Identification of c-di-GMP/FleQ-Regulated New Target Genes, Including cyaA, Encoding Adenylate Cyclase, in Pseudomonas putida

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ABSTRACT The bacterial second messenger cyclic diguanylate (c-di-GMP) modulates plankton-to-biofilm lifestyle transition of Pseudomonas species through its transcriptional regulatory effector FleQ. FleQ regulates transcription of biofilm- and flagellum-related genes in response to c-di-GMP. Through transcriptomic analysis and FleQ-DNA binding assay, this study identified five new target genes of c-di-GMP/FleQ in P. putida, including PP_0681, PP_0788, PP_4519 (lapE), PP_5222 (cyA), and PP_5586. Except lapE encoding an outer membrane pore protein and cyA encoding an adenylate cyclase, the functions of the other three genes encoding hypothetical proteins remain unknown. FleQ and c-di-GMP coordinately inhibit transcription of PP_0788 and cyA and promote transcription of PP_0681, lapE, and PP_5586. Both in vitro and in vivo assays show that FleQ binds directly to promoters of the five genes. Further analyses confirm that LapE plays a central role in the secretion of adhesin LapA and that c-di-GMP/FleQ increases lapE transcription, thereby promoting adhesin secretion and biofilm formation. The adenylate cyclase CyA is responsible for synthesis of another second messenger, cyclic AMP (cAMP). FleQ and c-di-GMP coordinate to decrease the content of cAMP, suggesting that c-di-GMP and FleQ coregulate cAMP by modulating cyA expression. Overall, this study adds five new members to the c-di-GMP/FleQ-regulated gene family and reveals the role of c-di-GMP/FleQ in LapA secretion and cAMP synthesis regulation in P. putida.

IMPORTANCE c-di-GMP/FleQ promotes the plankton-to-biofilm lifestyle transition at the transcriptional level via FleQ in Pseudomonas species. Identification of new target genes directly regulated by c-di-GMP/FleQ helps to broaden the knowledge of c-di-GMP/FleQ-mediated transcriptional regulation. Regulation of lapE by c-di-GMP/FleQ guarantees highly efficient LapA secretion and biofilm formation. The mechanism of negative correlation between c-di-GMP and cAMP in both P. putida and P. aeruginosa remains unknown. Our result concerning transcriptional inhibition of cyA by c-di-GMP/FleQ reveals the mechanism underlying the decrease of cAMP content by c-di-GMP in P. putida.

KEYWORDS c-di-GMP, FleQ, transcriptome sequencing, LapA secretion, cAMP, adenylate cyclase

Cyclic diguanylate (c-di-GMP) is a ubiquitous bacterial second messenger that participates in regulation of a wide range of cellular processes through its downstream receptors (1). Several types of c-di-GMP receptors have been identified, including PilZ proteins, GGDEF, and/or EAL domain-containing proteins, riboswitches, and transcriptional regulators. Transcriptional regulatory c-di-GMP effectors modulate various bacterial physiological processes by directly regulating target gene expression in response to c-di-GMP. For example, transcriptional regulatory c-di-GMP effectors, such as VpsT

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of *Vibrio cholerae*, FleQ of *Pseudomonas aeruginosa*, and MrkH of *Klebsiella pneumoniae*, regulate transcription of biofilm matrix- and flagellum-related genes, thereby modulating biofilm formation and bacterial motility (2–4). The c-di-GMP-responsive regulator Clp from *Xanthomonas campestris* and the c-di-GMP-responsive regulator Bcm1349 from *Burkholderia cenocepacia* are involved in the regulation of bacterial virulence-related genes (5, 6). Two c-di-GMP-responsive regulators, LtmA and HpoR from *Mycobacterium smegmatis*, regulate lipid metabolism/transport and antioxidant defense, respectively (7, 8).

Among all transcriptional regulatory c-di-GMP effectors identified to date, FleQ is the best characterized in terms of its action mechanism. FleQ, as an NtrC subfamily regulator protein, is widely distributed in *Pseudomonas* species, and it contains an N-terminal atypical REC domain (also named FleQ domain), a central AAA+ ATPase domain, and a C-terminal helix-turn-helix DNA-binding domain (9). Transverse dimers formed via the atypical REC domain are essential for FleQ to function as a c-di-GMP receptor and flagellum gene regulator (10). FleQ was identified at first as a master regulator to activate transcription of flagellar genes (11, 12), and then it was found to bind c-di-GMP and regulate the genes involved in biofilm formation (3). By inversely regulating biofilm and flagellar gene expression, FleQ helps to control the plankton-to-biofilm lifestyle transition in response to c-di-GMP.

FleQ functions as both a repressor and an activator to bind to two sites on the promoter of the exopolysaccharide pel operon, and it controls the activity of the pel promoter along with FleN (another ATPase) in response to c-di-GMP in *P. aeruginosa* (13). Upon binding ATP, FleN forms dimers and interacts with the two FleQ molecules bound to DNA, and then the obtained FleQ-FleN-DNA complex further forms a bridge to inhibit transcription of the promoter, leading to pel repression. In the presence of c-di-GMP, FleQ undergoes a conformational change and then switches into an activator. Crystal structure analysis shows that c-di-GMP binding to the AAA+ ATPase domain of FleQ leads to ATPase active site obstruction, hexameric ring destabilization, and quaternary structure transition disruption, thereby altering the transcriptional activity of FleQ (14). A schematic diagram shows the mechanism by which c-di-GMP and FleQ/KeN coregulate target genes (Fig. 1).

Identifying new members of the c-di-GMP/FleQ regulon helps to enrich knowledge of FleQ. Through the search for FleQ binding consensus sequences in *Pseudomonas* genome sequences, several potential new target genes of FleQ have been obtained (15), such as the siaABCD operon and bdiA gene in *P. aeruginosa*, responsible for cell aggregation and biofilm dispersal, lapA-like adhesins, and a homologue of gcbA encoding diguanylate cyclase. Studies of *P. putida* confirm that lapA and gcbA are directly regulated by FleQ in response to c-di-GMP (16, 17). Chromatin immunoprecipitation sequencing (ChIP-seq) analysis reveals that FleQ regulates iron homeostasis-related genes in *P. fluorescens* and *P. putida* and that FleQ shares some common target genes with another global regulator, AmrZ, in *P. fluorescens*, especially iron-related genes, indicating cross talk between these two regulators (18).

To identify new target genes of c-di-GMP/FleQ, we carried out transcriptomic analysis to investigate the influence of high-level c-di-GMP and fleQ deletion on transcriptomic profiles of *P. putida*. By verifying the results from transcriptomic analysis, we discovered five new target genes under the direct regulation of c-di-GMP/FleQ. Further study concerning the functions of two target genes, lapE and cyaA, revealed the role of c-di-GMP/FleQ in LapA secretion and cyclic AMP (cAMP) synthesis regulation.

**RESULTS**

**Identification of c-di-GMP-regulated genes through transcriptomic analysis.** WspR is a well-known diguanylate cyclase (DGC) with c-di-GMP-synthesizing activity in *Pseudomonas*, and a multicopy plasmid (pBBR1MCS5-wspR) containing wspR is used to increase intracellular c-di-GMP in wild-type KT2440, as previously reported (16, 19). The wild-type KT2440 harboring pBBR1MCS5-wspR is termed WT+wspR, and the wild-type
KT2440 harboring empty plasmid pBBR1MCS5 is termed WT control. pCdrA::gfpC-tet, a fluorescent reporter of c-di-GMP (20), was used to determine and compare the c-di-GMP levels in WT control and WT wspR. Normalized green fluorescent protein (GFP) fluorescence of WT wspR was about threefold as high as that of WT control (Fig. 2A), indicating that introducing pBBR1MCS5-wspR to the wild type provokes an increase in cellular c-di-GMP. To identify genes under the influence of c-di-GMP levels, more c-di-GMP-binding FleQ molecules exist, and such c-di-GMP binding inhibits its ATPase activity of FleQ, repressing transcription of flagellar genes. Meanwhile, c-di-GMP binding changes conformation of the FleQ hexamer and releases FleQ from the repression site of biofilm matrix promoters. FleQ at the activation site activates the transcription together with another sigma factor, possibly RpoD.

Identification of FleQ-regulated genes through transcriptomic analysis. To identify the genes under the influence of FleQ, transcriptomic analysis was performed by comparing the transcriptome profiles of WT wspR and WT control with three technical replicates for each strain. A total of 283 differentially expressed genes (DEGs) were identified (fold change, ≥2 or ≤−2) under high c-di-GMP levels. Among them, 187 genes exhibited upregulated expression (see Table S1 in the supplemental material), and the remaining 96 genes showed downregulated expression (Table S2).

We summarized the numbers of DEGs involved in major physiological processes (Fig. 2B). The 48 DEGs were found to be related to cell motility and secretion processes, which were the most well-known processes that c-di-GMP was involved in. Meanwhile, 39 DEGs were related to amino acid transport/metabolism processes, 34 DEGs to carbohydrate transport/metabolism process, and 20 DEGs to secretion and transport processes, and these were relatively less reported processes that c-di-GMP was involved in. In addition, 68 DEGs encode hypothetical proteins of unknown function.

Identification of FleQ-regulated genes through transcriptomic analysis. To identify the genes under the influence of FleQ, transcriptomic analysis was performed.
to compare the transcriptome profiles of an 
 fleQ deletion mutant (ΔfleQ) and wild-type 
KT2440 with three technical replicates for each strain. A total of 399 DEGs were identi- 
fied (fold change, ≥2 or ≤−2) in the fleQ mutant, of which 172 genes showed upregu-
lated expression (Table S3), and the remaining 227 genes showed downregulated 
expression (Table S4). Of the 399 DEGs, 68 DEGs were related to cell motility and secre-
tion processes, which were the most well-reported processes that FleQ was involved in 
(Fig. 2D). Some DEGs were found to be related to defense mechanisms (41 DEGs), 
amino acid transport/metabolism (34 DEGs), carbohydrate transport/metabolism (23 
DEGs), and signal transduction (21 DEGs). Meanwhile, 133 DEGs were responsible for 
encoding putative function-unknown proteins.

Identification of c-di-GMP/FleQ-regulated genes. c-di-GMP binds to FleQ to modu-
late gene transcription (3, 13, 14). Theoretically, genes regulated by FleQ are also 
influenced by c-di-GMP, but genes regulated by c-di-GMP are not necessarily influ-
enced by FleQ, since there are other potential c-di-GMP-responsive transcriptional reg-
ulators. The purpose of this study was to identify new target genes coregulated by 
c-di-GMP and FleQ. The first transcriptomic analysis (WT+wspR versus WT+control) 
described above identified the potential genes regulated by c-di-GMP, and the second 
transcriptomic analysis (ΔfleQ versus WT) identified the potential genes regulated by 
FleQ. Thus, the genes coregulated by c-di-GMP and FleQ should be found in both the 
first and the second transcriptomic analysis. Considering this, we matched the 283
DEGs identified in WT+wspR with the 399 DEGs identified in the fleQ mutant and found 133 common DEGs (Fig. 2C). Of these 133 DEGs, 47 DEGs were reported to be coregulated by c-di-GMP and FleQ, including 1 lapA gene, 7 bcs genes, and 39 flagellum- and motility-related genes (3, 11, 12, 16), and they were not investigated in the following studies. The remaining 86 DEGs were potentially new target genes of c-di-GMP/FleQ (Table 1), and they belonged to 68 operons in terms of their distribution in the KT2440 genome (GenBank accession no. NC_002947.3). To verify the results of transcriptomic analysis, we compared transcriptions of these 68 operons in WT+wspR, fleQ mutant harboring control vector (∆fleQ+control), and WT+control using quantitative PCR (qRT-PCR). One gene was chosen from each operon to test. Transcription levels of 50 out of the 68 genes exhibited significant differences between WT+control and WT+wspR or ∆fleQ+control (Fig. 3A and B), and these 50 genes exhibited transcription change trends similar to those obtained from the transcriptomic analysis. The qRT-PCR assay results indicated that transcription levels of the remaining 18 genes showed no obvious difference between WT+control and WT+wspR or ∆fleQ+control, suggesting that these 18 genes were not regulated by c-di-GMP/FleQ, and transcriptomic analysis results of these genes and related operons were false positive. Taken together, 50 new operons under the influence of c-di-GMP/FleQ were identified.

FleQ specifically binds with the upstream sequences of PP_0681, PP_0788, lapE, cyaA, and PP_5586. FleQ regulates transcription of target genes by directly binding to their promoters (11, 15). To determine those operons directly regulated by FleQ from the 50 identified operons, we carried out electrophoretic mobility shift assays (EMSAs) to test whether FleQ could bind upstream sequence of each of these 50 operons and found that 7 upstream sequence fragments, including PP_0681, PP_0788, PP_4519(lapEp), PP_4858, PP_5222(cyaAp), PP_5496, and PP_5586, exhibited a stepwise increase in the shifted DNA amount, with FleQ protein amount increasing from 100 nM to 300 nM (Fig. 4A). Binding of FleQ with the seven labeled DNAs was not disrupted by a pUC19 fragment (unlabeled nontarget DNA) (Fig. 4A). EMSA results indicated that the band shifts of PP_0681, PP_0788, lapEp, cyaAp, and PP_5586 were stronger than those of PP_4858 and PP_5496 and that the remaining 43 promoters showed no band shift (Fig. S1). These results indicated that FleQ bound to upstream sequences of PP_0681, PP_0788, lapE, PP_4858, cyaA, PP_5496, and PP_5586 in in vitro EMSA.

In previous studies, a bacterial one-hybrid assay (B1H) was applied to detect protein–DNA interactions in vivo using a reporter plasmid, pBXcmT, containing the selectable genes HIS3 and aadA and a plasmid, pTRG, harboring a gene encoding the alpha subunit of RNA polymerase. The regulator protein has been reported to be able to recruit RNA polymerase and stabilize its binding to the promoter on pBXcmT, activating the transcription of HIS3 and aadA, so that the reporter strains could survive on screening medium containing 3-amino-1,2,4-triazole (3-AT) and streptomycin only when this regulator protein interacts with the promoter on pBXcmT (21). Based on these findings, we performed B1H to verify our EMSA results. As shown in Fig. 4B, the reporter strains containing both pTRG-fleQ and pBXcmT with any of the five promoters (PP_0681, PP_0788, lapEp, cyaAp, and PP_5586) grew well, but the strain containing pTRG-fleQ and pBX-PP_4858 or pBX-PP_5496 exhibited no growth on the screening medium. The self-activation control strain containing either pTRG-fleQ or reporter plasmid alone also did not grow on the screening medium. These results indicated that FleQ specifically bound to the upstream sequences of PP_0681, PP_0788, lapE, cyaA, and PP_5586.

To determine the precise binding site of FleQ in the five promoters, a DNase I footprinting assay was performed. The results indicated that TGAGTCAATACAGGCGCTG (−165 to −145 bp relative to the translational start site) sequence in PP_0681, AAGGGGCGCTGG (−113 to −103 bp relative to the translational start site) and TATTGCGCTGCTAG (−67 to −53 bp relative to the translational start site) sequences in PP_0788, CAAAGTGACAATATTGCGCCAA sequence (−319 to −295 bp relative to the translational start site) in lapEp, and TGACATCGGATCGGT (−175 to −157 bp relative to the
### TABLE 1 Genes exhibiting significant differences in transcription levels in both WT+wspR versus WT+ control and ΔfeQ+ control versus WT+control

| Gene ID | Fold change in: | ΔfeQ mutant versus WT | Gene name | Description |
|---------|----------------|----------------------|-----------|-------------|
| PP_0089 | 2.081          | 7.48                 | osmC      | Stress-induced peroxiredoxin |
| PP_0115 | 2.244          | 10.86                | katE      | Hydroperoxidase |
| PP_0817 | -4.499         | -2.623               | alaC      | Aminotransferase |
| PP_1502 | 2.414          | 6.765                | OmpA family protein |
| PP_1895 | 2.896          | 5.952                | yadG      | ABC transporter ATP-binding protein |
| PP_1896 | 2.533          | 2.928                | yadH      | ABC transporter permease |
| PP_1970 | -6.342         | -9.33                | Lipoprotein |
| PP_2125 | 2.2            | 2.811                | yegS      | Lipid kinase |
| PP_2358 | -2.259         | -2.193               | Putative type 1 pilus subunit CsuA/B protein |
| PP_2359 | -2.406         | -2.078               | Putative type 1 pilus subunit CsuA/B protein |
| PP_2360 | 2.216          | 2.457                | Type I pilus subunit CsuA/B |
| PP_2362 | -2.104         | -2.303               | Usher protein |
| PP_2561 | 2.777          | 5.809                | Hemolysin-type calcium-binding bacteriocin |
| PP_2647 | 30.251         | 32.067               | MFS transporter |
| PP_2689 | 2.354          | 2.221                | Endoribonuclease |
| PP_2827 | 11.051         | 14.62                | Alcohol dehydrogenase |
| PP_3089 | 2.04           | 2.717                | proP      | Osmosensory proline/betaine/H⁺ permease |
| PP_3096 | -2.006         | 2.494                | tssG1     | TssG1 |
| PP_3097 | -2.407         | 2.535                | tssF1     | TssF1 |
| PP_3100 | -2.128         | 2.833                | tssB1     | TssB1 |
| PP_3260 | 2.242          | 8.681                | ligD      | DNA ligase D |
| PP_3360 | 2.218          | 6.175                | Membrane protein |
| PP_3425 | 1073.048       | 897.89               | RND family transporter MFP subunit |
| PP_3426 | 329.193        | 276.409              | mexF      | Multidrug RND transporter MexF |
| PP_3427 | 205.080        | 159.445              | opnN      | Multidrug RND transporter outer membrane protein OpnN |
| PP_3435 | -2.714         | -2.606               | Multidrug RND transporter membrane fusion protein |
| PP_3456 | -2.179         | -4.668               | mexB      | Multidrug resistance protein MexB |
| PP_3503 | 6.009          | 3.556                | Sigma-54 dependent transcriptional regulator |
| PP_3519 | 32.250         | 21.445               | Lipoprotein |
| PP_3541 | 2.027          | 2.404                | MgtC family transporter |
| PP_3613 | 2.069          | 4.476                | L- Sorbose dehydrogenase |
| PP_3878 | 2.257          | -2.87                | Minor capsip protein C |
| PP_3941 | 3.585          | 2.758                | nicF      | Maleamate amidohydrolase |
| PP_3942 | 3.891          | 3.150                | nicE      | Maleate isomerase |
| PP_3943 | 3.983          | 2.488                | nicD      | N-formylmaleamate deformylase |
| PP_3944 | 3.989          | 3.070                | nicC      | 6-hydroxynicotinate 3-monooxygenase |
| PP_3945 | 2.667          | 2.918                | nicX      | 2,5-dihydroxyprpyridine 5,6-dioxygenase |
| PP_4057 | 7.307          | 5.311                | membrane protein |
| PP_4434 | 5.950          | 2.479                | dadAl     | α-Amino acid dehydrogenase small subunit |
| PP_4519 | 2.893          | 7.296                | tolC      | Agglutination protein |
| PP_4856 | 2.167          | 6.544                | Dps family ferritin |
| PP_4983 | 2.550          | 3.045                | Amine oxidase |
| PP_5033 | 41.840         | -2.460               | hutU      | Urocanate hydratase |
| PP_5222 | -2.256         | 3.010                | cyaA      | Adenylate cyclase |
| PP_5269 | 11.139         | 4.010                | daA        | Alanine racemase |
| PP_5270 | 5.722          | 3.509                | dassAll    | α-Amino acidquinone oxidoreductase |
| PP_5298 | -2.615         | -2.316               | Glutamine amidotransferase |
| PP_5299 | -2.752         | -2.209               | psuAll     | Glutamate-putrescine ligase |
| PP_5084 | -2.54          | -4.18                | Methyl-accepting chemotaxis transducer |
| PP_1371 | 3.79           | 16.49                | pctA       | Methyl-accepting chemotaxis protein PctA |
| PP_1819 | -2.426         | -2.632               | Methyl-accepting chemotaxis transducer |
| PP_2249 | 4.663          | -11.056              | pctB       | Methyl-accepting chemotaxis protein PctB |
| PP_3557 | -2.710         | -3.439               | methyl-accepting chemotaxis transducer |
| PP_4888 | -2.570         | -4.793               | methyl-accepting chemotaxis transducer |
| PP_5020 | -4.702         | -13.313              | methyl-accepting chemotaxis protein |
| PP_0681 | 3.128          | 2.467                | Hypothetical protein |
| PP_0788 | -11.795        | 6.391                | Hypothetical protein |
| PP_1503 | 2.555          | 6.825                | Hypothetical protein |
| PP_1691 | 4.986          | 2.156                | Hypothetical protein |

(Continued on next page)
to the translational start site) and TATGGTGTCGGATCATTGA (−118 to −100 bp relative to the translational start site) sequences in PP_5586 were protected by FleQ protein (Fig. 4C). Unfortunately, we tried several times but failed to obtain the precise binding site(s) in the DNase I footprinting assay of the cyaA promoter. To locate the specific region of the cyaA promoter interacting with FleQ, three truncated fragments of cyaA promoters were amplified for EMSA. As shown in Fig. 4D, cyaApF2 and cyaApF3 DNA fragments produced a band shift on the gel, whereas no band shift was observed with cyaApF1, indicating that the binding site(s) is located between positions −2139 and −51 on the cyaA promoter relative to its translational start site.

Since c-di-GMP can change the oligomerization of FleQ and, thus, change FleQ/promoter binding (13, 14), we added c-di-GMP (80 μM) to the reaction solution to test the effect of c-di-GMP on the binding of FleQ to target promoters in a DNase I footprinting assay. However, we failed to observe any change in the binding sites with or without c-di-GMP (data not shown). We then added c-di-GMP (from 0 to 90 μM) to the reaction solution in EMSA. The EMSA results showed that c-di-GMP enhanced the binding of FleQ to PP_0681pro, lapEpro, and PP_5586pro, but it had no obvious influence on the binding of FleQ to PP_0788pro and cyaApro (Fig. 4E).

**c-di-GMP regulates expression of PP_0681, PP_0788, lapE, cyaA, and PP_5586 in FleQ-dependent manner.** To further determine whether c-di-GMP regulated the expression of the five new target genes via FleQ, we compared promoter activities of the five genes under different c-di-GMP levels in wild-type KT2440 and the fleQ mutant by using β-galactosidase (LacZ) promoter fusion reporters. High c-di-GMP levels were achieved by expressing WspR as described above, and low c-di-GMP levels were obtained by expressing the phosphodiesterase BifA as previously reported (Fig. 2A) (16). Promoter activities of PP_0681, PP_5586, and lapE were decreased in WT+wspR and increased in WT+wspR compared with those in WT+control, but promoter activities of PP_0788 and cyaA showed an opposite trend. In the fleQ deletion mutant, the promoter activity of each gene showed no obvious differences between ΔfleQ+wspR or

### TABLE 1 (Continued)

| Gene ID | Fold change in: | Gene name | Description |
|---------|-----------------|-----------|-------------|
| PP_1828 | −3.956          | −16.106   | Hypothetical protein |
| PP_2059 | 2.102           | 4.908     | Hypothetical protein |
| PP_2858 | 29.15           | −7.298    | Hypothetical protein |
| PP_3104 | −2.238          | 3.096     | Hypothetical protein |
| PP_3261 | 2.523           | 13.247    | Hypothetical protein |
| PP_3524 | 2.007           | 5.944     | Hypothetical protein |
| PP_3542 | 2.092           | 2.267     | Hypothetical protein |
| PP_3770 | 53.334          | 48.34     | Hypothetical protein |
| PP_3795 | −3.555          | −6.312    | Hypothetical protein |
| PP_3855 | 3.297           | −2.668    | Hypothetical protein |
| PP_3856 | 2.217           | −2.995    | Hypothetical protein |
| PP_3874 | 2.327           | −3.28     | Hypothetical protein |
| PP_3928 | 5.593           | 10.226    | Hypothetical protein |
| PP_4406 | −4.688          | −8.016    | Hypothetical protein |
| PP_4858 | 70.390          | 42.431    | Hypothetical protein |
| PP_5073 | −5.853          | −3.019    | Hypothetical protein |
| PP_5430 | 3.929           | −6.020    | Hypothetical protein |
| PP_5462 | 2.957           | −2.611    | Hypothetical protein |
| PP_5496 | 79.486          | 44.679    | Hypothetical protein |
| PP_5524 | 2.395           | 23.189    | Hypothetical protein |
| PP_5542 | 2.816           | 3.496     | Hypothetical protein |
| PP_5549 | 4.553           | −2.003    | Hypothetical protein |
| PP_5560 | 2.394           | 16.782    | Hypothetical protein |
| PP_5586 | 80.559          | 3.473     | Hypothetical protein |
| PP_5592 | 3.287           | 8.070     | Hypothetical protein |
| PP_5710 | −2.442          | −39.200   | Hypothetical protein |

*Positive values indicate the increased transcription levels, and negative values indicate the decreased transcription levels. Detailed information of gene names and descriptions can be obtained from the annotated genome database at [http://www.pseudomonas.com](http://www.pseudomonas.com) (48).
ΔfleQ−ΔbflA and ΔfleQ+control, indicating that the regulation of target genes by c-di-GMP was abolished (Fig. 5). These results suggested that c-di-GMP regulated expression of \textit{PP_0681}, \textit{PP_0788}, \textit{lapE}, \textit{cyaA}, and \textit{PP_5586} in a FleQ-dependent manner.

In this study, the overexpression of WspR resulted in high-level c-di-GMP, further changing the transcription of target genes, but transcription changes possibly were...
FIG 4 Analysis for interactions between FleQ and upstream sequences of PP_0681, PP_0788, lapE, cyaA, and PP_5586. (A) FleQ protein binds to upstream sequences of the seven genes in EMSA. The concentrations of FleQ and the amounts of unlabeled DNA used are shown. Unlabeled pUC19 fragment was used for competition experiments. (B) Bacterial one-hybrid assays of the interactions between FleQ and promoters of the seven target genes. Cotransformants containing plasmids pTRG-Rv3133 and pTRG1pBX were used as positive and negative controls, respectively. (C) DNase I footprinting assay with fragments containing the promoters of PP_0681, PP_0788, lapE, cyaA, and PP_5586 in the presence and absence of FleQ. The protected regions and sequences are boxed. (D) Binding of truncated cyaA promoter fragments to FleQ. Schematic (Continued on next page)
caused by unintended effects of overexpressing a protein. To test this possibility, we introduced a point mutation of the GGDEF motif of WspR (from GGEEF to GGAAF) to abolish the c-di-GMP synthesis ability of WspR. Western blot results showed that the point-mutated WspR was detected in both the wild-type strain and fluQ mutant with their molecular weight and signal intensity similar to those of the wild-type WspR, indicating that the point-mutated WspR was stable in both the wild type and fluQ mutant (Fig. S2). Normalized GFP fluorescence of the c-di-GMP reporter exhibited no obvious difference between WT+wspR GGAAF and WT+control (Fig. 2A), indicating that introduction of the point-mutated WspR to the wild type had no influence on cellular c-di-GMP.

The influence of the point-mutated WspR on activities of the five target promoters was investigated. The results showed that all five promoters showed no obvious difference in activity between WT+wspR GGAAF and WT+control (Fig. 2A), implying that the transcriptional changes were caused by c-di-GMP rather than by unintended effects of overexpression of WspR.

ATPase activity, RpoN binding ability, and c-di-GMP binding ability of FleQ are not required for the complementation of new target genes. Typically, FleQ interacts with the sigma factor RpoN bound to the promoter of the flagellar gene. This RpoN recruits RNA polymerase to the promoter, and then the ATPase domain of FleQ catalyzes ATP hydrolysis to form an RNA polymerase-promoter open complex and to initiate gene transcription (11). However, ATPase activity and RpoN binding ability of FleQ are not required for regulation of exopolysaccharide-synthesizing operons (15, 17). The key roles of mutants of amino acid residues T224 and D245 in abolishing RpoN binding ability and ATPase activity of FleQ are confirmed from P. aeruginosa (22). To check whether the positions of these amino acid residues were the same in P. aeruginosa and...
P. putida, we aligned the amino acid sequence of FleQ in P. putida KT2440 with that in P. aeruginosa PAO1. Results showed that FleQ in P. aeruginosa and FleQ in P. putida shared 84% amino acid sequence identity, and that positions of the two amino acid residues (T224 and D245) were the same in the two bacteria (Fig. S3). Based on this, the mutants FleQT224S and FleQD245A were separately introduced to construct the flcQ complementation plasmids, and the obtained complementation plasmids were transformed into flcQ mutant to obtain a complementation strain (termed cΔflcQ T224S/D245A).

Promoter activities of the five target genes in cΔflcQ T224S/D245A were tested and compared with those in the wild-type strain, flcQ mutant, and flcQ mutant complemented with wild-type FleQ (termed cΔflcQ WT). The results showed that transcriptional activities of all five target promoters were totally restored by the two point-mutated FleQ proteins to wild-type FleQ, indicating that point mutations of FleQT224S and FleQD245A had no obvious influence on complementation of all five new target genes (Fig. 6). These results suggested that neither the ATPase activity nor RpoN binding ability of FleQ were required for complementation of PP_0681, PP_0788, lapE, cyaA, and PP_5586.

Mutation of another conserved amino acid residue (K180A) in the ATPase domain of FleQ has been reported to abolish both c-di-GMP binding ability and ATPase activity of FleQ (22). Based on this finding, we introduced mutant FleQK180A to construct the flcQ complementation plasmid and transformed this complementation plasmid into flcQ mutant to obtain a complementation strain (termed cΔflcQ K180A). Promoter activities of the five target genes in cΔflcQ K180A were tested and compared with those in the wild-type strain, flcQ mutant, and flcQ mutant complemented with wild-type FleQ (termed cΔflcQ WT). The results showed that transcriptional activities of all five target promoters were totally restored by the two point-mutated FleQ proteins to wild-type FleQ, indicating that point mutations of FleQ K180A and FleQ D245A had no obvious influence on complementation of all five new target genes (Fig. 6). These results suggested that neither the ATPase activity nor RpoN binding ability of FleQ were required for complementation of PP_0681, PP_0788, lapE, cyaA, and PP_5586.

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Promoter activities of the five target genes in cΔflcQ T224S/D245A were tested and compared with those in the wild-type strain, flcQ mutant, and flcQ mutant complemented with wild-type FleQ (termed cΔflcQ WT). The results showed that transcriptional activities of all five target promoters were totally restored by the two point-mutated FleQ proteins to wild-type FleQ, indicating that point mutations of FleQ T224S and FleQ D245A had no obvious influence on complementation of all five new target genes (Fig. 6). These results suggested that neither the ATPase activity nor RpoN binding ability of FleQ were required for complementation of PP_0681, PP_0788, lapE, cyaA, and PP_5586.

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Transcriptional changes of lapE and cyaA lead to corresponding protein level changes. c-di-GMP/FleQ modulates the lifestyle transition from plankton to biofilm by regulating expression of biofilm- and flagellum-related genes (3, 15, 16). In this study, we identified five target genes coregulated by FleQ and c-di-GMP in P. putida through transcriptomics and protein-DNA binding assays. c-di-GMP/FleQ inhibited the transcription of PP_0788 and cyaA and promotes the transcription of PP_0681, lapE, and PP_5586. We further investigated the functions of the five genes in P. putida and the effect of c-di-GMP/FleQ on the phenotypes related to these genes. BLAST results revealed that PP_0681, PP_0788, and PP_5586 encoded putative function-unknown proteins but lapE and cyaA did not; thus, lapE and cyaA were further investigated.

Before we examined the influence of c-di-GMP/FleQ on the phenotypes related to lapE and cyaA, we first tested whether the transcriptional changes of lapE and cyaA lead to corresponding protein level changes. We fused the gfp gene to the end of lapE-cyaA to achieve fusion expression in a plasmid, and transcriptions of lapE and cyaA from the plasmid were under the control of their native promoters. We then transformed the plasmids into the wild-type strain, bifA mutant (ΔbifA), fleQ mutant (ΔfleQ), and fleQ-bifA double mutant (ΔfleQ ΔbifA) and further tested their GFP fluorescence intensity. Our previous study has shown that deletion of BifA (a phosphodiesterase with c-di-GMP degradation activity) causes increased c-di-GMP levels in P. putida (23). The results showed that GFP fluorescence intensity of the ΔbifA mutant containing the lapE-gfp fusion reporter was much higher than that of the wild type, whereas the fluorescence intensity of the ΔbifA mutant containing the cyaA-gfp fusion reporter was lower than that of the wild type (Fig. 7A). Fluorescence intensity of the ΔfleQ mutant containing either fusion reporter was higher than that of the wild type. The ΔfleQ ΔbifA mutant containing either fusion reporter showed fluorescence intensity similar to that of the ΔfleQ mutant, suggesting that the modulation of c-di-GMP on LapE and CyaA protein levels was FleQ dependent. These results indicated that under high c-di-GMP levels, transcriptional levels of lapE and cyaA affected the corresponding protein levels in an FleQ-dependent manner.

LapE is responsible for LapA secretion and biofilm formation. LapE (PF01_1462) in P. fluorescens Pf0-1, which shares about 73.3% amino acid sequence identity with LapE of P. putida, is responsible for secretion of LapA (an adhesin), which plays a vital role in initial attachment and biofilm formation (24–26). To test the role of LapE in LapA secretion and biofilm formation in P. putida, a lapE deletion mutant (ΔlapE) was constructed using wild-type KT2440 containing a 3 × hemagglutinin (HA) tag in LapA. Biofilm formation and LapA secretion of the mutant were assessed. Deletion of lapE abolished biofilm formation, and complementation with multicopy wild-type lapE (cΔlapE) restored biofilm formation of the ΔlapE mutant (Fig. 7B). Dot blot assays using anti-HA antibody revealed that deletion of lapE significantly reduced the content of cell surface-associated (CS) LapA, and complementation restored the content of CS LapA. In addition, the c-di-GMP level was elevated in the lapE mutant by introducing multiple copies of wspR, causing increased total cellular (TC) LapA content, but had no obvious influence on CS LapA content and biofilm formation, indicating that LapE was necessary for LapA secretion and biofilm formation even under high-level c-di-GMP conditions (Fig. 7B). The wild-type strain without the 3 × HA tag was used as a negative control (NC), and our data showed no detectable signal in both total cellular LapA and cell surface LapA in this strain. All these results suggested that LapE of P. putida was responsible for LapA secretion and biofilm formation.

To further test the role of FleQ and c-di-GMP in biofilm formation and LapA secretion, we constructed an fleQ deletion mutant (ΔfleQ) and an fleQ-ΔlapE double deletion mutant (ΔfleQ ΔlapE) using the WT-3 × HA strain. The wspR expression vector was introduced into the two mutants to increase c-di-GMP (ΔfleQ+ wspR and ΔfleQ ΔlapE+ wspR), and empty vector was introduced into the mutants as controls (ΔfleQ+ control and ΔfleQ ΔlapE+ control). The biofilm formation and LapA levels in these mutants were tested. ΔfleQ+ control showed defection in biofilm formation, as we previously reported (16), and ΔfleQ+ wspR exhibited slightly more biofilm formation.
than ΔfleQ+control (Fig. 7B). Biofilm formed in ΔfleQ ΔlapE+control was almost undetectable, and ΔfleQ ΔlapE+wspR showed a similar biofilm phenotype (Fig. 7B). Dot blot assays revealed that ΔfleQ+control and ΔfleQ+wspR exhibited less surface LapA and total LapA than the wild-type strain, which might be attributed to the decreased lapA transcription in these two fleQ mutants (16). Surface LapA content was undetectable in ΔfleQ ΔlapE+control and ΔfleQ ΔlapE+wspR, and their total LapA level was significantly lower than that in the wild type but similar to that in ΔfleQ+control (Fig. 7B). These results demonstrated that FleQ was essential for c-di-GMP-mediated biofilm formation and LapA secretion.

**c-di-GMP decreases cAMP content in FleQ-dependent manner.** cyaA, encoding an adenylate cyclase, is responsible for synthesis of another second messenger, cAMP, in *P. putida* (27). Both a previous study in *P. aeruginosa* and our recent study in *P. putida* revealed that high-level c-di-GMP can decrease the content of cAMP, but their correlation mechanism remains unclear (28, 29). In this study, our result showed that c-di-GMP regulated the expression of *cyaA* in an FleQ-dependent manner. Based on this result, we hypothesized that c-di-GMP decreases cAMP content via FleQ. To test this hypothesis, we measured the cAMP levels in WT+control, WT+wspR, ΔfleQ+control, and ΔfleQ+wspR strains by performing an enzyme-linked immunosorbent assay (ELISA). As shown in Fig. 7C, ΔfleQ+control exhibited higher cAMP content than
WT+control, which may be attributed to the increased cyaA expression in the ΔfleQ mutant. WT+wspR showed lower cAMP levels than WT+control. However, ΔfleQ+wspR showed cAMP levels similar to those of ΔfleQ+control, indicating that the influence of c-di-GMP on cAMP was abolished in the ΔfleQ mutant. This result revealed that c-di-GMP lowered cAMP content in an FleQ-dependent manner.

**DISCUSSION**

By performing transcriptomic analysis, we identified 68 differentially expressed operons coregulated by c-di-GMP and FleQ in *P. putida* (Table 1). However, only 50 of them were confirmed to be positive by subsequent qRT-PCR assay (Fig. 3). We speculated that the major reason for this obvious discrepancy was that the three RNA copies used for the transcriptomic assay were extracted from three technical replicates of a single sample, while the RNAs used for the qRT-PCR assay were extracted from three biological replicates of each sample. Therefore, there existed a difference in RNAs between the two assays, and we considered the qRT-PCR result more accurate and reliable in this case. In protein-DNA binding assays (EMSA and B1H), we identified five new target genes under the direct regulation of FleQ. Through ChIP-seq analysis, a previous study identified 160 putative target genes of FleQ in *P. putida* KT2440, including several iron homeostasis-related genes (18). However, these iron homeostasis-related genes have not been identified in this study using transcriptomic analysis. Likewise, cyaA and PP_5586, identified in this study, have not been identified with ChIP-seq analysis. This result discrepancy may be caused by the different types of applied methods and procedures of transcriptomic analysis versus ChIP-seq analysis. ChIP-seq identifies potential target genes by screening FleQ-bound promoters in vivo first and then examining the influence of FleQ on expression of potential target genes, while transcriptomic analysis shows potential target genes by screening differentially expressed genes under the influence of FleQ. Whether FleQ can bind to target promoters is tested in subsequent assays.

FleQ is a global transcriptional regulator, and finding an FleQ-specific binding motif will help to improve target gene identification. Previous studies have reported an FleQ binding motif in *P. aeruginosa* (GTCaNTAAAtTGAC) based on 13 binding sites, and a FleQ binding motif in *P. putida* KT2440 (GTCAaAAAatTGAC) was identified based on the promoter regions of 15 selected genes and conserved sequence in *P. aeruginosa* (15, 30). In this study, FleQ-binding sites on the promoters of PP_0681, PP_0788, lapE, and PP_5586 were identified by footprinting assay (Fig. 4C), but no conserved sequence was found from the identified binding sites and no significant similarity was found to the abovementioned two conserved FleQ-binding sequences reported in previous studies. Consistent with this conclusion, the previous ChIP-seq analysis has not found a robust FleQ-binding motif in *P. putida* KT2440 (18). FleQ can form dimers, tetramers, and hexamers, and binding of c-di-GMP to FleQ leads to hexameric ring destabilization and quaternary structure transition disruption, resulting in FleQ existence mainly in the form of monomers and dimers (14, 22). It is possible that oligomerization influences DNA binding preferences of FleQ, which means that FleQ may bind to different sites under different oligomeric states. This hypothesis can explain why no robust FleQ-binding motif has been identified from all the target promoters.

Our data indicated that high-level c-di-GMP and fleQ deletion had opposite effects on transcriptions of some target genes (Table 1). For instance, fleQ deletion increased transcription of cyaA but high-level c-di-GMP decreased transcription of cyaA (Fig. 3), and a similar trend was observed in PP_0788. The effects of high-level c-di-GMP and fleQ deletion on the transcription levels were inconsistent among different genes. For example, for lapA, high c-di-GMP caused increased lapA transcription, and fleQ deletion caused decreased lapA transcription (16); for the bcs operon, both high-level c-di-GMP and fleQ deletion increased bcs transcription (16); and for fleR, both high-level c-di-GMP and fleQ deletion decreased fleR transcription (3). One possible explanation is that FleQ functions as both a repressor and an activator to control gene expression by...
binding to two sites on the target promoter and that c-di-GMP changes the binding of FleQ to one site, resulting in transcriptional changes under high-level c-di-GMP, as previously described for pel regulation in *P. aeruginosa* (13). Consistent with this explanation, our footprinting assay revealed that both *PP_0788pro* and *PP_5586pro* contained two FleQ-binding sites and that both *PP_0681pro* and *lapEpro* had one relatively larger FleQ-binding site (Fig. 4C); this larger binding site might consist of two adjacent binding sites that cannot be distinguished due to experimental limitations.

The adhesin LapA is required for cell surface interactions and biofilm formation in *P. fluorescens* and *P. putida* (21, 31). LapA needs to be retained at the cell surface in a “half-secreted” status to perform its function, and the retention of LapA relies on the outer membrane pore LapE (26). Thus, there might exist a mechanism to ensure matching the expression of LapE and the expression of LapA. This speculation was confirmed by our finding that *lapE* and *lapA* were both positively coregulated by c-di-GMP and FleQ. Promotion of *lapE* transcription by c-di-GMP/FleQ favored LapA secretion and biofilm formation. Under high-level c-di-GMP, LapA was upregulated by the c-di-GMP effector FleQ at the transcription level, while LapA cleavage was inhibited by a c-di-GMP-dependent signaling system at the posttranscriptional level (16, 32–35). Our results in this study demonstrated that LapA was regulated by c-di-GMP at the secretion level. It is these multilevel regulatory mechanisms that guarantee high efficiency of LapA expression/localization and biofilm formation under certain c-di-GMP levels.

Our investigation of another target gene, *cyaA*, reveals the role of c-di-GMP/FleQ in cAMP synthesis regulation, which is in line with the previous findings that high-level c-di-GMP decreased cAMP content in *P. aeruginosa* and *P. putida* (28, 29). However, the mechanism behind this negative correlation between the 2 second messengers remains unknown. Our results showed that transcription of the adenylate cyclase encoded by *cyaA* was directly coinhibited by c-di-GMP and FleQ and that the c-di-GMP-mediated lowering of cAMP content was FleQ dependent, suggesting that c-di-GMP and FleQ coregulate *cyaA* expression and further modulate cAMP in *P. putida*. However, the c-di-GMP-mediated lowering of cAMP content was not caused by inhibition of adenylate cyclase transcription in *P. aeruginosa* (28). The inconsistent results in *P. putida* and *P. aeruginosa* imply that although the same inhibition of cAMP by c-di-GMP was observed in both strains, the inhibition mechanisms are different. After all, functions of cAMP in *P. aeruginosa* and *P. putida* are different. For example, cAMP in *P. aeruginosa* positively regulates the expression of acute virulence factors, including type II and III secretion systems and type IV pili (36, 37), whereas cAMP in *P. putida* is involved in the utilization of amino acids as N sources but plays no roles in virulence regulation (27, 38).

The remaining three target genes, *PP_0681*, *PP_0788*, and *PP_5586*, encode hypothetical proteins with unknown functions. Since c-di-GMP/FleQ is mainly reported to modulate the bacterial plankton-to-biofilm lifestyle transition, the three genes are assumed to belong to unidentified biofilm- or flagellum-related genes, or they may play a role in bacterial plankton-to-biofilm lifestyle transition. Our observation provides evidence for this assumption that *PP_5586* was closely located upstream of the exopolysaccharide Pea synthesizing/transporting operon, implying that *PP_5586* is related to Pea synthesis/transport (39).

In conclusion, we investigated the influence of high-level c-di-GMP and *fleQ* deletion on the transcriptomic profile of *P. putida* and identified five new target genes directly regulated by c-di-GMP/FleQ. We further characterized the function and regulation of two target genes, *lapE* and *cyaA*. The results of this study extend our knowledge of c-di-GMP-mediated FleQ-dependent transcriptional regulation, LapA adhesin secretion, and cAMP synthesis regulation in *P. putida*.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** All bacterial strains and plasmids used in this study are listed in Table S5 in the supplemental material. Planktonic cultures of *Escherichia coli* and *P. putida* strains were routinely grown in Luria-Bertani (LB) broth at 37°C and 28°C, respectively, with 180-rpm
shaking. For agar plates, LB medium was solidified with 1.5% (wt/vol) agar. Antibiotics were used, when required, for plasmid maintenance or transformant screening at the following concentrations: kanamycin (50 mg · liter⁻¹), chloramphenicol (25 mg · liter⁻¹), gentamicin (20 mg · liter⁻¹ for E. coli or 40 mg · liter⁻¹ for P. putida), and tetracycline (10 mg · liter⁻¹).

**Plasmid and strain construction.** All DNA manipulations were performed by following standard protocols (40). Primers used for plasmid and strain construction are listed in Table S6. All cloning steps involving PCR were verified by commercial sequencing (Tsingke, Wuhan, China). Gene deletion mutants were constructed by following a previously described method (16). Briefly, to construct a *P. putida* lapE deletion mutant, ~800 bp from the chromosomal regions flanking *lapE* were PCR amplified with oligonucleotide pair *lapE_UpS* and *lapE_UpA* (upstream region) or *lapE_DwS* and *lapE_DwA* (downstream region). The PCR products were ligated into a suicide vector, pBBR401, yielding pBBR401-UP-DOWN. A kanamycin resistance cassette was amplified from plasmid pTnmod-RKm and ligated into pBBR401-UP-DOWN, generating the final plasmid pBBR401-UP-Km-DW. The final plasmid was transferred to *P. putida* KT2440 by electroporation. Selection of the kanamycin resistance strain was performed on a kanamycin and chloramphenicol double antibiotics plate. The structure of the deleted *lapE* locus was verified by PCR and sequencing. Other deletion mutants were constructed with the same method.

The strain containing the 3 × HA tag-labeled LapA was constructed with a method similar to that for the isogenic mutant, except that a 3 × HA epitope-encoding sequence instead of a kanamycin resistance cassette was ligated into the suicide vector containing two adjacent homologous sequences of *lapA*. After electroporation, selection of the integration gentamicin resistance strain was performed on gentamicin resistance strain was performed on gentamicin resistance strain was performed on gentamicin resistance strain was performed on gentamicin resistance strain was performed on gentamicin resistance strain was performed on gentamicin resistance strain was performed on gentamicin resistance strain was performed on gentamicin resistance strain was performed on gentamicin resistance strain was performed on gentamicin resistance strain was performed on gentamicin resistance strain was performed on gentamicin resistance strain was performed on gentamicin resistance strain was performed on gentamicin resistance strain was performed on gentamicin resistance strain was performed on gentamicin resistance strain was performed on gentamicin resistance strain was performed on gentamicin resistance strain was performed on gentamicin resistance strain was performed on gentamicin resistance strain 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for 10 min. Excess crystal violet was washed off with distilled water 3 times, and then tubes were left at room temperature for drying for 4 h before taking digital photographs.

**Assays for β-galactosidase activity.** β-Galactosidase activity was measured as described in a previous study (46). Overnight cultures were inoculated (1:100 dilution) in fresh LB medium supplied with tetracycline and grown for 24 h. Two milliliters of culture was pelleted by centrifuging at 4°C, and the pellet was resuspended with the same volume of chilled 2× buffer (0.06 mol liter⁻¹ Na₂HPO₄ · 7H₂O, 0.04 mol liter⁻¹ NaH₂PO₄ · H₂O, 0.01 mol liter⁻¹ KCl, 0.001 mol liter⁻¹ MgSO₄ · 0.05 mol liter⁻¹ β-mercaptoethanol, pH 7.0). After measurement of the optical density at 600 nm (OD₆₀₀), 0.2 ml (V) of the resuspended cell culture was added to permeabilize bacterial cells for 5 min. The β-galactosidase activity reaction was started by adding 0.2 ml of 20 mM o-nitrophenyl-β-D-galactopyranoside (in Z buffer). After the reaction solution turned yellowish at 28°C, 0.5 ml of 1 M Na₂CO₃ was added to stop the reaction, and reaction time (T), in minutes, was record. The reaction time was limited to within 30 min, and if the β-galactosidase activity was too low to turn the reaction solution yellowish within 30 min (such as cynA-proA-lacZ), more cell culture (0.5 ml) would be used in the reaction mixture. Finally, we spun the reaction solution for 5 min at 15,000 × g to remove debris and chloroform, and the optical densities of the supernatant at 420 nm and 550 nm were measured with a spectrophotometer (INESA, Shanghai, China). The unit of enzyme activity was calculated using the following equation: Miller units = 1,000 × [(OD₄₂₀ − 1.75 × OD₅₅₀)]/(T × V × OD₀₁₀). The wild-type strain with multiple wspR genes was repeatedly blown with a pipette until the culture became well distributed before analysis, since the strain formed clumps and strong biofilm. Measurements were repeated at least in triplicate with two technical repeats per sample.

**Fluorescence measurements.** Overnight cultures bearing reporter plasmid lapE-gfp or cynA-gfp or pCdRA-gfp-tet (a derivative of pCdrA-gfp⁶, with its gentamicin resistance gene replaced by a tetracycline resistance gene) were diluted in 5 ml LB to an OD₆₀₀ of 0.01. Cultures were incubated for 24 h at 28°C with shaking. For GFP fluorescence measurements, culture samples were transferred to 96-well microtiter dishes, and OD₆₀₀ and fluorescence were determined on an Envision multimode plate reader (PerkinElmer, Germany). Fluorescence was measured from 150-μl samples using a 485-nm band pass filter and a 520-nm emission filter on a black 96-well plate (Costar, USA). Specific fluorescence was calculated by dividing the fluorescence reading by the OD₆₀₀ reading and then normalized by subtracting the autofluorescence of wild-type or bifA mutant culture not bearing a GFP plasmid. The WT + wspR and bifA mutant cultures were repeatedly blown with a pipette until the culture became well distributed before analysis, since the strain formed clumps and strong biofilm. For each measurement, 4 biological replicates were assayed in sextuplicate.

**Dot blot assay and Western blot assay.** Dot blot assay was performed as described in a previous study (47). Briefly, aliquots of exponentially growing cultures were pelleted, washed once with phosphate-buffered saline (PBS), and then resuspended in PBS. For cell surface-associated LapA assays, cell suspensions were adjusted to equivalent OD values (OD₆₀₀ ≈ 1), and then 5-μl aliquots of each suspension were spotted onto a nitrocellulose membrane. For total cellular LapA assays, cells were lysed with a JBIO pressure cell breaking apparatus, and protein concentration was determined by bichinchoninic acid (BCA) assay. The same amount of protein (5 μg) was spotted onto a nitrocellulose membrane. After drying at room temperature, membranes were probed for LapA-3×HA using an anti-HA mouse antibody (Signalway Antibody). A horseradish peroxidase-conjugated secondary antibody (goat anti-mouse; Signalway Antibody) was used for chemiluminescent detection of bound ligands. Detection was carried out using Western ECL reagents (Bio-Rad). Images of dot blots were digitized using a Tanon 5200 scanner (Shanghai, China). For Western blot assay, strains harboring strep II-tagged WspR were cultured and lysed with the method described above for the dot blot assay. The same amount of protein from different samples was resolved by 12.5% SDS-PAGE. After transferring the proteins onto a nitrocellulose membrane, WspR protein expression was measured by chemiluminescent detection of bound ligands. Detection was carried out using Western ECL reagents (Bio-Rad). Images of dot blots were digitized using a Tanon 5200 scanner (Shanghai, China). For Western blot assay, strains harboring strep II-tagged WspR were cultured and lysed with the method described above for the dot blot assay. The same amount of protein from different samples was resolved by 12.5% SDS-PAGE. After transferring the proteins onto a nitrocellulose membrane, WspR protein concentration was determined by BCA assay.

**Expression and purification of His-tagged FleQ.** E. coli BL21 carrying pET28a-fleQ was grown overnight in LB, diluted 1:100, and grown for 4 h at 37°C. The expression of His-tagged FleQ was induced with addition of 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside), followed by incubation at 16°C overnight. Cultures were harvested by centrifugation at 6,000 × g for 10 min and resuspended in lysing buffer (10 mM Tris·Cl [pH 7.8], 300 mM KCl, and 10% glycerol); Cells were lysed with a JBIO pressure cell breaking apparatus, followed by centrifugation at 15,000 × g for 5 min. The lysate was filtered through a 0.22-μm-pore-size filter before it was loaded onto a NiSO₄ column and collected from the column with elution buffer (10 mM Tris·Cl [pH 7.8], 300 mM KCl, 10% glycerol, 250 mM imidazole). Protein concentration was determined by BCA assay.

**EMSA.** Fragments of target promoters used in EMSA were generated by PCR using 6-FAM (6-carboxyfluorescein phosphoramidate)-labeled primers (Tsingke, Wuhan, China). Equal amounts of labeled DNA fragments were added to binding reactions with various amounts of FleQ in binding buffer (10 mM Tris, pH 7.8, 10 mM magnesium acetate, 50 mM KCl, 5% glycerol, 250 ng ml⁻¹ bovine serum albumin, 20 μl total reaction volume). FleQ was incubated with DNA for 30 min at 25°C. Reaction mixtures containing c-di-GMP were performed as described above, except that c-di-GMP was incubated with FleQ for 10 min before addition of DNA. All reaction solutions were loaded onto a 5% acrylamide gel containing 10 mM Tris·Cl (pH 7.8), 400 mM glycine, 5 mM EDTA and electrophoresed at 100 V at room temperature for 1 to 1.5 h. Gels were dried and exposed to a phosphorimaging screen.
**Bacterial one-hybrid assay.** FleQ was cloned into pTRG vector. Promoters of target genes were amplified and cloned into pBXcmT vector (21). Primers used for amplifying target promoters are listed in Table S6. E. coli XL1-Blue MRF+ Kan (Stratagene) was used for routine propagation of all pBXcmT and pTRG recombinant plasmids. **Bacterial one-hybrid assays** were carried out as described previously (21). Positive growth cotransformants were selected on a selective screening medium plate containing 10 mM 3-AT, 10 μg/ml streptomycin, 10 μg/ml tetracycline, 25 μg/ml chloramphenicol, and 30 μg/ml kanamycin. The plates were incubated at 30°C for 3 days. Cotransformants containing the pBXcv3133 plasmids (21) served as a positive control, and cotransformants containing empty vectors pBXcmT and pTRG served as negative controls.

**DNase I footprinting assay.** The 6-FAM-labeled target promoters were PCR amplified using 6-FAM-labeled primers. In a 600-μl reaction system, 1,000 ng of labeled DNA fragment was bound to 600 nM FleQ (final concentration; bovine serum albumin was used instead of FleQ in the control experiment) in buffer containing 10 mM Tris-HCl (pH 7.8), 10 mM MgCl2, 1 mM CaCl2, 0.4 mM dithiothreitol, 100 mM KCl, and 5% glycerol and incubated for 30 min at room temperature. After binding, 0.03 U of RNase-free DNase I (Roche, Basel, Switzerland) was added and allowed to react for 3 min at 23°C. The reaction was stopped and precipitated with ethanol. Samples were analyzed in a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA), and the electropherograms were aligned with GeneMapper v3.5 (Applied Biosystems).

**cAMP content measurement.** Intracellular cAMP contents of *P. putida* were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Abcam). Cells were grown in M9 medium supplemented with 0.4% glucose as a carbon source for 24 h. Nucleotides were extracted with extraction solution (methanol-acetonitrile-water, 2:2:1, vol/vol/vol) and a heating method described previously (28). The samples were assayed for cAMP by following the manufacturer’s protocol. The protein concentrations of bacterial samples were measured by the BCA protein assay. cAMP contents were normalized to the total protein per milliliter of culture.

**Statistical analysis.** For analysis of the significance of differences in gene expression and cAMP concentrations, Student’s t test was used for comparison of two groups of data. For analysis of the significance of differences in β-galactosidase activity, analysis of variance was used for comparison of three or more groups of data. A P value of less than or equal to 0.05 was considered statistically significant.

**Data availability.** Transcriptomic data were deposited in the SRA database under accession numbers SRR8959867, SRR8959868, SRR8959869, SRR8959870, SRR8959871, SRR8959872, SRR8959874, SRR8959875, and SRR8959873.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**FIG S1**, TIF file, 1.9 MB.
**FIG S2**, TIF file, 0.3 MB.
**FIG S3**, TIF file, 2.2 MB.
**TABLE S1**, DOC file, 0.3 MB.
**TABLE S2**, DOC file, 0.2 MB.
**TABLE S3**, DOC file, 0.3 MB.
**TABLE S4**, DOC file, 0.3 MB.
**TABLE S5**, DOC file, 0.1 MB.
**TABLE S6**, DOC file, 0.2 MB.

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