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Original Article

FleQ of *Pseudomonas putida* KT2440 is a multimeric cyclic diguanylate binding protein that differentially regulates expression of biofilm matrix components

María Antonia Molina-Henares, María Isabel Ramos-González, Abdelali Daddaoua, Ana María Fernández-Escamilla, Manuel Espinosa-Urgel*

Department of Environmental Protection, Estación Experimental del Zaidín, CSIC, Profesor Albareda, 1, Granada 18008, Spain

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Abstract

The intracellular signal molecule cyclic di-GMP (c-di-GMP) is an important element in regulation of biofilm formation by bacteria. In *Pseudomonas aeruginosa*, FleQ functions as a c-di-GMP-dependent transcriptional regulator of expression of flagellar genes and the exopolysaccharide (EPS) Pel, a component of the biofilm extracellular matrix. In the plant-beneficial bacterium *Pseudomonas putida* KT2440, a mutation in *fleQ* reduces biofilm formation and colonization of plant surfaces. Using isothermal titration calorimetry and electrophoretic mobility shift assays, we show in this work that FleQ of *P. putida* interacts with c-di-GMP and directly binds the promoter regions of flagellar and EPS genes. Data obtained by analytical gel filtration and ultracentrifugation indicate that FleQ is in multiple oligomeric states in solution (dimers, tetramers and hexamers), which do not show altered equilibrium in the presence of c-di-GMP. DNA binding is independent of c-diGMP, although it is favored by the second messenger in the case of the promoter of the operon responsible for synthesis of the species-specific EPS Pea. Analysis of expression using transcriptional fusions showed an influence of FleQ upon two of the four EPS operons under regular growth conditions. Finally, a consensus sequence for promoter recognition by FleQ in *P. putida* is also proposed.

Keywords: Biofilm regulation; Exopolysaccharide; Isothermal titration microcalorimetry; Electrophoretic mobility shift assay

1. Introduction

The bacterial second messenger cyclic diguanilate (c-di-GMP) has emerged in the last few years as a key intracellular signal molecule governing transition between planktonic single-cell life and the development of sessile multicellular communities, or biofilms [1,2]. The turnover of c-di-GMP is well characterized at the biochemical level; diguanylate cyclases responsible for its synthesis and phosphodiesterases participating in its degradation have been described in many bacteria [3,4]. Often, several genes encoding proteins with diguanylate cyclase and/or phosphodiesterase domains are present in a bacterial genome, a fact that has led to the notion that these enzymes could act at localized sites in the cell and be active in response to different environmental or cellular cues not yet well understood.

The molecular mechanism by which c-di-GMP exerts its messenger role, leading to genetic reprogramming of the bacterial lifestyle, is beginning to be unraveled. In the opportunistic pathogen *Pseudomonas aeruginosa*, the transcriptional regulator FleQ, which was initially identified as involved in flagellar gene expression, has been described as one of the main elements responsible for transducing the signal and participating in the switch between motile and

* Corresponding author.
E-mail addresses: nene.molina@eez.csic.es (M.A. Molina-Henares), maribel.ramos@eez.csic.es (M.I. Ramos-González), abdelali.daddaoua@eez.csic.es (A. Daddaoua), anamfe@eez.csic.es (A.M. Fernández-Escamilla), manuel.espinosa@eez.csic.es (M. Espinosa-Urgel).

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Table 1
Strains and plasmids used in this study.

Strain or plasmid	Genotype or relevant characteristics[a]	Source/reference
P. putida		
KT2440	Derivate of *P. putida* mt-2, cured of pWWO	[22]
mus-69	*fleQ* mutant derivative of KT2440 obtained by mini-Tn*5* insertion; Km^r	[7]
E. coli		
DH5αF′	F′ *hsdR17 recA1 gyrA*	[23]
BL21 (DE3)	F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm* (DE3)	Stratagene
pET28b (+)	Protein expression vector; Km^r	Novagen
pMBL-T	cloning vector	Molbiolab
pMBL::FleQ	*fleQ* gene cloned in pMBL vector	This work
pET28b::FleQ	*fleQ* gene in pET28b, for His-tagged FleQ expression; Km^r	This work
pMBL::P*fliE*	pMBL containing the *fliE* promoter; Ap^r	This work
pMBL::P*bcs*	pMBL containing the *bcs* promoter; Ap^r	This work
pMBL::P*pea*	pMBL containing the *pea* promoter; Ap^r	This work
pMBL::P*peb*	pMBL containing the *peb* promoter; Ap^r	This work
pMBL::P*alg*	pMBL containing the *alg* promoter; Ap^r	This work
pMP220	broad host range promoter-probe vector; *′lacZ*, Tc^r	[24]
pMP-bcs	*bcs*::*lacZ* transcriptional fusion in pMP220	This work
pMP-pea	*pea*::*lacZ* transcriptional fusion in pMP220	This work
pMP-peb	*peb*::*lacZ* transcriptional fusion in pMP220	This work
pMP-alg	*alg*::*lacZ* transcriptional fusion in pMP220	This work

[a] Ap^r, Km^r, Tc^r stand for resistance to ampicillin, kanamycin and tetracycline respectively.

sessile lifestyles. In this organism, FleQ controls the expression of certain exopolysaccharide biosynthesis operons in a c-di-GMP-dependent manner; binding of c-di-GMP by FleQ relieves transcriptional repression exerted by this protein and allows activation of expression of the *pel* operon [5], a key component of the extracellular matrix of *P. aeruginosa* biofilms. On the other hand, the interaction between the second messenger and FleQ, in combination with the activity of a second protein, FleN, causes downregulation of flagellar genes [6]. FleQ belongs to the AAA + ATPase family of proteins, and has a C-terminal helix-turn-helix DNA binding domain and a domain characteristic of proteins that interact with the sigma factor σ^{54} (RpoN).

Previous work carried out on the plant-beneficial bacterium *Pseudomonas putida* KT2440 revealed that FleQ plays a positive role in bacterial adhesion to plant surfaces and biofilm formation [7,8]. Expression of the main *P. putida* adhesin, LapA, turned out to be increased in response to high intracellular levels of c-di-GMP and was reduced in a *fleQ* mutant regardless of the amount of second messenger [9], indicating that, in the case of LapA, FleQ acts as a positive regulator, transducing the c-di-GMP signal and triggering bacterial attachment to abiotic and plant surfaces.

Besides the large surface-associated protein LapA, other structural elements have been described in the buildup of *P. putida* biofilms. These include LapF, a second large extracellular protein involved in cell–cell interactions [10], and several exopolysaccharides (EPSs) that appear to have different contributions and relevance depending on the environmental conditions. Thus, two strain-specific EPSs, Pea and Peb, seem to be the main elements in the matrix of biofilms grown under flow conditions in the laboratory [11], whereas alginate (Alg) and a cellulose-like polymer (Bcs) may be relevant in water-limiting environments [12,13]. Alg and Pea are the most relevant for *P. putida* fitness in the rhizosphere [14], and Pea is also a key to development of crinkly colony morphology, characteristic of *P. putida* KT2440 harboring a diguanylate cyclase in multicopy [15]. This phenotype also requires a functional FleQ [8].

All this information led us to analyze whether FleQ of *P. putida* functions as its *P. aeruginosa* homolog in terms of biochemical and structural characteristics, and its direct or indirect role in expression of biofilm matrix components.

2. Material and methods

2.1. Strains, plasmids and growth conditions

Strains and plasmids used in this work are detailed in Table 1. Unless otherwise specified, strains were cultured at 30 °C (*P. putida*) or 37 °C (*Escherichia coli*) in liquid LB or

Table 2
Primers used in this study.

Primer	Sequence (5′ → 3′)	Use
FleQ-1	CATATGTGGCGTGAAACCAAGATTCTG	Cloning of *fleQ* in pET28b (+)
FleQ-2	GGATCCTCAATCCTCCGCCTGG	Cloning of *fleQ* in pET28b (+)
Alg-1	ATACGCATCGCTATCACCT	EMSA
Alg-2	GCGGAGTGAGCCTGTCAC	EMSA
Cel-1	TTGCTTGGCCAATGCCCTGC	EMSA
Cel-2	GAAACGGTTTGAAGGCGGAA	EMSA
Peb-1	CATAGACATGGCGTTGTTAA	EMSA
Peb-2	ATGGTATCCATACTGAGGTA	EMSA
Pea-1	TCGCTGAGCGGAATCAGGT	EMSA
Pea-2	TGAAGCCACCTGCGAGACC	EMSA

LB-agar plates [16] with appropriate antibiotics at the following concentrations (in μg/mL): ampicillin, 100; kanamycin, 25; tetracycline, 10. Where indicated, D-cycloserine was used at 75 μg/mL.

2.2. Recombinant expression of FleQ in E. coli

To produce polyhistidine-tagged FleQ, the *fleQ* gene was amplified from *P. putida* KT2440 chromosomal DNA using primers FleQ-1 and FleQ-2 (Table 2), which contain restriction sites for NdeI and BamHI, respectively. The fragment was cloned into pMBL to yield pMBL::FleQ and sequenced to ensure the absence of mutations. The NdeI/BamHI fragment was subsequently cloned into plasmid pET28b (+) to obtain pET28b::FleQ, which was used to express and purify FleQ fused to an N-terminal hexahistidine tag. To this end, *E. coli* BL21 (DE3) transformed with pET28b::FleQ was grown in 2 l Erlenmeyer flasks containing 250 mL of LB supplemented with 25 μg/mL kanamycin. Cultures were incubated at 30 °C with shaking until a turbidity at 600 nm (OD_{600}) of 0.6 was reached, and then 1 mM isopropyl-ß-D-thiogalactopyranoside (IPTG) was added to induce expression of *fleQ* from the plasmid P_{lac} promoter. After incubation at 18 °C overnight, cells were harvested by centrifugation (30 min at 20,000 × *g*) and stored at −80 °C until use for protein purification.

For FleQ purification, cells were resuspended in 25 mL of buffer A (50 mM Tris−HCl, pH 7.8, 500 mM NaCl, 1 mM dithiothreitol (DTT), 10 mM imidazole, 10% glycerol [vol/vol]) supplemented with a tablet of complete EDTA-free protease inhibitor mixture. Cells were lysed by three passes through a French press at a pressure of 1000 psi. The cell suspension was then centrifuged at 20,000 × *g* for 1 h. The pellet was discarded and the supernatant was filtered and loaded onto a 5-mL His-Trap chelating column (Amersham Bioscience) previously equilibrated with buffer A.

The FleQ protein was eluted with a 10−500 mM gradient of imidazole in buffer A. The protein concentration was determined by the Bradford assay, and protein purity was verified by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The protein was dialyzed overnight against buffer B (50 mM Tris−HCl, pH 7.8, 500 mM NaCl, 1 mM DTT, 10% glycerol (v/v)), quantitated and aliquots were stored at 4 °C.

2.3. Isothermal titration calorimetry

Microcalorimetric experiments were carried out at 25 °C using a VP-microcalorimeter (Microcal, Amherst, MA). Protein at 4 μM was introduced into the sample cell and titrated with aliquots of effector solution at 200 μM, prepared by dissolving the compound to be tested in 1% NaCl (w/v). Cyclic di-GMP was obtained from the Biolog Life Science Institute. Other nucleotides (GTP, ATP) were from Sigma. The mean enthalpies measured from the injection of effector into the buffer were subtracted from raw titration data prior to data analysis with the Microcal version of ORIGIN. Data were fitted with the two-binding site model of ORIGIN.

2.4. Analytical gel filtration chromatography

Analytical gel filtration chromatography was done using an Akta FPLC system (Amersham Biosciences). Purified FleQ was loaded onto a Superdex-200 10/300 GL column (Amersham Biosciences) equilibrated in buffer B; it was eluted at a constant flow rate of 0.7 mL/min, and the absorbance of the eluate was monitored at 280 nm. The molecular mass of FleQ was estimated from a plot of elution volume against the logarithm of the molecular masses of the following protein standards (Sigma): carbonic anhydrase (29 kDa), albumin from bovine serum (monomer, 66 kDa), cytochrome C (12.4 kDa), alcohol dehydrogenase (150 kDa) and β-amylase (200 kDa).

2.5. Analytical ultracentrifugation

Analysis of FleQ was performed at the Analytical Ultracentrifugation and DLS facility of the Centro de Investigaciones Biológicas. Sedimentation velocity runs were carried out at 48,000 rpm and 20 °C in an XL-I analytical ultracentrifuge (Beckman−Coulter Inc.) with a UV−visible light optics detection system, with an An50Ti rotor and 12 mm double-sector centerpieces using a protein concentration of 10 μM. Absorbance scans were run at 290 nm. Sedimentation coefficient (*S*) distributions were calculated by least-squares boundary modeling of sedimentation velocity data using the *c*(*s*) method [17] as implemented in the SEDFIT program. The *S* values were corrected to standard conditions (water, 20 °C, and infinite dilution) using the SEDNTERP program ($S_{20,w}$) [18].

The sedimentation equilibrium runs were carried out at multiple speeds (24,000 and 8000 rpm) and wavelengths (280 and 295 nm) with a column (85 μL), using the same experimental conditions and instruments as for the sedimentation velocity experiments. After the equilibrium scans, a high-speed centrifugation run (43,000 rpm) was done to estimate the corresponding baseline offsets. The weight-average buoyant molecular weight of FleQ was determined by fitting data to the single species model using either the MATLAB program based on the conservation-of-signal algorithm (kindly provided by Allen Minton, NIH), or the HeteroAnalysis program (available at http://www.biotech.uconn.edu/auf). The corresponding protein molecular weight was determined from experimental buoyant mass using 0.733 cm^3/g as the partial specific volume of FleQ (calculated from the amino acid composition using the SEDTERP program [18]).

2.6. Electrophoresis mobility shift assays (EMSAs)

The promoter regions of *fliE* and the operons for exopolysaccharide production (*pea*, *peb*, *bcs* and *alg*) were amplified by PCR using *P. putida* KT2440 chromosomal DNA and the primer pairs indicated in Table 2 and cloned into pMBL-T (Molbiolab), rendering pMBL-T::P_{fliE}, pMBL-T::P_{pea}, pMBL-T::P_{bcs}, and pMBL-T::P_{peb}, respectively. Standard protocols were used for DNA manipulation [19]. These plasmids, once sequenced to ensure their integrity, were used as templates for

further PCR amplification of the promoters. After electrophoresis, the amplified fragments (ranging from 240 to 600 bp, approximately) were isolated from agarose gels and end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. A 10 μL sample containing ~2 nM-labeled DNA (1.5×10^4 cpm) was incubated with increasing concentrations of purified FleQ for 1 h in 10 μL of binding buffer (50 mM Tris–HCl, pH 7.8, 50 mM KCl, 100 mM NaCl, 8 mM magnesium acetate, 5% [wt/vol] glycerol) containing 20 μg/mL of poly-d (IC) and 200 μg/mL bovine serum albumin. The DNA-protein complexes were resolved by electrophoresis in 4% (wt/vol) non-denaturing polyacrylamide gels in 1 × Tris-borate-EDTA (TBE) buffer. Reactions containing c-di-GMP were incubated with FleQ for 30 min before DNA addition. Gels were dried, exposed to a phosphorimaging screen overnight and visualized on a Storm phosphorimager using ImageQuant software (GE Healthcare).

2.7. Analysis of β-galactosidase activity

The pMBL-T derivatives obtained in the previous section were used to excise P_{alg}, P_{pea}, P_{bcs}, and P_{peb} and clone them in pMP220, to obtain transcriptional fusions to *'lacZ*. These constructs were introduced in KT2440 and its *fleQ* mutant derivative mus-69 [7], and β-galactosidase activity was measured during growth in LB using a modified version of Miller's method [20]. In brief, 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, to ensure proper dilution of β-galactosidase accumulated after overnight growth. Experiments were repeated at least three times with two technical repeats per sample. Averages and standard deviations are presented.

2.8. Bioinformatics

The SWISS-MODEL tool (https://swissmodel.expasy.org) was used to build a 3D model of the *P. putida* FleQ domain (amino acids 6–123) and AAA + domain (amino acids 142–307). The corresponding structures of the equivalent *P. aeruginosa* protein domains [21,22] were used as templates (Protein Data Bank id. 4WXM and 5EXP). For the prediction of a consensus sequence for promoter recognition by FleQ in *P. putida*, the binding site proposed in *P. aeruginosa* [23] was used in a multiple comparison with upstream sequences of the indicated genes using CLUSTALW (http://embnet.vital-it.ch/software/ClustalW.html), followed by visual inspection. Sequences were obtained from the Pseudomonas Genome Database (www.pseudomonas.com). The logo for the *P. putida* consensus was created using WebLogo (http://weblogo.berkeley.edu/logo.cgi).

3. Results

3.1. FleQ of P. putida specifically binds c-di-GMP

Analysis of the 491 amino acid sequence of FleQ of *P. putida* showed that it is organized in the same domains as its *P. aeruginosa* counterpart (Supplementary Fig. 1). It contains an N-terminal domain corresponding to σ^{54}-dependent transcriptional activators, spanning residues 6–123, annotated as the FleQ domain. This domain resembles REC domains of response regulators, but it lacks the canonical phosphorylation residue (aspartic acid), and it has been shown to participate in dimerization [21]. It is followed by the AAA + conserved domain (residues 147–300) characteristic of this class of ATPases. Within this domain, a Walker A motif is present, although the last amino acid in the consensus signature (GxxGxGK [S/T]) is different in FleQ, where a glutamic acid residue is found instead (**GESGTGKE**). Overlapping the final part of the AAA + domain and expanding 16 additional residues, a Walker B motif is found. In different ATPases, this motif is important for Mg^{2+}binding, which is required for their activity. Adjacent to the AAA + domain, there is an arginine finger, an AAA + family-specific motif that senses nucleotide binding and hydrolysis and transmits conformational changes. The C-terminal part of FleQ contains a helix-turn-helix DNA binding domain (residues 437–478) of the Fis family.

For biochemical characterization of FleQ, the complete gene was amplified and cloned in an expression vector, rendering a His-tagged fusion protein that was purified by affinity chromatography. Freshly purified FleQ was used to analyze binding to c-di-GMP by isothermal titration calorimetry (ITC) assays and the corresponding curve is provided in Fig. 1. FleQ binds the second messenger molecule and was characterized by two events with K_D values of 8.35 ± 0.8 and 2.80 ± 1.4 μM, and enthalpy changes of −0.21 ± 0.1 and −20.54 ± 0.3 kcal/mol, respectively. In contrast, the titration of FleQ with other nucleotides like GTP and ATP gave rise to small even peaks that can be entirely attributed to dilution heats (data not shown), indicating that this protein specifically binds c-di-GMP.

3.2. Multiple oligomeric forms of FleQ coexist in solution

In *P. aeruginosa*, FleQ is mainly purified as dimers [21] and can form tetramers and hexamers in solution [6,23]. In order to determine the possible oligomeric state of FleQ, analytical gel filtration chromatography was first done. Two elution peaks corresponding to FleQ were obtained, with apparent masses of 299 and 132 kDa, respectively (Fig. 2). This indicated that FleQ is, in fact, a multimer, but that different forms of oligomers coexist; given the predicted size of the protein based on its primary sequence (55.5 kDa), the results suggest a combination of hexamers and dimers or trimers. The integrity of the protein was confirmed by gel electrophoresis under denaturing conditions, rendering a band of the expected size (data not shown).

To further explore the different forms in which FleQ is organized and to determine if c-di-GMP may alter the oligomeric state of the protein, analytical ultracentrifugation was done with purified FleQ. Fig. 3 shows sedimentation velocity data for a 10 μM FleQ solution in the absence and in the presence of 200 μM c-di-GMP. The main species (>93%)

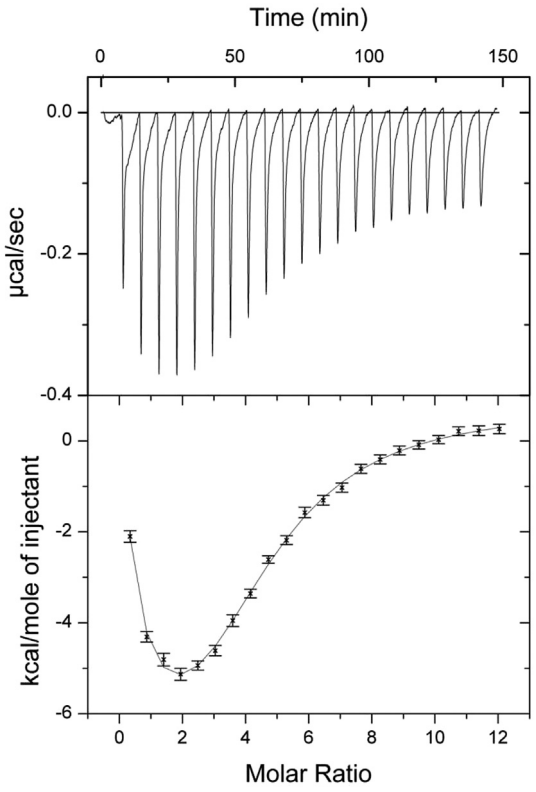

Fig. 1. Microcalorimetric binding studies of purified FleQ to di-GMPc. 4 µM of FleQ were titrated with aliquots of 200 µM of di-GMPc. Upper panel contains titration raw data and lower panel shows the integrated and dilution-corrected peak areas of the raw data. Data were fitted with the "two-binding-site model" of the MicroCal version of ORIGIN.

sedimented with a standard $S_{20,w}$ value of 3.2 S (±0.1) in the absence and 3.4 S (±0.1) in the presence of c-di-GMP, respectively. These values were, in both cases, compatible with a theoretic dimer of FleQ (calculated frictional ratios $f/f_o = 1.6$ and 1.4 respectively).

Sedimentation equilibrium experiments were also carried out in parallel as an additional method to determine the molecular weight (Fig. 4), as described above in Materials and methods. The sedimentation equilibrium gradient of FleQ fitted best with a single species with a molecular weight of 204.7 ± 1.2 KDa (FleQ without c-di-GMP) and 215.5 ± 0.9 KDa (FleQ in the presence of c-di-GMP), which are compatible with a FleQ tetramer.

Overall, the biophysical characterization of FleQ of *P. putida* indicates that this protein can form dimers, tetramers and hexamers in solution and that c-di-GMP does not favor any of the different states.

3.3. FleQ directly binds to the promoter regions of EPS operons in vitro

The potential binding of FleQ to the promoters of the four EPS operons identified in *P. putida* was examined by EMSA. Radiolabelled fragments containing the promoter regions of *pea*, *peb*, *bcs* and *alg* were incubated with increasing concentrations of FleQ in the reaction buffer and assayed for formation of protein-DNA complexes causing mobility retardation. The promoter of the *P. putida* flagellar gene *fliE*, one of the known targets of FleQ in *P. aeruginosa*, was included as positive control. Mobility retardation due to complex formation was observed upon incubation of *fliE*, *pea*, *peb* and *bcs* fragments with FleQ (Fig. 5). In the case of the *alg* promoter, the DNA fragment was retained in the well or showed very low mobility even in the absence of FleQ (data not shown), so the influence of the protein could not be defined. As a negative control, a fragment containing the promoter of the *ttgGHI* showed no retardation in the presence of FleQ, with or without addition of c-di-GMP (Supplementary Fig. 2), indicating that the mobility shift observed in the other promoters was not due to specific interactions. The concentration of FleQ required to observe a complete shift in DNA mobility varied, but, in all

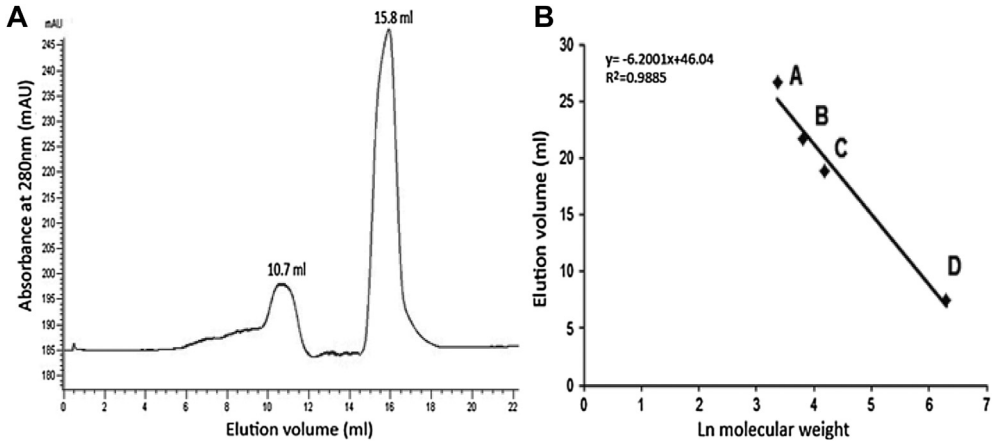

Fig. 2. Determination of the oligomeric state of FleQ. A. Analytical gel filtration elution profile of FleQ. B. Calibration curve of the analytical gel filtration column using the following marker proteins: carbonic anhydrase (29 kDa), albumin from chicken egg white (45 kDa), albumin from bovine serum monomer (66 kDa) and urease (545 kDa). The Ln of the molecular weight of the protein was plotted versus the corresponding elution volumes, which were fitted by linear regression curve. The elution volume determined for FleQ is indicated and the corresponding molecular weight (298.85 kDa and 131.29 kDa, respectively) was calculated by extrapolating from the elution volume.

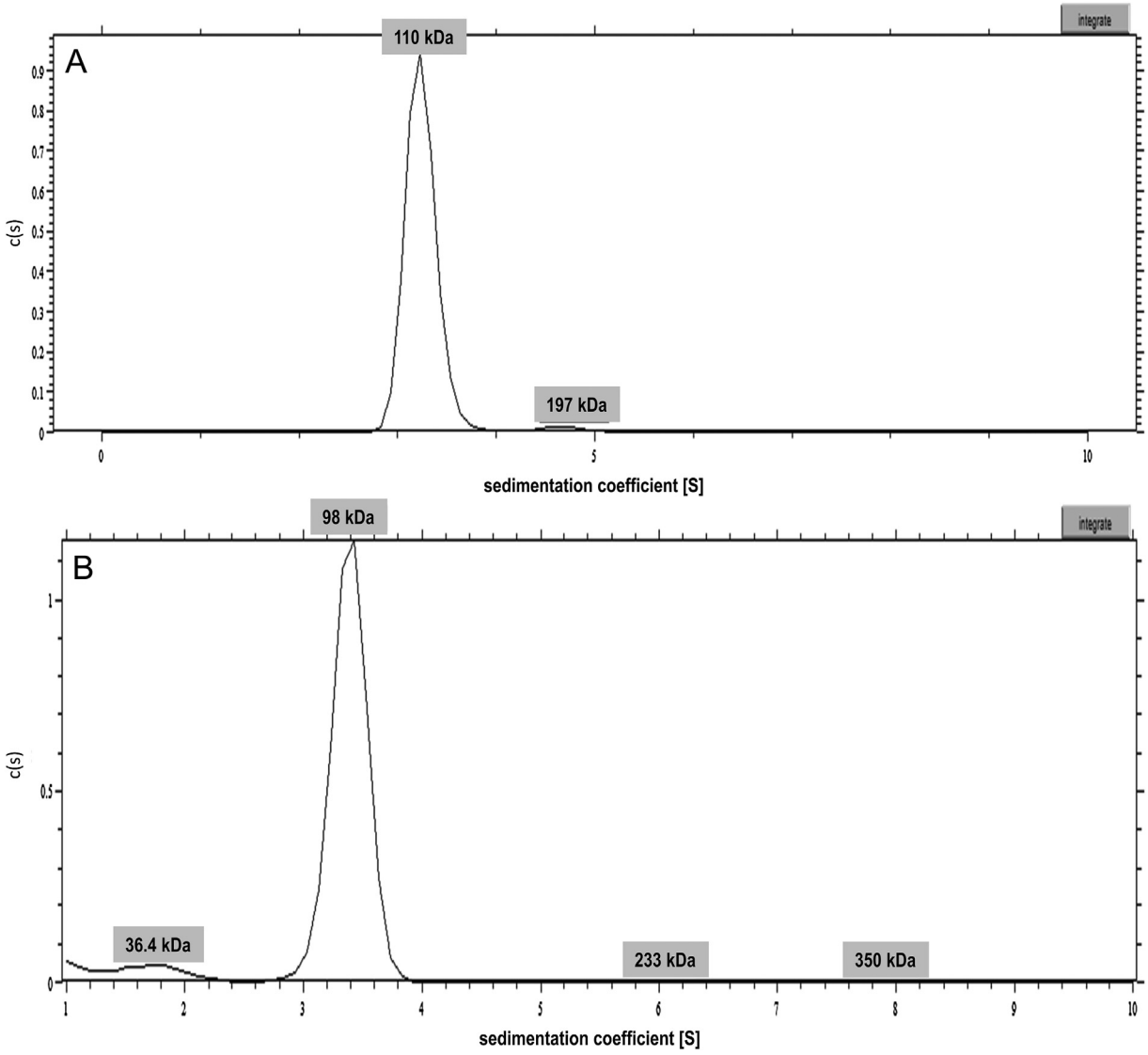

Fig. 3. Sedimentation velocity analysis of FleQ. Sedimentation coefficient distributions, c(s), at 48,000 rpm, 20 °C, corresponding to 10 μM FleQ alone (A) and in the presence of 200 μM c-di-GMP (B). Estimated weights of detected peaks are indicated.

cases, 14 μM was sufficient. Addition of c-di-GMP did not promote formation of protein-DNA complexes at lower FleQ concentrations, as shown when the *fliE* promoter region was incubated with 9, 10 and 12 μM FleQ in the presence or absence of c-di-GMP (Supplementary Fig. 2). Interestingly, addition of c-di-GMP at concentrations of 10 μM or 100 μM did not influence *fliE* and *bcs* mobility, but the higher concentration resulted in an increase in the mobility shift caused by FleQ binding to the promoters of *pea* and *peb* (Fig. 5).

3.4. Influence of FleQ on expression from promoters of EPS genes

To evaluate whether binding of FleQ to the promoters of EPS genes observed in vitro correlated with an influence of the regulator on their expression in vivo, transcriptional fusions of each promoter region with '*lacZ* were constructed, as indicated in Materials and methods, and introduced in KT2440 and its *fleQ* mutant derivative mus-69, which shows reduced biofilm formation and adhesion to plant surfaces [7]. β-galactosidase activity was analyzed during growth in liquid LB (Fig. 6). The expression pattern of *pea*::*lacZ* was very similar in the two strains, although the levels of activity were slightly higher in the wild type than in the *fleQ* mutant. This fusion showed no activity during the exponential phase, with its expression increasing as growth slowed down and during stationary phase (Fig. 6A). In the case of *peb*::*lacZ*, large differences were observed between KT2440 and mus-69 (Fig. 6B). Expression of this fusion in the wild type started earlier than that of *pea*::*lacZ* fusion, although it also increased significantly at the end of the exponential phase. This increase was greatly attenuated in mus-69, indicating that FleQ is a positive modulator of *peb* expression. Intriguingly, the mutation in *fleQ* had no impact on expression of *bcs*::*lacZ* fusion which, in both strains, showed the same pattern of activation until mid-exponential growth, followed by a sharp decline in

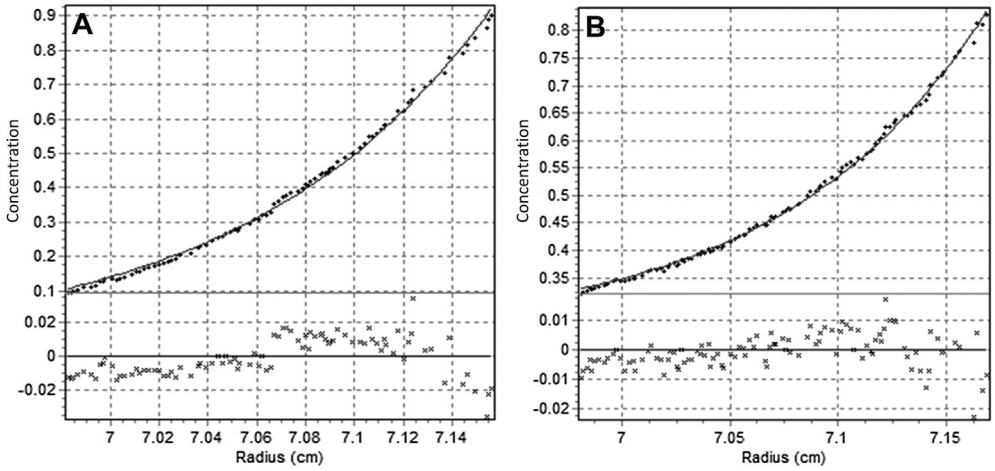

Fig. 4. Sedimentation equilibrium analysis of the association state of FleQ in the absence (A) and presence (B) of c-di-GMP. Sedimentation equilibrium absorbance gradients were carried out at 24,000 rpm during the first 2 h and at 8000 rpm the remaining time. The solid lines show corresponding best-fit gradients for a single sedimenting species at equilibrium with a solution average molar mass of 200 KDa. The residuals (difference between the experimental date and the fitted data for each point) are shown at the bottom of each panel.

activity to basal levels that were nonetheless rather high (Fig. 6C). Finally, analysis of *alg*::*lacZ* fusion showed that this promoter is silent under regular growth conditions in LB (data not shown). However, antibiotics and stressors targeting the cell wall, such as D-cycloserine, have been reported to trigger alginate expression [24]. We therefore performed the same experiments in the presence of 75 μM D-cycloserine, added after 3 h of growth, a concentration that was tested beforehand to minimize growth impairment while allowing activation of the *alg* promoter. Results shown in Fig. 6D indicate that, in this case, FleQ functions as a negative regulator, with expression of the *alg*::*lacZ* fusion being much higher in the mutant than in the wild type.

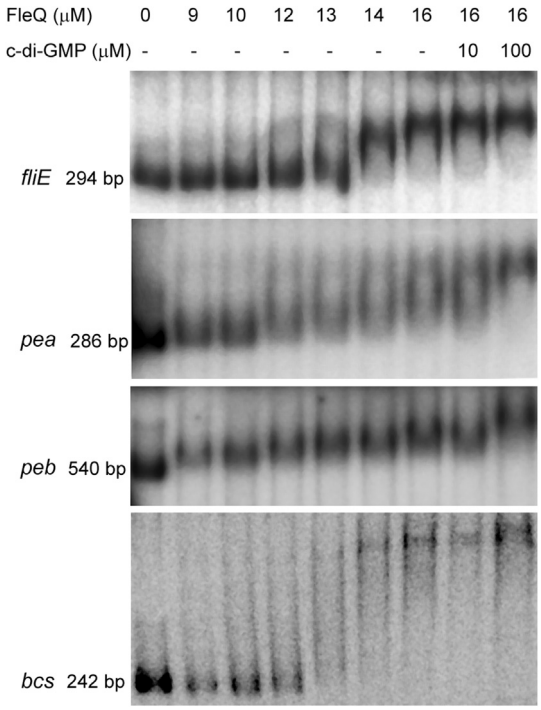

Fig. 5. Electrophoresis mobility shift assays for the binding of FleQ to different promoters: P_{fliE}, P_{pea}, P_{peb} and P_{bcs}. EMSAs were conducted using increased concentrations of purified protein with 2 nM of the promoter DNA end-labeled with [γ-P^{32}] in the presence or in the absence of c-di-GMP.

3.5. Identification of putative FleQ binding sites in P. putida promoter regions

A consensus for the DNA sequence recognized by FleQ has been recently proposed in *P. aeruginosa* [23]. Using this consensus, we were able to identify similar sites in *P. putida* promoters regulated or bound in vitro by FleQ. As shown in Fig. 7, the predicted consensus for *P. putida* is slightly different than that of *P. aeruginosa* and consists of a 14 base site that includes a palindrome (GTCAAAAAATTGAC). Several promoter regions show more than one potential FleQ site, the most noticeable being *lapA*, with three sites. In some cases the predicted sequence is found also in the complementary strand. The distance from the putative FleQ sites and the initiation codon varies notably.

4. Discussion

Among the regulatory elements responsible for the transition between planktonic and sessile lifestyles in bacteria, the transcriptional regulator FleQ is emerging as a key player in the signaling cascade controlled by the second messenger c-di-GMP. The biochemical study of FleQ of *P. putida* presented in this work reveals similar characteristics with its *P. aeruginosa* counterpart in terms of its binding to c-di-GMP and its potentially multimeric nature. Recently, the crystal structure of the AAA + domain of *P. aeruginosa* FleQ has been described, exposing hexameric assemblies of this domain [22], whereas the structure of the "FleQ domain" has been shown to be involved in dimerization [21]. Those results are consistent with our observations based on analytical FPLC, where the

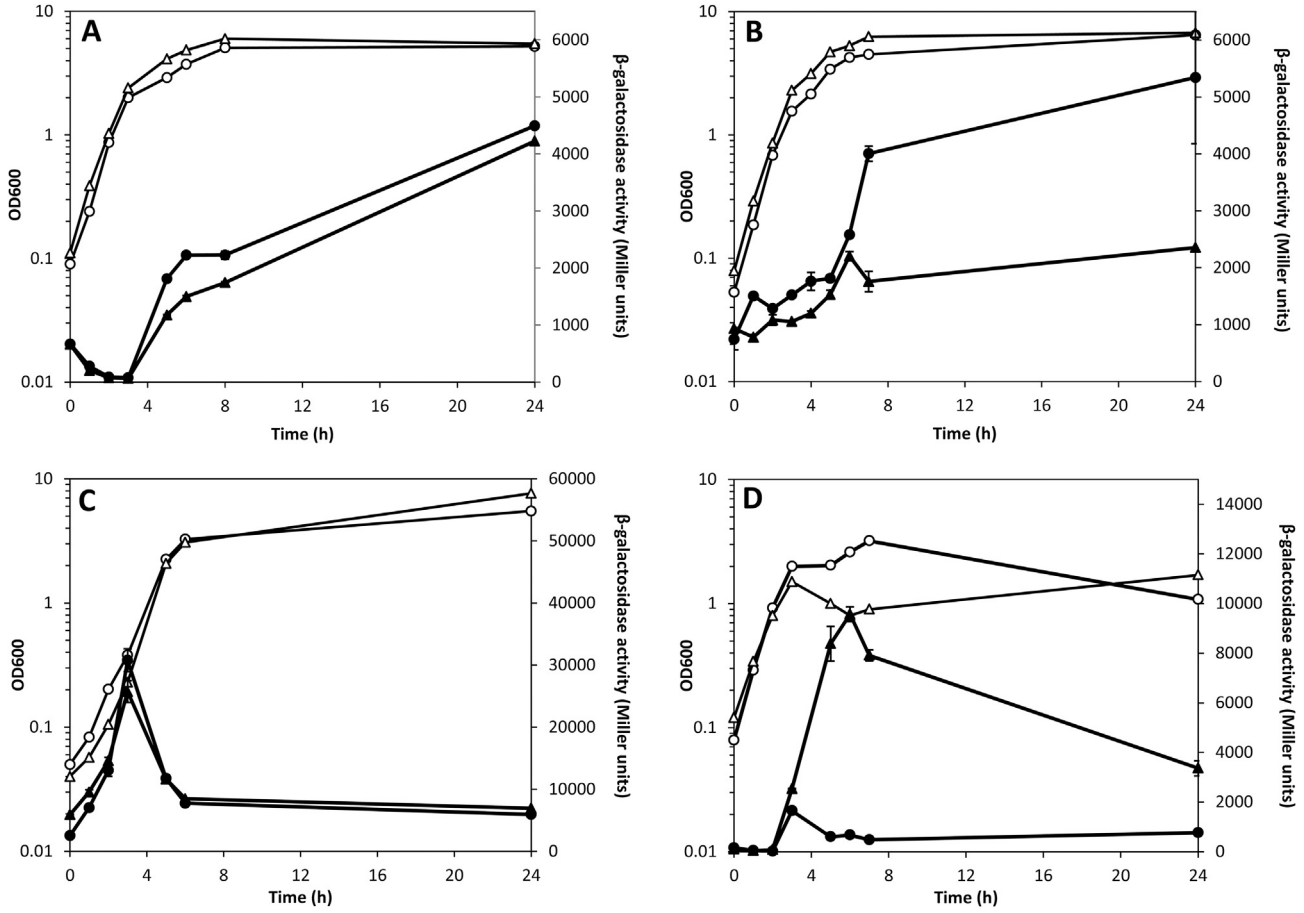

Fig. 6. Growth (open symbols) and β-galactosidase activity (filled symbols) of KT2440 (circles) and the *fleQ* mutant mus-69 (triangles) carrying the transcriptional fusions *pea*::*lacZ* (A), *peb*::*lacZ* (B), *bcs*::*lacZ* (C) and *alg*::*lacZ* (D). In panel D, D-cycloserine was added immediately after the 3 h time point.

calculated molecular weight of one of the two FleQ species of *P. putida* obtained during purification corresponds to hexamers, whereas analytical ultracentrifugation data showed also the existence of dimeric and tetrameric forms of the protein.

Matsuyama et al. [22] have elegantly shown that c-di-GMP influences the oligomeric state of the protein, causing an increase in the proportion of monomer and dimer species with respect to higher oligomeric forms. However, in our results from analytical ultracentrifugation, the oligomeric state of FleQ does not seem to be influenced by the presence of the second messenger, and monomers were not detected by any of the analytical methods used here. Also, it is worth mentioning that we did not detect binding of other nucleotides in our ITC experiments. Thus, there may be subtle differences in the way each protein interacts with c-di-GMP despite the close homology between them and the fact that motifs relevant for this interaction are conserved in both proteins. These differences are not self-evident and would require further characterization. A structural comparison between the FleQ and AAA + domains of the *P. aeruginosa* and *P. putida* proteins shows small differences (Supplementary Fig. 3), except for a slightly different length of β-strands. It is possible that some of the contrasting data are due to different technical and purification conditions and/or to structural differences between the purified domains and the conformation of the native protein as a whole, which to date has not been resolved.

The analysis of the in vitro interaction of FleQ with its potential targets revealed that c-di-GMP does not alter binding of FleQ to promoter regions of *fliE* and *bcs*, although it does influence the interaction between the protein and the *pea* and *peb* promoters. The *alg* promoter rendered inconclusive results that could be due to the presence in this DNA region of extensive secondary structure that may interfere with migration. Intriguingly, the observed binding to the *bcs* and *pea* promoters does not correlate with any detectable effect of the lack of FleQ on expression of a *bcs*::*lacZ* transcriptional fusion, and only a minor reduction in the case of *pea*::*lacZ*. This suggests participation of additional elements in regulation of these genes that mask the influence of FleQ. On the other hand, in the case of *peb*::*lacZ*, FleQ does have a prominent effect as positive regulator. Yet the fact that activity is not completely abolished in the mutant indicates the participation of other regulatory elements. It also seems likely that the role of FleQ may depend on specific environmental conditions. In fact, *alg*::*lacZ* fusion shows no activity under regular growth conditions regardless of the presence or absence of an intact *fleQ* gene. It is only under cell wall stress when the lack of FleQ leads to a large increase in activity with respect to the

promoter	sequence	bases to ATG
fliE	GTCAAAAAATGCG	21
*lapA*1	GTCAATAGTTGGC	139
*lapA*2	GACGGAATATTGAC	100
*lapA*3	GTCAAAAACGTCAA	86
algD	ATGAGAAAATCGAC	349
algD(-)	GTCAAAAAAGGTAT	625
bcs	GTCAAAAAACGAC	72
bcs(-)	GCTGTATAATTGAC	32
*pea*1	ACCAGGTAATTGAC	392
*pea*2	GAAACTATAGTGAC	29
*peb*1	GTCCGGAAGCTGAC	443
*peb*2	GTCAAAAAAACCGC	300
fleS(-)	GTCATAAAACGGTG	23
flhA	GTCAAAGTTTTGCA	15
flhA(-)	TGCAAAACTTTGAC	15
P. putida	**GTCAaAAAAtTGAC**	
P. aeruginosa	**GTCaNTAAAtTGAC**	

Fig. 7. Predicted FleQ binding site in *P. putida* promoter regions. The promoters used in this work and others previously described as regulated by FleQ were surveyed using the *P. aeruginosa* consensus. Bases identical to this consensus are shown in bold, and bases identical to the deduced *P. putida* consensus are shaded in gray. The data were used to build a sequence logo for the FleQ binding site in *P. putida*, shown at the bottom.

wild type, indicating that, in this case, it functions as a negative regulator.

Specific roles have been ascribed to the four EPS operons in *P. putida* in biofilm formation, rhizosphere colonization, colony morphology and water stress [11–15]. The different effects that FleQ can exert on expression of each EPS may be indicative of this protein acting as a multiple switch that modulates the biofilm matrix composition in response to environmental changes. How this modulation takes place and what environmental or cellular signals may be involved, other than changes in c-di-GMP levels, needs to be further explored.

Conflict of interest

No conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.resmic.2016.07.005.

Appendix B.

During processing of this article, direct regulation of the lapA and bcs promoters by FleQ has been independently reported in a pre-print by Xiao et al. (http://dx.doi.org/10.1111/1758-2229.12419), further supporting the conclusions of this work and of our previous data on lapA regulation by FleQ [9].

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