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

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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

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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, YgrR (Boehm et al., 2010).

The c-di-GMP is synthesized from two molecules of GTP by diguanylate cyclases (DGCs) and degraded into 5’-phosphoguanosyl-(3’-5’)-guanosine (pGpG) by specific phosphodiesterases (PDEs); later, pGpG was split into two GMP molecules by ribonuclease Orn (Orr et al., 2015; Stellitano 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, 3-glucose-5 g/L, bacit 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 were 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. OD₆₀₀ 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 (∆PA0169, ∆PA0847, ∆PA1120, ∆PA4332, ∆PA3177, and ∆PA3702) 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 (OD₅₆₀), 5.82; Psl, 29 µg/OD₆₀₀; swarm zone, 29 mm; and swim zone, 32 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.
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, 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.

### Table 1

| Contribution index | Swarming | Swimming | Biofilm | Psl | Overall |
|--------------------|----------|----------|---------|-----|---------|
| ∆PA0169 (siaD)     | 1.79     | 1.91     | 4.98    | 3.66| 12.34  |
| ∆PA4843 (adcA)     | 1.80     | 1.71     | 2.50    | 1.61| 7.62   |
| ∆PA0338            | 1.78     | 1.85     | 2.60    | 1.35| 7.58   |
| ∆PA3702 (wspR)     | 1.13     | 1.94     | 2.75    | 1.37| 7.19   |
| ∆PA1120 (yfiN)     | 1.70     | 1.59     | 2.29    | 1.40| 6.98   |
| ∆PA2870            | 1.67     | 1.93     | 1.54    | 1.65| 6.78   |
| ∆PA4332 (sadC)     | 1.55     | 2.00     | 1.53    | 1.63| 6.71   |
| ∆PA3177            | 1.54     | 1.99     | 1.70    | 1.38| 6.61   |
| ∆PA3343            | 1.75     | 1.93     | 1.37    | 1.55| 6.60   |
| ∆PA0290            | 1.81     | 1.87     | 1.10    | 1.70| 6.48   |
| ∆PA1107 (roeA)     | 1.68     | 1.90     | 1.45    | 1.38| 6.40   |
| ∆PA4929            | 1.41     | 2.21     | 1.40    | 1.30| 6.33   |
| ∆PA0847            | 1.73     | 1.93     | 1.08    | 1.45| 6.18   |
| ∆PA5487            | 1.67     | 2.00     | 1.03    | 1.39| 6.09   |
| ∆PA1851            | 1.53     | 1.78     | 1.41    | 1.37| 6.09   |
| ∆PA4396            | 1.37     | 1.90     | 1.30    | 1.08| 5.65   |
| PA01               | 1.00     | 1.00     | 1.00    | 1.00| 4.00   |

### Table 2

| 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   |

### 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 Pseudomonas 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 (yfR), 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, YfIR (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.

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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 Tathiere 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.

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

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### 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 | GACGCCGTAGAGGAGCCAGTTGCTTCAGCCGA |  |
| PA0169-DWFw | TCCTCTGAGCAAACTGCTCCTCAGGCTTCGTC |  |
| PA0169-DWRv | GCTCTAGAGCCAGCGTTTCATGGGCGGC | XbaI |  |
| PA0290-UPFw | GCTCTAGACATCGTACCCGTGGTCCTTCTTC | XbaI | Generation of pEX18Gm-PA0290 |
| PA0290-UPRv | GTGACGCTGATCTGGCTTCGAGGACCTGATGGATG |  |
| PA0290-DWFw | ATGGGACCTCCTAACGCGAAGGACCTGCTTGGTCTCAG | XbaI |  |
| PA0290-DWRv | CCCAAGCTGATGAGGGTCTGCAGGTCAA | Hind III |  |
| PA0338-UPFw | GCTCTAGACTCTACGACTACCTGCATCGGTCCTCC | XbaI | Generation of pEX18Gm-PA0338 |
| PA0338-UPRv | GCCATCATTCACGCTAGGAGCACACCTTCCTTCTTCAGGGT |  |
| PA0338-DWFw | CTTGGCCTCGTAGAGTGCAAGCATGCTGACTCCC | XbaI | Generation of pEX18Gm-PA3343 |
| PA0338-DWRv | CCCAAGCTTTCAAGGTTTCAGGTGAAC | Hind III |  |
| PA3702-UPFw | GCTCTAGACCTATCGTATGGGTGATGGTGACC | XbaI | Generation of pEX18Gm-PA3702 |
| PA3702-UPRv | GTCCGCCTCATCAGGAGCCATGCTTCTCTTCTTCAGGGT |  |
| PA3702-DWFw | CAACCTCTGTAGCTGCTTGCAGGAGATGCTGCTGCTTGGC | Hind III |  |
| PA3702-DWRv | CCCAAGCTACCCAGTACCTTCTTGAGG | XbaI |  |
| PA3343-UPFw | GCTCTAGACCTATCGTATGGGTGATGGTGACC | XbaI | Generation of pEX18Gm-PA3343 |
| PA3343-UPRv | GCCATCATTCACGCTAGGAGCACACCTTCCTTCTTCAGGGT |  |
| PA3343-DWFw | CTTGGCCTCGTAGAGTGCAAGCATGCTGACTCCC | XbaI | Generation of pEX18Gm-PA3343 |
| PA3343-DWRv | CCCAAGCTTTCAAGGTTTCAGGTGAAC | Hind III |  |
| PA1377-UPFw | GCTCTAGACCTATCGTATGGGTGATGGTGACC | XbaI | Generation of pEX18Gm-PA3177 |
| PA1377-UPRv | GTCCGCCTCATCAGGAGCCATGCTTCTCTTCTTCAGGGT |  |
| PA1377-DWFw | CAACCTCTGTAGCTGCTTGCAGGAGATGCTGCTGCTTGGC | Hind III |  |
| PA1377-DWRv | CCCAAGCTTTCAAGGTTTCAGGTGAAC | Hind III |  |
| PA2870-UPFw | GCTCTAGACCTATCGTATGGGTGATGGTGACC | XbaI | Generation of pEX18Gm-PA2870 |
| PA2870-UPRv | GTCCGCCTCATCAGGAGCCATGCTTCTCTTCTTCAGGGT |  |
| PA2870-DWFw | CAACCTCTGTAGCTGCTTGCAGGAGATGCTGCTGCTTGGC | Hind III |  |
| PA2870-DWRv | CCCAAGCTTTCAAGGTTTCAGGTGAAC | Hind III |  |
| PA1851-UPFw | GCTCTAGACCTATCGTATGGGTGATGGTGACC | XbaI | Generation of pEX18Gm-PA1851 |
| PA1851-UPRv | GTCCGCCTCATCAGGAGCCATGCTTCTCTTCTTCAGGGT |  |
| PA1851-DWFw | CAACCTCTGTAGCTGCTTGCAGGAGATGCTGCTGCTTGGC | Hind III |  |
| PA1851-DWRv | CCCAAGCTTTCAAGGTTTCAGGTGAAC | Hind III |  |
| PA1120-UPFw | GCTCTAGACCTATCGTATGGGTGATGGTGACC | XbaI | Generation of pEX18Gm-PA1120 |
| PA1120-UPRv | GTCCGCCTCATCAGGAGCCATGCTTCTCTTCTTCAGGGT |  |
| PA1120-DWFw | CAACCTCTGTAGCTGCTTGCAGGAGATGCTGCTGCTTGGC | Hind III |  |
| PA1120-DWRv | CCCAAGCTTTCAAGGTTTCAGGTGAAC | Hind III |  |
| PA1107-UPFw | GCTCTAGACCTATCGTATGGGTGATGGTGACC | XbaI | Generation of pEX18Gm-PA1107 |
| PA1107-UPRv | GTCCGCCTCATCAGGAGCCATGCTTCTCTTCTTCAGGGT |  |
| PA1107-DWFw | CAACCTCTGTAGCTGCTTGCAGGAGATGCTGCTGCTTGGC | Hind III |  |
| PA1107-DWRv | CCCAAGCTTTCAAGGTTTCAGGTGAAC | Hind III |  |
| PA0847-UPFw | GCTCTAGACCTATCGTATGGGTGATGGTGACC | XbaI | Generation of pEX18Gm-PA0847 |
| PA0847-UPRv | GTCCGCCTCATCAGGAGCCATGCTTCTCTTCTTCAGGGT |  |
| PA0847-DWFw | CAACCTCTGTAGCTGCTTGCAGGAGATGCTGCTGCTTGGC | Hind III |  |
| PA0847-DWRv | CCCAAGCTTTCAAGGTTTCAGGTGAAC | Hind III |  |

(Continues)
| Primer name | Primer sequence (5′-3′) | Restriction sites | Application |
|-------------|-------------------------|------------------|-------------|
| PA4332-UPFw | GGAATTCGCCGACCCCGTATGCTA | EcoRI | Zhu et al. (2016) |
| PA4332-UPRv | GGGTGACCCGACCCCGTATGCTA | | |
| PA4332-DwFw | GGGTGACCCGACCCCGTATGCTA | Hind III | |
| PA4332-DwRv | GGGTGACCCGACCCCGTATGCTA | Hind III | |
| PA4396-UPFw | GCTCTAGAGCTCCTGGATACGACCGAACC | XbaI | Generation of pEX18Gm-PA4396 |
| PA4396-UPRv | GCTCTAGAGCTCCTGGATACGACCGAACC | | |
| PA4396-DwFw | GCTCTAGAGCTCCTGGATACGACCGAACC | Hind III | |
| PA4396-DwRv | GCTCTAGAGCTCCTGGATACGACCGAACC | Hind III | |
| PA4843-UPFw | GCTCTAGAGCTCCTGGATACGACCGAACC | XbaI | Generation of pEX18Gm-PA4843 |
| PA4843-UPRv | GCTCTAGAGCTCCTGGATACGACCGAACC | | |
| PA4843-DwFw | GCTCTAGAGCTCCTGGATACGACCGAACC | Hind III | |
| PA4843-DwRv | GCTCTAGAGCTCCTGGATACGACCGAACC | Hind III | |
| PA4929-UPFw | GCTCTAGAGCTCCTGGATACGACCGAACC | XbaI | Generation of pEX18Gm-PA4929 |
| PA4929-UPRv | GCTCTAGAGCTCCTGGATACGACCGAACC | | |
| PA4929-DwFw | GCTCTAGAGCTCCTGGATACGACCGAACC | Hind III | |
| PA4929-DwRv | GCTCTAGAGCTCCTGGATACGACCGAACC | Hind III | |
| PA5487-UPFw | GCTCTAGAGCTCCTGGATACGACCGAACC | XbaI | Generation of pEX18Gm-PA5487 |
| PA5487-UPRv | GCTCTAGAGCTCCTGGATACGACCGAACC | | |
| PA5487-DwFw | GCTCTAGAGCTCCTGGATACGACCGAACC | Hind III | |
| PA5487-DwRv | GCTCTAGAGCTCCTGGATACGACCGAACC | Hind III | |
| PA0169-Fw | GGGTGACCCGACCCCGTATGCTA | KpnI | Generation of pPA0169 |
| PA0169-Rv | GGGTGACCCGACCCCGTATGCTA | XbaI | |
| PA0290-Fw | GGGTGACCCGACCCCGTATGCTA | KpnI | Generation of pPA0290 |
| PA0290-Rv | GGGTGACCCGACCCCGTATGCTA | XbaI | |
| PA0338-Fw | GGGTGACCCGACCCCGTATGCTA | KpnI | Generation of pPA0338 |
| PA0338-Rv | GGGTGACCCGACCCCGTATGCTA | XbaI | |
| PA3433-Fw | GGGTGACCCGACCCCGTATGCTA | XbaI | Generation of pPA3433 |
| PA3433-Rv | GGGTGACCCGACCCCGTATGCTA | XbaI | |
| PA3177-Fw | GGGTGACCCGACCCCGTATGCTA | KpnI | Generation of pPA3177 |
| PA3177-Rv | GGGTGACCCGACCCCGTATGCTA | XbaI | |
| PA2807-Fw | GGGTGACCCGACCCCGTATGCTA | KpnI | Generation of pPA2807 |
| PA2807-Rv | GGGTGACCCGACCCCGTATGCTA | XbaI | |
| PA1851-Fw | GGGTGACCCGACCCCGTATGCTA | KpnI | Generation of pPA1851 |
| PA1851-Rv | GGGTGACCCGACCCCGTATGCTA | XbaI | |
| PA1120-Fw | GGGTGACCCGACCCCGTATGCTA | KpnI | Generation of pPA1120 |
| PA1120-Rv | GGGTGACCCGACCCCGTATGCTA | XbaI | |
| PA1107-Fw | GGGTGACCCGACCCCGTATGCTA | KpnI | Generation of pPA1107 |
| PA1107-Rv | GGGTGACCCGACCCCGTATGCTA | XbaI | |
| PA0847-Fw | GGGTGACCCGACCCCGTATGCTA | KpnI | Generation of pPA0847 |
| PA0847-Rv | GGGTGACCCGACCCCGTATGCTA | XbaI | |
| PA4332-Fw | GGGTGACCCGACCCCGTATGCTA | KpnI | Generation of pPA4332 |
| PA4332-Rv | GGGTGACCCGACCCCGTATGCTA | XbaI | |
| PA4396-Fw | GGGTGACCCGACCCCGTATGCTA | KpnI | Generation of pPA4396 |
| PA4396-Rv | GGGTGACCCGACCCCGTATGCTA | XbaI | |

(Continues)
| Primer name          | Primer sequence (5′-3′)                                      | Restriction sites | Application           |
|---------------------|----------------------------------------------------------------|------------------|-----------------------|
| PA4843-Fw           | GCTCTAGACGAGTAGTTACGAC                                       | Xbal             | Generation of pPA4843 |
| PA4843-Rv           | CCCAAGCTTCTGACTCTGTCGACAGC                                   | Hind III         |                       |
| PA4929-Fw           | GCTCTAGAGAGAACGGACCTTCTCTGTCC                                  | Xbal             | Generation of pPA4929 |
| PA4929-Rv           | CCCAAGCTTCTGACTCTGTCGACAGC                                   | Hind III         |                       |
| PA5487-Fw           | GGGGTACCCGACCTGTCATCTGAA                                      | KpnI             | Generation of pPA5487 |
| PA5487-Rv           | GCTCTAGACGAGTAGTTACGAC                                       | Xbal             |                       |

**TABLE A1 (Continued)**

**TABLE A2**

| 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 0847 gene | 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          |

**TABLE A2 (Continued)**

| Strains | Relevant phenotype               | References of source |
|---------|----------------------------------|----------------------|
| 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)