Expression of the phosphodiesterase BifA facilitating swimming motility is partly controlled by FliA in Pseudomonas putida KT2440

Yujie Xiao\textsuperscript{1} | Huizhong Liu\textsuperscript{1} | Hailing Nie\textsuperscript{1} | Shan Xie\textsuperscript{1} | Xuesong Luo\textsuperscript{1,2} | Wenli Chen\textsuperscript{1} | Qiaoyun Huang\textsuperscript{1,2}

Abstract
Flagella-mediated motility is an important capability of many bacteria to survive in nutrient-depleted and harsh environments. Decreasing the intracellular cyclic di-GMP (c-di-GMP) level by overexpression of phosphodiesterase BifA promotes flagellar-mediated motility and induces planktonic lifestyle in Pseudomonas. The mechanism that regulates expression of \textit{bifA} gene was poorly studied. Here we showed that expression of BifA was partly controlled by flagellar sigma factor FliA ($\sigma_{28}$) in \textit{Pseudomonas putida} KT2440. FliA deletion led to an approximately twofold decrease in transcription of \textit{bifA}. 5′ race assay revealed two transcription start points in \textit{bifA} promoter region, with the putative $\sigma_{70}$ and $\sigma_{28}$ promoter sequences upstream, respectively. Point mutation in $\sigma_{28}$ promoter region reduced transcriptional activity of the promoter in wild-type KT2440, but showed no influence on that in \textit{fliA} deletion mutant. FliA overexpression decreased the intracellular c-di-GMP level in a BifA-dependent way, suggesting that FliA was able to modulate the intracellular c-di-GMP level and BifA function was required for the modulation. Besides, FliA overexpression enhanced swimming ability of wild-type strain, while made no difference to the \textit{bifA} mutant. Our results suggest that FliA acts as a negative regulator to modulate the c-di-GMP level via controlling transcription of \textit{bifA} to facilitate swimming motility.

KEYWORDS
BifA, C-di-GMP, feedback regulation, FliA, swimming motility

1 | INTRODUCTION
Flagella-mediated motility is an important capability of many bacteria to explore the environment for nutrients and escape from hostile environmental conditions. In most flagellated bacteria, more than 50 genes are typically involved in flagella production, a complex process that requires coordination in space and time (Liu & Ochman, 2007). In the genus \textit{Pseudomonas}, the master regulator FleQ, together with RpoN ($\sigma_{54}$), induces expression of the class II flagellar genes including \textit{fleSR}, which encode a two-component system that in turn activates class III genes in concert with RpoN. The sigma factor FliA is required for transcription of class IV flagellar genes including \textit{fliC}, which encodes the major flagellin subunit (Dasgupta et al., 2003; Liu & Ochman, 2007). Although the flagellar machinery endows cells with a phenominal survival device, it also consumes much of the metabolic currency to synthesize and fuel such a vigorous nanomotor (Martínez-García, Nikel, Chavarria, & de Lorenzo, 2014). Several feedback regulations in flagella synthesis and rotation have been reported and believed...
to function as insurances to avoid costly production of unnecessary flagellar subunits or flagellar rotation under unnecessary conditions (Aldridge & Hughes, 2002). For example, the interaction between FliA and the cytoplasmic anti-sigma factor FlgM depresses transcription of class IV flagellar genes by inhibiting the FliA-RNA polymerase association until completion of the hook-basal body complex, at which point the anti-sigma factor is secreted (Chadsey, Karlinsky, & Hughes, 1998; Frisk, Jyt, Arora, & Ramlal, 2002). Besides, the c-di-GMP-binding protein, YcgR interacted with the flagellar switch-complex proteins FlIG and FlIM, caused reduced flagellar rotation and swimming ability, most strongly in the presence of c-di-GMP in enteric bacteria (Fang & Gomelsky, 2010; Paul, Nut, Carlquist, Blair, & Harsey, 2010).

In diverse bacterial species, the secondary message c-di-GMP contributes to regulate bacterial swimming motility (Henghe, 2009; Jenal & Malone, 2006). Increasing the concentration of intracellular c-di-GMP by overexpression of diguanylate cyclase (DGC) enzymes promotes expression of genes for biofilm components and leads to biofilm formation, while low levels caused by phosphodiesterase (PDE) activity induces a planktonic lifestyle with highly motile cells. It has been reported that c-di-GMP modulates the transition between planktonic and biofilm life styles through the transcription factor FlgQ in Pseudomonas species (Baraquet & Harwood, 2015; Hickman & Harwood, 2008; Martinez-Gil, Ramos-González, & Espinosa-Urgel, 2014; Xiao et al., 2016). BifA encoding a genus-specific PDE, which had been identified in Pseudomonas fluorescens (Martinez-Granero et al., 2014). Pseudomonas aeruginosa (Kuchma et al., 2007), and P. putida (Jiménez-Fernández, López-Sánchez, Calero, & Govantes, 2015), were found to regulate the intracellular c-di-GMP pool. BifA overexpression caused increased swimming motility and decreased biofilm formation. This suggested that this gene play a central role in regulating bacterial motility via the c-di-GMP pathway. Therefore, figure out the regulation of bifA will deepen our understanding on the regulating of P. putida KT2440 motility.

Among the known sigma factors, the flagellar regulator FliA control numerous genes other than those involved in flagellar biogenesis in several bacterial species (Claret et al., 2007; Byoung-Mo Koo, Rhodius, Campbell, & Gross, 2009; Yu, Kibler, & Tan, 2006). For example, in Escherichia coli strain LF82, accumulation of FliA leads to a comitant strong induction of yhiH, which encodes a PDE. This implied that FliA putatively functions as a c-di-GMP regulator. It is interesting to see that in a previous microarray analysis showed that the transcription of bifA in a P. putida fliA mutant decreased approximately twofold (Rodríguez-Herva et al., 2010). Therefore, we hypothesized that the expression of BifA was putatively directly regulated by FliA and FliA possessed an ability to modulate the intracellular c-di-GMP level via regulating BifA expression in P. putida.

In this study, a set of experiments was carried out to test the hypothesis. Based on the experimental results, we confirm that expression of the BifA is partly controlled by the flagellar sigma factor FliA in P. putida KT2440, and FliA overexpression decreases intracellular c-di-GMP level in a FliA-dependent way. By enhancing expression of BifA, FliA acted as a negative regulator to modulate the c-di-GMP level to facilitate swimming motility. Our results imply a positive feedback function of FliA in swimming ability regulation.

2 | EXPERIMENTAL PROCEDURES

2.1 | Bacterial strains, plasmids, media, and culture conditions

All bacterial strains, plasmids, and primers used in this study are listed in Table 1. The P. putida KT2440 and E. coli DH5α, S17-1/pXir strains were routinely cultured at 28°C and 37°C, respectively, on Luria-Bertani (LB) broth medium, which was solidified with 1.5% agar when necessary. Gentamicin was used at 40 μg/ml for P. putida and at 20 μg/ml for E. coli. Kanamycin was used at 50 μg/ml and chloramphenicol at 25 μg/ml. For expression plasmids harboring the pta promoter, isopropyl β-D-thiogalactoside (IPTG) was added to cultures at a 0.4 mM final concentration when needed. Gentamicin and kanamycin were used for plasmid maintenance in P. putida.

2.2 | Plasmid and strain construction

To construct a markerless P. putida fliA deletion mutant, ~1,000 bp from the chromosomal regions flanking fliA were PCR-amplified with oligonucleotide pairs fliAups and fliAupa (upstream region), or fliAdown and fliAdowna (downstream region). The PCR products were ligated into pBBS401 (Xiao et al., 2016), yielding pBBS401-fliAUP-DW. Then the final plasmid was transferred to P. putida KT2440 by electroporation. Selection of integration gentamicin resistance strain was performed on gentamicin and chloramphenicol double antibiotics plates. After subculture the integration strain in LB medium without antibiotic for ten generations, single colonies were obtained by plate streaking. Then the identity of delete mutants were confirmed by PCR and sequencing. Markerless bifA mutant was constructed using the same method.

A DNA fragment containing the complete fliA coding region was PCR amplified using oligonucleotides fliAS and fliAA as primers. The product was digested with EcoRI and BamHI and ligated to pVLT33 to yield pVLT33-fliA. Complementation was accomplished by introducing pVLT33-fliA into fliA mutant. To generate the lacZ reporter plasmid, about 500-bp fragment containing the promoter region of relevant gene was obtained by PCR. The PCR product was cloned into vector pBBR-lacZ, which harbors a promoterless lacZ gene.

Mutations in the promoter region were generated by inverting PCR with oligonucleotides containing the mutation (-35bifAs and -10bifAs, -10bifAs, and -10bifAs, middlebifAs and middlebifAa), and pBBR-bifApro-lacZ was used as template. The fragment was then cycled by adding DNA ligase and transformed into E. coli S17-1. Plasmid was transferred to E. coli or P. putida strains by transformation, biparental mating, or electroporation. All cloning steps involving PCR were verified by sequencing (Tsingke).

2.3 | Swimming motility assay

Swimming assays were carried out as reported previously (Kuchma et al., 2007). Swimming plates (0.3% agar) consisted of M63 medium supplemented with glucose, MgSO₄, and CAA were
toothpick-inoculated with fresh colonies and incubated for 20 h at 28°C. Then digital photographs were taken, and the diameters of the swim zones were measured in millimeters and averaged over four replicate platings.

### 2.4 Biofilm formation analysis

The quantitative analysis of biofilm forming was performed using crystal violet (CV) staining as previously described (O’Toole & Kolter, 1998).
1998). The total of 1% (v/v) of the bacterial culture (OD$_{600}$ = 1.0) was inoculated into 50 ml LB medium, after 8 h growth at 28°C with 180 rpm shaking, 0.4 mM (final concentration) IPTG was added to the culture. Then the bacterial culture (OD$_{600}$ = 1.5) was aliquoted (2 ml) into glass tubes, and biofilms were allowed to develop at 28°C, with 40 rpm shaking. After 4 h, the supernatants were taken out and the tubes were rinsed three times with distilled water. Then 2.5 ml 0.1% CV was added into each tube to stain for 10 min. The excess stain was washed off three times with distilled water. The CV that stained the biofilm was destained with 3 ml of 95% ethanol by leaving it at room temperature for 20 min. Then optical density (OD$_{590}$) of the destained solution was examined, using a spectrophotometer (INESA). The absorbance value was positively correlated to the amount of biofilm. All tests were performed in triplicate and the absorbance readings were averaged.

2.5 | Assays for β-galactosidase activity

β-galactosidase activity was determined from sodium dodecyl sulfate- and chloroform-permeabilized cells as described (Miller, 1992). Overnight cultures were inoculated (1:100 dilution) in fresh LB medium and grown for 1.5 h; cultures were diluted 1:1 three times (every half hour) before the start of sample collection. These steps were done to ensure proper dilution of β-galactosidase accumulated after overnight growth. Experiments were repeated at least three times with two technical repeats per sample, and data are given in Miller units.

2.6 | RNA extraction and primer extension

Total RNA from exponentially growing cells in LB medium was extracted with total RNA extraction reagent (Vazyme) as recommended by the manufacturer. Following extraction, total RNA was treated with DNasel, which was later heat-inactivated at 70°C for 15 min. The transcriptional start site was determined using the 5’ rapid amplification of cDNA ends (5’ RACE) system, as recommended by the supplier (Clontech, wwwbdbiosciences.com) with 3 μg of total RNA (DNA-free) obtained above. The gene-specific primer bif-α-race (Table 1) was used to initiate the first-strand cDNA synthesis. Small aliquots of cDNA as templates were amplified using SMART PCR primer mix, UP, and gene-specific primer bif-α-race (Table 1). The resulting PCR product was cloned into the T-easy vector (Takara) according to the manufacturer’s instructions before being sequenced.

2.7 | RNA extraction, preparation of cDNA and qRT-PCR

Total RNA from exponentially growing cells in LB medium was extracted with total RNA extraction reagent (Vazyme) as recommended by the manufacturer. Reverse transcription reactions to generate the corresponding cDNA were performed with 1 μg of RNA using PrimeScriptTM RT reagent kit (Takara RR047A). RpoD was used as an internal control for normalization (Savil et al., 2003). Sequence of primer used for qRT-PCR (qpcrbifAs, qpcrbifAa) was listed in Table 1. The results were analyzed by means of the comparative threshold cycle method to determine the relative expression of bifA gene in target strains with respect to the wild type with empty vector. Three individual replicates were performed with three independent cultures grown on different days. Standard errors were calculated from these independent replicates.

2.8 | Extraction and quantification of intracellular c-di-GMP

C-di-GMP was extracted and quantitated as previously described (Morgan, Kohn, Hwang, Hassett, & Sauer, 2006; Petrova, Schurr, Schurr, & Sauer, 2011; Roy, Petrova, & Sauer, 2013). Briefly, c-di-GMP was extracted in triplicate from wild-type and mutant strains grown planktonically to exponential phase using heat and ethanol precipitation followed by centrifugation. Supernatants were combined, dried using a Speed-Vac and resuspended in deionized water. Samples (30 μl) were analyzed using an Agilent 1260 HPLC equipped with an autosampler, degasser, and Targa column (2.1 × 40 mm; 5 μm) at a detector set to 253 nm, and separated using a reverse-phase C$_{18}$ flow rate of 0.2 ml/min with the following gradient: 0 to 6.3 min, 2% B; 6.3 to 11 min, 10% B; 11 to 13.5 min, 90% B; 13.5 to 18 min, 2% B (buffer A, 10 mM ammonium acetate; buffer B, methanol plus 10 mM ammonium acetate). Commercially available c-di-GMP was used as a reference for the identification and quantification of c-di-GMP in cell extracts. C-di-GMP levels were normalized to total protein per ml of culture.

2.9 | Statistical analysis

All statistical analyses were performed in Microsoft Excel, using a two-tailed Student’s t test, assuming equal variance, or single-factor analysis of variance (ANOVA). All experiments were performed at least three times. A p value less than or equal to 0.05 was considered as statistically significant.

3 | RESULTS AND DISCUSSION

3.1 | Transcription of bifA decreased in fliA deletion mutant

To test whether transcription of bifA was controlled by FliA, we used allelic replacement to construct an unmarked deletion of fliA, and then promoter activity of bifA was compared between wild type and the fliA deletion mutant using promoter-lacZ fusion plasmid. The mutant was nonmotile, as expected, and the motility defect was complemented with wild-type copy of FliA expressed by the IPTG-inducible lacI/P tac promoter of pVLT33 (Fig. 1A). Result of β-galactosidase activity showed that deletion of fliA in P. putida, while not impacting growth rates in liquid LB broth (Fig. 1B), resulted in an about twofold decrease in activity of bifA promoter relative to the wild type. QRT-PCR data revealed that complementation with wild-type fliA restored the transcription level of bifA in the fliA mutant to a higher level than
that observed in wild-type strain with empty vector pVLT33 (Fig. 1C). Besides, overexpression of fliA induces the expression of bifA in both wild-type and fliA deletion mutant strains (Fig. 1C).

FliA was one of 24 alternative σ-factors of P. putida KT2440 (Martínez-Bueno, Tobes, Rey, & Ramos, 2002) that conferred promoter-recognition specificity to core RNA polymerase (RNAP) (Koo et al., 2009; Kuznedelov et al., 2002). In order to further verify the conclusion that FliA-RNAP control transcription of bifA, an E. coli reporter strain was constructed by introducing the promoter-lacZ fusion plasmid pBBR-bifApro-lacZ to E. coli DH5α. Then FliA expression vector pVLT33-fliA and empty vector pVLT33 were introduced into the E. coli reporter strain, respectively, to generate E. coli pE and E. coli pC (control) strains. Although the promoter activity in E. coli was rather low relative to that in P. putida, after 3 hr induction with 0.4 mM IPTG, the β-galactosidase activity of the strain E. coli pE was approximately fivefolds higher than that of the strain E. coli pC (Fig. 1D). Taken together, these results suggest that FliA control transcription of bifA.

Consistent with previously published results showing that transcription of bifA in a fliA mutant decreased approximately twofold in a microarray analysis (Rodríguez-Herva et al., 2010), we observed twofold lower levels of bifA promoter transcription activity in fliA mutant compared with that of the wild type (Fig. 1B). Similar to the function of FliA in E. coli, FliA from P. putida also controlled expression of yhjH, coding a PDE involved in c-di-GMP turnover. In addition to yhjH, FliA controls expression of the c-di-GMP receptor YcgR in E. coli K-12 and Chlamydia trachomatis (Claret et al., 2007; Shen et al., 2006; Yu et al., 2006).

### 3.2 Identify the transcription start point of bifA

Promoter-lacZ fusion assay revealed that FliA deletion led to a twofold decrease rather than a totally abolish to the bifA promoter activity, indicating that besides FliA other sigma factors may also be involved in bifA transcription. By analyzing the promoter sequence of bifA, we found a putative σ^70 promoter sequence ahead of the putative σ^28 promoter sequence (Mclean, Wiseman, & Kropinski, 1997; Rodríguez-Herva et al., 2010) (Fig. 2), thus we hypothesized that transcription of bifA was controlled by two sigma factors that start from two or more transcription start points (TSPs). To test our hypothesis, 5'-race was utilized to identify the TSP of bifA. As expected, two TSPs were found in the promoter region, located on 103 and 40 nt upstream of the translation initiation codon, and with the putative σ^70 and σ^28 promoter sequences upstream, respectively (Fig. 2). Location of the two TSPs indicates that transcription of bifA is controlled by σ^28 and σ^70, but whether σ^70 participates in controlling bifA transcription or not still requires further verification.
Several examples of promoter sites that can be recognized by two or more sigma factors had been reported (Cao & Helmann, 2002; Jervis, Thackray, Houston, Horsburgh, & Moir, 2007; Minnig, Barblan, Kehl, Möller, & Mauël, 2003). The overlap between sigma factors may function as a regulation mode to modulate gene expression. We infer that σ\(^{70}\) acts as a housekeeping sigma factor to give a basic bifA expression level, while σ\(^{28}\) functions as an enhancement factor to increase transcription of bifA under certain conditions or at certain periods.

A recent study in P. aeruginosa PAO1 revealed that the FliA gene modulates the c-di-GMP concentration via bifA to regulate motility and phenazine pigment production (Lo et al., 2016), and suggested that this regulation may be indirect due to the lack of a canonical FliA promoter sequence in the promoter region of bifA. We also tried to compare the upstream regions of bifA in P. putida KT2440 with those in P. aeruginosa PAO1, but no significant similarity was found between the two sequences, and no σ\(^{28}\) consensus sequence was found in the bifA promoter region of the PAO1 strain (sequences of the two promoter were shown in Data S1). We infer that while function of FliA in controlling bifA may be conserved in the two Pseudomonas species, the σ\(^{28}\) promoter consensus sequences may be different. Future studies on determining the FliA promoter consensus sequence in PAO1 would help to answer this question.

3.3 | Mutations in the putative σ\(^{28}\) promoter sequence decreased the bifA promoter activity

To further characterize the capacity of FliA in regulating bifA, we created point mutations in the putative σ\(^{28}\) promoter sequence of the bifA promoter region and analyzed the influences of these mutations on promoter activity. As shown in Fig. 3, mutations located in -35, -10, and spacer (shorted from 13 to 5 bp) regions of the putative σ\(^{28}\) promoter were constructed, respectively, and then activities of these promoters were analyzed by the promoter-lacZ fusion reporter plasmid and compared with that of the wild-type promoter. Result of β-galactosidase activity showed that the mutation in -35 region had no obvious impact on the promoter activity, the mutated promoter showed a transcriptional activity close to that of the wild-type promoter in both wild-type strain and the fliA deletion strain. However, both mutations in -10 and spacer regions significantly reduced the promoter activity in wild-type strain background, indicating that the two mutations impaired the function of the promoter in the wild-type strain background. Besides, both mutations in -10 and spacer regions showed no obvious influence on the promoter activity in the fliA deletion strain. Compared with the mutations in -35 and -10 regions, mutation in the spacer region reduced about half of the transcriptional activity of the promoter in wild-type KT2440, a level close to that caused by fliA deletion, indicating that the σ\(^{28}\) promoter was totally abolished by the mutation. These results further proved the function of the σ\(^{28}\) promoter and FliA controlled transcription of bifA.
FliA overexpression decreased intracellular c-di-GMP level via BifA

BifA was a PDE involved in the regulation of the intracellular c-di-GMP pool in KT2440 (Jiménez-Fernández et al., 2015). Since the expression of bifA was partly under the control of FliA, loss or overexpression of FliA may influence the intracellular c-di-GMP level. To confirm our presumption, c-di-GMP levels of the wild-type, fliA deletion mutant, and FliA overexpressing strains were measured using HPLC. Unexpectedly, no obvious difference in c-di-GMP concentrations was observed between wild-type (23.33 pmol/mg protein) and the fliA mutant (20.13 pmol/mg protein) (Fig. 4). But in both strains, FliA overexpression caused an about 35% decrease in the c-di-GMP level. To determine whether modulation of c-di-GMP by FliA was BifA dependent, an unmarked bifA deletion mutant was constructed, and FliA overexpression vector or empty vector was introduced into the bifA mutant. Thereafter c-di-GMP levels were quantified. C-di-GMP concentration of bifA mutant was nearly twice as that in the wild type, about 45.73 pmol/mg protein. With the same amount of IPTG added to the culture, no obvious difference in c-di-GMP levels was observed between the FliA overexpression bifA mutant strain (42.98 pmol/mg protein) and the control bifA mutant strain (45.73 pmol/mg protein). This result indicated that bifA deletion abolished the influence of FliA on the c-di-GMP level.

Although expression of bifA was under the control of FliA, FliA deletion showed no obvious influence on the c-di-GMP level, the reason may lie in the functional redundancy of DGCs and PDEs in the strain. The genome of P. putida KT2440 encodes 43 polypeptides potentially involved in the c-di-GMP turnover (Ulrich & Zhulin, 2007). Relationship, expression, and coordination of these proteins must be a complex and extensive system. The effect of decreased bifA expression on the c-di-GMP level in the fliA mutant may be compensated by the function of other DGCs or PDEs.

Activity of FliA was inhibited by FlgM until the assembly of the hook-basal body (HBB) was completed through which the FlgM was secreted (Chadsey et al., 1998). We speculated that the accumulation of FliA before HBB formation would cause a FliA overexpression effect after secretion of FlgM. Then free FliA drives the transcription of class FIGURE 4  Quantification of intracellular c-di-GMP. C-di-GMP was extracted and measured as described in Materials and Methods. Strains are wild-type KT2440, fliA mutant and bifA mutant, harboring pVLT33 or pVLT33-fliA, respectively. Data represent averages of three independent cultures. *Significantly different between FliA overexpression strain (harboring pVLT33-fliA) and the control strain (harboring pVLT33) (p < .05)

FIGURE 5  Influence of FliA overexpression on swimming ability and biofilm formation of wild-type and bifA mutant. Wild-type KT2440 (A) and bifA mutant (B), harboring pVLT33 or pVLT33-fliA respectively, were spotted onto swimming plates contained 0.4 mM IPTG. The photograph was taken 20 hr after inoculation at 28°C. (C) Quantitative measurements of swimming zone areas are presented for four replicates of the assay shown in paned A and B. (D) Biofilm formation on glass tube surface was quantified by staining attached cells with the crystal violet method. The results are the average of three independent assays. **Statistically significant difference between FliA overexpression strain and the control strain (p < .05)
IV flagellar genes to form complete flagella with other components. Meanwhile, free FliA enhances expression of BifA, and lowered the intracellular c-di-GMP level.

3.5 FliA overexpression enhanced swimming ability in wild-type strain, but not in bifA mutant strain

It has been reported that reducing the intracellular c-di-GMP level promotes swimming ability and inhibits biofilm formation of P. putida (Martínez-Granero et al., 2014; Xiao et al., 2016). Since FliA overexpression decreased the c-di-GMP level in the wild type via BifA, we assumed that FliA overexpression could enhance swimming ability and inhibit biofilm formation in wild-type strain, but not in the bifA mutant strain. To test our hypothesis, swimming ability and biofilm formation of wild type and bifA mutant both harboring pVLT33-fliA was tested, strains harboring empty plasmid pVLT33 were used as control. As expected, swimming ability of wild type was enhanced by FliA overexpression (swimming zone measurements: WT + pVLT33 = 17.83 ± 1.61 mm, WT + pVLT33-fliA = 26.17 ± 1.76 mm) (Fig. 5A and C), while that of the bifA mutant was not affected (swimming zone measurements: ΔbifA + pVLT33 = 13 ± 1.32 mm, ΔbifA + pVLT33-fliA = 13.67 ± 1.26 mm) (Fig. 5B and C). The bifA mutant strain with an empty vector showed weaker swimming motility than that of the wild type, probably due to the higher c-di-GMP level in the mutant. Biofilm formation assay showed that FliA overexpression did not influence biofilm formation in both the wild-type strain and the bifA mutant, as the strains harboring FliA overexpression vector or control vector showed similar biofilm formation ability (Fig. 5D).

In general, increasing the cellular c-di-GMP levels induces the production of extracellular matrix components and increases biofilm formation, whereas low levels promote swimming motility. But little is known about the dosage effect of c-di-GMP in promoting motility or biofilm formation. Swimming motility is pushed by flagellar rotation, while biofilm formation is done by secretion of several matrix components and attaching to surfaces. In contrast to swimming ability, we confer that the effect of c-di-GMP reduction caused by FliA overexpression on biofilm formation is too weak to detect.

In line with a recent study in P. aeruginosa PA01 revealed that FliA modulates the c-di-GMP concentration via bifA to regulate motility (Lo et al., 2016), we also found that FliA acted as a negative regulator to modulate the c-di-GMP level via controlling the transcription of bifA to facilitate motility in P. putida KT2440. Together with the former finding in E. coli (Claret et al., 2007), it seems that modulation of the c-di-GMP level by controlling expression of PDE coding genes is a conservative function of FliA and its homologs.

In summary, we show that the expression of PDE BifA was partly controlled by the flagellar sigma factor FliA and FliA overexpression decreases the intracellular c-di-GMP level in P. putida KT2440. By enhancing the expression of BifA, FliA acted as a negative regulator to modulate the c-di-GMP level to facilitate swimming motility (Fig. 6). Our results indicated a positive feedback function of FliA in the swimming ability regulation.

ACKNOWLEDGMENTS

The research was financially supported by the National Natural Science Foundation of China (41571230, 41230854, J1103510), the National Basic Research Program of China (973, grant No. 2015CB150504), and the Fundamental Research Funds for the Central Universities (2662015PY016, 2662015PY116). We thank Congzhi Li for help with the HPLC analysis.

FUNDING INFORMATION

The research was financially supported by the National Natural Science Foundation of China (41571230, 41230854, J1103510), the National Basic Research Program of China (973, grant No. 2015CB150504), and the Fundamental Research Funds for the Central Universities (2662015PY016, 2662015PY116).
CONFLICT OF INTEREST

The authors declare that they have no competing interests.

REFERENCES

Aldridge, P. & Hughes, K. T. (2002). Regulation of flagellar assembly. Current Opinion in Microbiology, 5, 160–165.

Bagdasarian, M., Lurz, R., Rückert, B., Franklin, F. C. H., Bagdasarian, M. M., Frey, J. & Timmis, K. N. (1981). Specific-purpose plasmid cloning vectors II. Broad host range, high copy number, RSP 1010-derived vectors, and a host-vector system for gene cloning in Pseudomonas. Gene, 16, 237–247.

Baraquet, C. & Harwood, C. S. (2015). A FleQ DNA binding consensus sequence revealed by studies of FleQ-dependent regulation of biofilm gene expression in Pseudomonas aeruginosa. Journal of Bacteriology, 197, 178–186.

Cao, M. & Helmann, J. D. (2002). Regulation of the Bacillus subtilis bccC bacitracin resistance gene by two extracytoplasmic function sigma factors. Journal of Bacteriology, 184, 6123–6129.

Chadsey, M. S., Karlinsky, J. E. & Hughes, K. T. (1998). The flagellar anti-sigma factor FlgM actively dissociates Salmonella typhimurium sigma28 RNA polymerase holoenzyme. Genes & Development, 12, 3123–3136.

Claret, L., Miquel, S., Vieille, N., Ryjenkov, D. A., Gomelsky, M. & Darfeuille-Michaud, A. (2007). The flagellar sigma factor FilA regulates adhesion and invasion of Crohn disease-associated Escherichia coli via a cyclic dimeric GMP-dependent pathway. Journal of Biological Chemistry, 282, 33275–33283.

Dasgupta, N., Wolfgang, M. C., Goodman, A. L., Arora, S. K., Jyot, J., Lory, S. & Ramphal, R. (2003). A four-tiered transcriptional regulatory circuit controls flagellar biogenesis in Pseudomonas aeruginosa. Molecular Microbiology, 50, 809–824.

De Lorenzo, V., Eltis, L., Kessler, B. & Timmis, K. N. (1993). Analysis of Pseudomonas gene products using lacIq/Ptrp-lac plasmids and transposons that confer conditional phenotypes. Gene, 123, 17–24.

Fang, X. & Gomelsky, M. (2010). A post-translational, c-di-GMP-dependent mechanism regulating flagellar motility. Molecular Microbiology, 76, 1295–1305.

Frisk, A., Jyot, J., Arora, S. K. & Ramphal, R. (2002). Identification and functional characterization of FlgM, a gene encoding the anti-sigma 28 factor in Pseudomonas aeruginosa. Journal of Bacteriology, 184, 1514–1521.

Hengge, R. (2009). Principles of c-di-GMP signalling in bacteria. Nature Reviews Microbiology, 7, 263–273.

Hickman, J. W. & Harwood, C. S. (2008). Identification of FleQ from Pseudomonas aeruginosa as a c-di-GMP-responsive transcription factor. Molecular Microbiology, 69, 376–389.

Jenal, U. & Malone, J. (2006). Mechanisms of cyclic-di-GMP signaling in bacteria. Annual Review of Genetics, 40, 385–407.

Jervis, A. J., Thackray, P. D., Houston, C. W., Horsburgh, M. J. & Moir, A. (2007). SigM-responsive genes of Bacillus subtilis and their promoters. Journal of Bacteriology, 189, 4534–4538.

Jiménez-Fernández, A., López-Sánchez, A., Calero, P. & Govantes, F. (2015). The c-di-GMP phosphodiesterase BifA regulates biofilm development in Pseudomonas putida. Environmental Microbiology Reports, 7, 78–84.

Koo, B. M., Rhodius, V. A., Campbell, E. A. & Gross, C. A. (2009). Mutational analysis of Escherichia coli sigma28 and its target promoters reveal recognition of a composite -10 region, comprised of an "extended -10" motif and a core -10 element. Molecular Microbiology, 72, 830–843.

Kuchna, S. L., Brothers, K. M., Merritt, J. H., Liberati, N. T., Ausubel, F. M. & O’Toole, G. A. (2007). BifA, a cyclic-Di-GMP phosphodiesterase, inversely regulates biofilm formation and swarming motility by Pseudomonas aeruginosa PA14. Journal of Bacteriology, 189, 8165–8178.

Kuznedelov, K., Minakhin, L., Niedzla-Majka, A., Dove, S. L., Rogulja, D., Nickels, B. E., ... Severinov, K. (2002). A role for interaction of the RNA polymerase flap domain with the sigma subunit in promoter recognition. Science, 295, 855–857.

Liu, R. & Ochman, H. (2007). Stepwise formation of the bacterial flagellar system. Proceedings of the National Academy of Sciences USA, 104, 7116–7121.

Lo, Y. L., Shen, L., Chang, C. H., Bhujaw, M., Chiu, C. H. & Chang, H. Y. (2016). Regulation of motility and phenazine pigment production by FI5A is cyclic-di-GMP dependent in Pseudomonas aeruginosa PA01. PLoS ONE, 11, e0155397.

Martínez-Bueno, M. A., Tobes, R., Rey, M. & Ramos, J. L. (2002). Detection of multiple extracytoplasmic function (ECF) sigma factors in the genome of Pseudomonas putida KT2440 and their counterparts in Pseudomonas aeruginosa PA01. Environmental Microbiology, 4, 842–855.

Martínez-García, E., Nikel, P. I., Chavarria, M. & de Lorenzo, V. (2014). The metabolic cost of flagellar motion in Pseudomonas putida KT2440. Environmental Microbiology, 16, 291–303.

Martínez-Gil, M., Ramos-González, M. I. & Espinosa-Urgel, M. (2014). Roles of cyclic Di-GMP and the Gac system in transcriptional control of the genes coding for the Pseudomonas putida adhesin LapA and LapF. Journal of Bacteriology, 196, 1484–1495.

Martínez-Granero, F., Navazo, A., Barahona, E., Redondo-Nieto, M., González de Heredia, E. & Baena, I., et al. (2014). Identification of flgZ as a flagellar gene encoding a PilZ domain protein that regulates swimming motility and biofilm formation in Pseudomonas. PLoS ONE, 9, e87608.

Mclean, B. W., Wiseman, S. L. & Kropinski, A. M. (1997). Functional analysis of σ70 consensus promoters in Pseudomonas aeruginosa and Escherichia coli. Canadian Journal of Microbiology, 43, 981–985.

Miller, J. H. (1992). A short course in bacterial genetics: a laboratory manual and handbook for Escherichia coli and related bacteria. New York: Cold Spring Harbor Laboratory Press.

Minnig, K., Barblan, J. L., Kehl, S., Möller, S. B. & Maulé, C. (2003). In Bacillus subtilis W23, the duet σX σM, two sigma factors of the extracytoplasmic function subfamily, are required for septum and wall synthesis under batch culture conditions. Molecular Microbiology, 49, 1435–1447.

Morgan, R., Kohn, S., Hwang, S. H., Hassett, D. J. & Sauer, K. (2006). BdiA, a chemotaxis regulator essential for biofilm dispersion in Pseudomonas aeruginosa. Journal of Bacteriology, 188, 7335–7343.

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

Paul, K., Nieto, V., Carluquist, W. C., Blair, D. F. & Harshey, R. M. (2010). The c-di-GMP binding protein YcgR controls flagellar motor direction and speed to affect chemotaxis by a “backstop brake” mechanism. Molecular Cell, 38, 128–139.

Petrova, O. E., Schurr, J. R., Schurr, M. J. & Sauer, K. (2011). The novel Pseudomonas aeruginosa two-component regulator BfmR controls bacteriophage mediated lysis and DNA release during biofilm development through PhdA. Molecular Microbiology, 81, 767–783.

Rodríguez-Hervá, J. J., Duque, E., Molina-Henares, M. A., Navarro-Avilés, G., Van Dillewijn, P. & De La Torre, J., et al. (2010). Physiological and transcriptomic characterization of a ΔfliA mutant of Pseudomonas putida KT2440. Environmental Microbiology Reports, 2, 373–380.

Roy, A. B., Petrova, O. E. & Sauer, K. (2013). Extraction and quantification of cyclic di-GMP from Pseudomonas aeruginosa. Bio-protocol, 3, e828.

Savli, H., Karadenizli, A., Kolayli, F., Gundes, S., Ozbek, U. & Vahaboglu, H. (2003). Expression stability of six housekeeping genes: a proposal for resistance gene quantification studies of Pseudomonas aeruginosa by real-time quantitative RT-PCR. Journal of Medical Microbiology, 52, 403–408.
Shen, L., Feng, X., Yuan, Y., Luo, X., Hatch, T. P. & Hughes, K. T., et al. (2006). Selective promoter recognition by chlamydial sigma28 holoenzyme. *Journal of Bacteriology*, 188, 7364–7377.

Simon, R., Priefer, U. & Pühler, A. (1983). A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. *Nature Biotechnology*, 1, 784–791.

Ulrich, L. E. & Zhulin, I. B. (2007). MiST: a microbial signal transduction database. *Nucleic Acids Research*, 35, D386–D390.

Xiao, Y., Nie, H., Liu, H., Luo, X., Chen, W. & Huang, Q. (2016). C-di-GMP regulates the expression of *lapA* and *bcs* operons via FleQ in *Pseudomonas putida* KT2440. *Environmental Microbiology Reports*, doi:10.1111/1758-2229.12419.

Yu, H. H. Y., Kibler, D. & Tan, M. (2006). In silico prediction and functional validation of σ28-regulated genes in Chlamydia and Escherichia coli. *Journal of Bacteriology*, 188, 8206–8212.

### SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

**How to cite this article:** Xiao, Y., Liu, H., Nie, H., Xie, S., Luo, X., Chen, W. and Huang, Q. Expression of the phosphodiesterase BifA facilitating swimming motility is partly controlled by FliA in *Pseudomonas putida* KT2440. *MicrobiologyOpen*. 2017;6:e00402. https://doi.org/10.1002/mbo3.402