10.1038/35023079

Ueda, A., & Wood, T. K. (2009). Connecting quorum sensing, c-di-GMP, pel polysaccharide, and biofilm formation in *Pseudomonas aeruginosa* through tyrosine phosphatase TpbA (PA3885). *PLoS Path*, *5*(6), e1000483. https://doi.org/10.1371/journal.ppat.1000483

Valentini, M., & Filloux, A. (2016). Biofilms and cyclic di-GMP (c-di-GMP) signaling: Lessons from *Pseudomonas aeruginosa* and other bacteria. *Journal of Biological Chemistry*, *291*(24), 12547–12555. https://doi.org/10.1074/jbc.R115.711507

Wei, Q., & Ma, L. Z. (2013). Biofilm matrix and its regulation in *Pseudomonas aeruginosa*. *International Journal of Molecular Sciences*, *14*(10), 20983–21005. https://doi.org/10.3390/ijms141020983

Zhu, B., Liu, C., Liu, S., Cong, H., Chen, Y., Gu, L., & Ma, L. Z. (2016). Membrane association of SadC enhances its diguanylate cyclase activity to control exopolysaccharides synthesis and biofilm formation in *Pseudomonas aeruginosa*. *Environmental Microbiology*, *18*(10), 3440–3452. https://doi.org/10.1111/1462-2920.13263

How to cite this article: Bhasme P, Wei Q, Xu A, Naqvi STA, Wang D, Ma LZ. Evaluation and characterization of the predicted diguanylate cyclase-encoding genes in *Pseudomonas aeruginosa*. *MicrobiologyOpen*. 2020;9:e975. https://doi.org/10.1002/mbo3.975

APPENDIX A

TABLE A1 Primers used in this study

Primer name	Primer sequence (5′-3′)	Restriction sites	Application
PA0169-UPFw	GGGGTACCGACCTGCACATACCCGGCAC	KpnI	Generation of pEX18Gm-PA0169
PA0169-UPRv	GACGCCTGAGGAGGCCAGTTGCTCCAGCGA		
PA0169-DWFw	TCGCTGGAGCAACTGGCCTCCTCAGGCGTC		
PA0169-DWRv	GCTCTAGAGCGCAGGCGTTCATCGCCGC	XbaI	
PA0290-UPFw	GCTCTAGACATCGCTACCCTGGTCTTC	XbaI	Generation of pEX18Gm-PA0290
PA0290-UPRv	GATGCAGTCCTTGCCGGTTAGGTCGTCCATGGATC		
PA0290-DwFw	ATGGACGACCTAACCGGCAAGGACTGCATCGTCGT		
PA0290-DwRv	CCCAAGCTTGAACGGAAGCGTGTGCATG	Hind III	
PA0338-UPFw	GCTCTAGACTCTACGACTACCTGCATCC	XbaI	Generation of pEX18Gm-PA0338
PA0338-UPRv	GCCATCATTCAGCACCAGGCAGTCTATACGCTTGC		
PA0338-DwFw	CGTATAGACTGCCTGGTGCTGAATGATGGCGAGAC		
PA0338-DwRv	CCCAAGCTTGCTTCAACCTGCTCATTGGC	Hind III	
PA3702-UPFw	GCTCTAGACGGTTCGCTGGTGTACACC	XbaI	Generation of pEX18Gm-PA3702
PA3702-UPRv	GTCGGCCATCTCGATGAGCTCTCATGAGGGTTG		
PA3702-DwFw	CAACCCTCATGAGAGCTCATCGAGATGGCCGAC		
PA3702-DwRv	CCCAAGCTTGAACTCCAGCTTCTCGAGG	Hind III	
PA3343-UPFw	GCTCTAGACCTTCCTCGATGTCCTCAC	XbaI	Generation of pEX18Gm-PA3343
PA3343-UPRv	GGCATGCTCGTAGAGGCACACCTCTTCTCTTGGAG		
PA3343-DwFw	AGAGAAGAGGTGTGCCTCTACGAGCATGCCGATC		
PA3343-DwRv	CCCAAGCTTCTGGTAGAGGTAGACGAAGC	Hind III	
PA3177-UPFw	GCTCTAGAGCACAAGGCCTTCATCATCG	XbaI	Generation of pEX18Gm-PA3177
PA3177-UPRv	GTCCAGTTCCTCCACGGTATTGGACTGCTTGGGAG		
PA3177-DwFw	AAGCAGTCCAATACCGTGGAGGAACTGGACTACC		
PA3177-DwRv	CCCAAGCTTCACAGGATCCTGGCTATCG	Hind III	
PA2870-UPFw	GCTCTAGACGTCTTTCTCTGTTGCCTGG	XbaI	Generation of pEX18Gm-PA2870
PA2870-UPRv	GCTCACGGTCATTCGCAGCTTTGTCGGATCGAGC		
PA2870-DwFw	GATCCGACAAAGCTGCGAATGACCGTGAGCATCG		
PA2870-DwRv	CCCAAGCTTGGAGTTCACTCGCTACATCG	Hind III	
PA1851-UPFw	GCTCTAGATCACGTCCAGGCTCATCAC	XbaI	Generation of pEX18Gm-PA1851
PA1851-UPRv	CTTGGCCTCGTAGAGTGCAAGCATGCTGACTCCC		
PA1851-DwFw	GTCAGCATGCTTGCACTCTACGAGGCCAAGGATG		
PA1851-DwRv	CCCAAGCTTGAGACCGACTCCCATGAAC	Hind III	
PA1120-UPFw	GCTCTAGAGGACGTCGATCGAGCAAC	XbaI	Generation of pEX18Gm-PA1120
PA1120-UPRv	GCATGCTCGGGATAGAGGCGATGGATTGGAACC		
PA1120-DwFw	GGTTCCAATCCATCGCCTCTATCCCGAGCATGC		
PA1120-DwRw	CCCAAGCTTCGAGCGCAGGATTACTCG	Hind III	
PA1107-UPFw	GCTCTAGACGATTCCCAACCTGCTGG	XbaI	Generation of pEX18Gm-PA1107
PA1107-UPRv	GCTGTAGAGCTGCTCGTAGGACGAATTCACCTCG		
PA1107-DwFw	CGAGGTGAATTCGTCCTACGAGCAGCTCTACAGC		
PA1107-DwRv	CCCAAGCTTGAGGTTGGAGGTCCAGTTG	Hind III	
PA0847-UPFw	GCTCTAGACTGCAACTGAAGCAGTGGTC	XbaI	Generation of pEX18Gm-PA0847
PA0847-UPRv	GAAGTCGATGCGTTCGAAGTTGATGGCCTTCTCG		
PA0847-DwFw	AAGGCCATCAACTTCGAACGCATCGACTTCGTCAC		
PA0847-DwRv	CCCAAGCTTGATCATCGCCAGCAGTTCG	Hind III	

(Continues)

TABLE A1 (Continued)

Primer name	Primer sequence (5′-3′)	Restriction sites	Application
PA4332-UPFw	GGAATTCCGCCGACCCGTATGTCA	EcoRI	Zhu et al. (2016)
PA4332-UPRv	GGGGTACCGAAGACCTGCTGCCGAACC		
PA4332-DwFw	GGGGTACCGCAAGCCACCACCAGGG		
PA4332-DwRv	CCCAAGCTTCACGGTCCTCTTCAGGCG	Hind III	
PA4396-UPFw	GCTCTAGAGCTCCTTGATCAGACCAACG	XbaI	Generation of pEX18Gm-PA4396
PA4396-UPRv	CAGGCTGATGAAGCCGTTCCGTCTGTGAACCCTG		
PA4396-DwFw	GTTCACAGACGGAACGGCTTCATCAGCCTGAAAGC		
PA4396-DwRv	CCCAAGCTTCTGCAGCAACAGCAGTTCG	Hind III	
PA4843-UPFw	GCTCTAGAGTAGGTACGCACATCATCCAC	XbaI	Generation of pEX18Gm-PA4843
PA4843-UPRv	GTGGATCTCGGCGAAGTCATCGTGCTCGGTCATC		
PA4843-DwFw	ACCGAGCACGATGACTTCGCCGAGATCCACTACC		
PA4843-DwRv	CCCAAGCTTGGACCATTGTTCCAGGTTGC	Hind III	
PA4929-UPFw	GCTCTAGAGGAACGGACCTTCTTGTCC	XbaI	Generation of pEX18Gm-PA4929
PA4929-UPRv	GATTCAGGAGATACGCAGTAGCAGGAAGATGACG		
PA4929-DwFw	ATCTTCCTGCTACTGCGTATCTCCTGAATCAGTGG		
PA4929-DwRv	CCCAAGCTTACCTCAGTGCTGCCGAACTG	Hind III	
PA5487-UPFw	GCTCTAGAGCTGGAAGATCATCCGTTGC	XbaI	Generation of pEX18Gm-PA5487
PA5487-UPRv	GAAATGGAACGGACACTCCTGCTGCTCGATGTTC		
PA5487-DwFw	ATCGAGCAGCAGGAGTGTCCGTTCCATTTCAAGGG		
PA5487-DwRv	CCCAAGCTTTGAGGAAGAGCAGCAGCAG	Hind III	
PA0169-Fw	GGGGTACCGATGGACATCCTCGACCTGC	KpnI	Generation of pPA0169
PA0169-Rv	GCTCTAGACGTCCTGACCCTGGAGTTG	XbaI	
PA0290-Fw	GGGGTACCCAGGGACACGAAGAACGACC	KpnI	Generation of pPA0290
PA0290-Rv	GCTCTAGACCCAAAGAGGTATTCCGTGG	XbaI	
PAO338-Fw	GCTCTAGAGAGCATCTGCGGCGAGATG	XbaI	Generation of pPA0338
PA0338-Rv	CCCAAGCTTCAGCGTCGTACGGGTACACG	Hind III	
PA3702-Fw	GGGGTACCCTCAAGCAGATGCGCGAG	KpnI	Generation of pPA3702
PA3702-Rv	GCTCTAGACAGGGTTTCCACCACCTG	XbaI	
PA3343-Fw	GCTCTAGAGCAGCGAGAAGAACGTCTAC	XbaI	Generation of pPA3343
PA3343-Rv	CCCAAGCTTGGATCAAGCGCTGCATGG	Hind III	
PA3177-Fw	GGGGTACCGCTACTGACGATCTTCGTGC	KpnI	Generation of pPA3177
PA3177-Rv	GCTCTAGAGACGGCAAGGAAACCAGC	XbaI	
PA2870-Fw	GGGGTACCGAACGACTACTCGGCGATC	KpnI	Generation of pPA2870
PA2870-Rv	GCTCTAGACCCATGGAGGTTTCCCAC	XbaI	
PA1851-Fw	GGGGTACCCACCAACAGGGTGTCCAG	KpnI	Generation of pPA1851
PA1851-Rv	GCTCTAGAGTCCGTAGTCGATCGCAC	XbaI	
PA1120-Fw	GGGGTACCGACGTCGATCGAGCAACG	KpnI	Generation of pPA1120
PA1120-Rv	GCTCTAGACAGCACAAGGCTGAACAGG	XbaI	
PA1107-Fw	GCTCTAGACGATTCCCAACCTGCTGG	XbaI	Generation of pPA1107
PA1107-Rv	CCCAAGCTTGGTTCTCCCCTGGTAATGC	Hind III	
PA0847-Fw	GCTCTAGACCATCTCGTCCACCTCACG	XbaI	Generation of pPA0847
PA0847-Rv	CCCAAGCTTGGTTCTCCGATGGGATGC	Hind III	
PA4332-Fw	GCTCTAGACAGTCGACGATCGAGTCG	XbaI	Generation of pPA0847
PA4332-Rv	CCCAAGCTTCATCCAGCAGGTTGCTGC	Hind III	
PA4396-Fw	GCTCTAGAGCTTGCGCTCCTTGATCAG	XbaI	Generation of pPA4396
PA4396-Rv	CCCAAGCTTGCTGACGATAGGGCGAAG	Hind III	

(Continues)

TABLE A1 (Continued)

Primer name	Primer sequence (5′-3′)	Restriction sites	Application
PA4843-Fw	GCTCTAGACGAAGTAGGTACGCAC	XbaI	Generation of pPA4843
PA4843-Rv	CCCAAGCTTGAGTCTCGTCCGACTACG	Hind III	
PA4929-Fw	GCTCTAGAGAGGAACGGACCTTCTTGTCC	XbaI	Generation of pPA4929
PA4929-Rv	CCCAAGCTTGCTTCTGAGCAAGGTCACG	Hind III	
PA5487-Fw	GGGGTACCCGACCCTGCTGGAAGATC	KpnI	Generation of pPA5487
PA5487-Rv	GCTCTAGACGAGTCGTCGGAATGTGC	XbaI	

TABLE A2 Strains used in this study

Strains	Relevant phenotype	References of source
PAO1	Prototroph	Holloway (1955)
PAO1Δ0169	PAO1, In-frame deletion of 0169 gene	This study
PAO1Δ0290	PAO1, In-frame deletion of 0290 gene	This study
PAO1Δ0338	PAO1, In-frame deletion of 0338 gene	This study
PAO1Δ0847	PAO1, In-frame deletion of 0847gene	This study
PAO1Δ1107	PAO1, In-frame deletion of 1107 gene	This study
PAO1Δ1120	PAO1, In-frame deletion of 1120 gene	This study
PAO1Δ1851	PAO1, In-frame deletion of 1851 gene	This study
PAO1Δ2870	PAO1, In-frame deletion of 2870 gene	This study
PAO1Δ3177	PAO1, In-frame deletion of 3177 gene	This study
PAO1Δ3343	PAO1, In-frame deletion of 3343 gene	This study
PAO1Δ3702	PAO1, In-frame deletion of 3702 gene	This study
PAO1Δ4332	PAO1, In-frame deletion of 4332 gene	Zhu et al. (2016)
PAO1Δ4396	PAO1, In-frame deletion of 4396 gene	This study
PAO1Δ4843	PAO1, In-frame deletion of 4843 gene	This study
PAO1Δ4929	PAO1, In-frame deletion of 4929 gene	This study
PAO1Δ5487	PAO1, In-frame deletion of 5487 gene	This study
PAO1/pPA0169	Overexpression of PAO169 gene in PAO1	This study
PAO1/pPA0290	Overexpression of PA0290 gene in PAO1	This study
PAO1/pPA0338	Overexpression of PA0338 gene in PAO1	This study
PAO1/pPA0847	Overexpression of PA0847gene in PAO1	This study
PAO1/pPA1107	Overexpression of PA1107 gene in PAO1	This study
PAO1/pPA1120	Overexpression of PA1120 gene in PAO1	This study
PAO1 pPA1851	Overexpression of PA1851 gene in PAO1	This study
PAO1/pPA2870	Overexpression of PA2870 gene in PAO1	This study
PAO1/pPA3177	Overexpression of PA3177 gene in PAO1	This study
PAO1/pPA3343	Overexpression of PA3343 gene in PAO1	This study
PAO1/pPA3702	Overexpression of PA3702 gene in PAO1	This study
PAO1/pPA4332	Overexpression of PA4332 gene in PAO1	This study
PAO1/pPA4396	Overexpression of PA4396 gene in PAO1	This study
PAO1/pPA4843	Overexpression of PA4843 gene in PAO1	This study
PAO1/pPA4929	Overexpression of PA4929 gene in PAO1	This study
PAO1/pPA5487	Overexpression of PA5487 gene in PAO1	This study

(Continues)