The FleQ protein from *Pseudomonas aeruginosa* functions as both a repressor and an activator to control gene expression from the *pel* operon promoter in response to c-di-GMP

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ABSTRACT

Bis-(3′–5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) modulates the transition between planktonic and biofilm life styles. In response to c-di-GMP, the enhancer binding protein FleQ from *Pseudomonas aeruginosa* derepresses the expression of Pel exopolysaccharide genes required for biofilm formation when a second protein, FleN is present. A model is that binding of c-di-GMP to FleQ induces its dissociation from the *pelA* promoter allowing RNA polymerase to access this site. To test this, we analyzed *pelA* DNA footprinting patterns with various combinations of FleQ, FleN and c-di-GMP, coupled to *in vivo* promoter activities. FleQ binds to two sites called box 1 and 2. FleN binds to FleQ bound at these sites causing the intervening DNA to bend. Binding of c-di-GMP to FleQ relieves the DNA distortion but FleQ remains bound to the two sites. Analysis of wild type and mutated versions of *pelA-lacZ* transcriptional fusions suggests that FleQ represses gene expression from box 2 and activates gene expression in response to c-di-GMP from box 1. The role of c-di-GMP is thus to convert FleQ from a repressor to an activator. The mechanism of action of FleQ is distinct from that of other bacterial transcription factors that both activate and repress gene expression from a single promoter.

INTRODUCTION

Bis-(3′–5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) is an intracellular secondary messenger that modulates the transition between planktonic and biofilm lifestyles in many gram-negative bacteria (1–8).

In addition, c-di-GMP modulates virulence in some bacteria (9,10). High intracellular c-di-GMP is often associated with the production of biofilms and the formation of adhesive organelles such as pili or stalks, whereas reduced intracellular c-di-GMP is often correlated with increased motility and virulence. C-di-GMP is synthesized by diguanylate cyclases (DGC) with GGDEF motifs, degraded by phosphodiesterases (PDE) with EAL or HD-GYP motifs and bound by a wide variety of effectors (11–15). Proteins containing PilZ domains (16–19), or harboring degenerate GGDEF or EAL domains (20,21) bind c-di-GMP. C-di-GMP is also a riboswitch ligand (22). The binding of c-di-GMP to these effectors impacts at different cellular and molecular levels including enzymatic activity, protein/protein interaction, transcription and translation. The mechanisms involved in c-di-GMP control at each of these levels are just coming into focus.

Several transcriptional regulators have been described that respond to c-di-GMP (23–26). One of these, FleQ from the opportunistic pathogen *Pseudomonas aeruginosa* (27), binds c-di-GMP to regulate the expression of genes involved in biofilm formation. These include the *pel* and *psl* operons, which direct the synthesis of Pel and Psl exopolysaccharides, and the *cdrA* gene coding for an adhesin essential for Psl exopolysaccharide structure (27,28). FleQ represses the expression of these genes in the absence of c-di-GMP whereas in the presence of c-di-GMP the repression is relieved (27). FleQ is also the master regulator of flagella gene expression in *P. aeruginosa* (29,30). It activates the expression of the *flhA* and *fliLMNOPQ* genes involved in flagellar export, the *flhF-fleN* genes involved in regulation and localization of the flagellar apparatus, the *fleSR* genes encoding a two-component system that regulates other flagella genes and the *fliEFG* operon encoding structural components of the flagellar basal body and motor switch complex (30,31). Transcriptome analysis showed that expression of most of the FleQ-regulated flagellar genes is downregulated when

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intracellular c-di-GMP is high (27,32). However, the effect of c-di-GMP on flagella gene expression is modest and clear evidence that the effect observed is directly mediated by FleQ is missing.

FleQ is an enhancer binding protein (EBP), belonging to the NtrC family of bacterial transcription factors. It contains an N-terminal FleQ domain, a central AAA$^+$/ATPase σ^{54}-interaction domain and a C-terminal helix-turn-helix DNA-binding domain. EBPs typically bind >100 nt upstream of the transcription start site of a target promoter and then interact with a σ^{54}-RNA polymerase complex by a DNA looping mechanism. RNA polymerase open complex formation is driven by ATP hydrolysis catalyzed by the EBP. A specific motif, GG and GC, located at positions −24 and −12 relative to the transcription start site, is recognized by the σ^{54}-RNA polymerase complex. Whereas, expression of all FleQ-regulated operons involved in flagella biosynthesis depends on σ^{54} (30,31), the expression of FleQ regulated genes involved in biofilm formation does not, suggesting a different mechanism of regulation (30). FleQ works in concert with another protein, FleN, which is a putative ATPase with a deviant Walker A motif (33). Mutation of *fleN* leads to an upregulation of FleQ-dependant flagella genes (27) and *fleN* mutants are multiflagellate (34). FleN is required for full expression of *pel*, *psl* and *cdr* operons in the presence of high levels of c-di-GMP (27). FleN alone does not bind to DNA but FleQ and FleN interact and a FleQ/FleN/DNA complex has been observed to form at *flhA* and *pel* promoters in gel shift assays (27,35).

Hickman and Harwood (27) suggested based on gel shift data, that FleQ represses *pel* gene expression by a simple roadblock mechanism in which FleQ sits at the *pelA* promoter and prevents RNA polymerase from binding. They further suggested that the binding of c-di-GMP to FleQ induces dissociation of FleQ from the *pelA* promoter allowing the RNA polymerase to access DNA and form an active transcription complex. To test this hypothesis and to further probe the mechanism FleQ and FleN action in response to c-di-GMP, we analyzed *pelA* promoter DNA footprinting patterns with various combinations of FleQ, FleN and c-di-GMP, coupled to *in vivo* promoter activities. Our results show that the *pelA* promoter contains two FleQ binding sites on either side of its transcription start site. In addition, we provide evidence that FleQ is not only a repressor of *pel* gene expression in the absence of c-di-GMP but also an activator of *pel* expression in the presence of c-di-GMP. It appears that the two FleQ binding sites at the *pel* promoter are independently dedicated to activation and repression.

MATERIALS AND METHODS

Strains, media and compounds

Pseudomonas aeruginosa and *E. coli* strains were routinely grown on LB medium at 37°C. Antibiotics were added at the following concentrations: 60 μg/ml gentamycin or tetracycline for *P. aeruginosa* and 10 μg/ml gentamycin or tetracycline for *E. coli*. C-di-GMP and ATPγS were purchased from BIOLOG Life Science Institute

(Bremen, Germany). *Pseudomonas aeruginosa* strains PAO1Δ*wspF*, PAO1Δ*fleQ*, PAO1Δ*pel*, PAO1Δ*pel*Δ*psl*, PAO1Δ*pel*Δ*psl*Δ*fleN*, PAO1Δ*pel*Δ*psl*Δ*fleQ* and PAO1Δ*pel*Δ*psl*Δ*fleQ*Δ*fleN* were constructed using standard protocols for allelic exchange in *P. aeruginosa*. Deletions of *pel*, *psl* and *wspF* were constructed as previously described (1,32,36). To construct the chromosomal deletion of *fleQ* and *fleN*, DNA flanking the *fleQ* or the *fleN* genes was PCR-amplified and cloned into pEX19Gm (37). The resulting plasmid was used to transform *E. coli* S17-1, and was then mobilized into different *P. aeruginosa* strains. Transconjugants were selected on M63 media with succinate (10 mM) and containing gentamycin, followed by recovery of deletion mutants on LB plates containing 5% sucrose. Candidate deletion mutants were screened by PCR. Plasmids pJN1120 and pJN105 were electroporated into strains PAO1Δ*pel*Δ*psl*, PAO1Δ*pel*Δ*psl*Δ*fleQ* PAO1Δ*pel*Δ*psl*Δ*fleN* and PAO1Δ*pel*Δ*psl*Δ*fleQ*Δ*fleN* by a standard electroporation method (27,38,39).

DNase I footprinting

DNase I footprint analysis was performed using a non-radiochemical capillary electrophoresis method (40). Two DNA fragments containing the *pelA* promoter were used in this study. The fragments were generated by PCR using two 6-FAM (6-carboxyfluorescein phosphoramidate) primers: pelA-R1 ACTCGATGGGTCGAAG GATGTTAC, which paired to the RNA 49- to 73-nt downstream from the *pelA* translation start site or pelA-R2, TCGGAAGCATGGATGCGGTTTC, which paired to the RNA 43- to 65-nt upstream from the *pelA* translation start site and the primer pelA-F TTCCTCGC ACGCAACTGAAG, which paired 479- to 499-nt upstream from the *pelA* translation start site, leading to a PCR products labeled with FAM of 572 and 456 bases, respectively. The template for the PCR reaction was PAO1 chromosomal DNA except when different mutations of the *pel* promoter were studied; in these cases the templates were the plasmids mini-CTX-*lacZ-pelA*mut1, 2, 3 or 4 and the primer pelA-R2 was necessarily used. The footprint assays were performed as follows: FleQ and/or FleN proteins were incubated with ATP (10 μM) or c-di-GMP (100 μM or 1 mM) and binding buffer [10 mM Tris-HCl, pH 7.8, 50 mM KCl, 8 mM Mg acetate, 50 ng/μl bovine serum albumin (BSA) and 5% glycerol] for 10 min before adding the labeled PCR fragment (0.45 pmol) and incubating for 30 min. Reaction mixtures were treated for 2 min with DNase I (0.3 units, Promega). The samples were phenol extracted and ethanol precipitated. Fragments were analyzed with an Applied Biosystems 3730×l Genetic Analyzer (Genewiz, Inc or Genomic Resources at Fred Hutchinson Cancer Research Center). DNA fragment sizes were determined using ABI Peak Scanner software.

RNA extraction and primer extension

Pseudomonas aeruginosa strains were grown in LB until the culture reached an OD$_{600}$ of 0.5–0.7. Cells were then harvested and total RNA was extracted and purified by using an RNeasy Mini kit (Qiagen). Reverse transcription
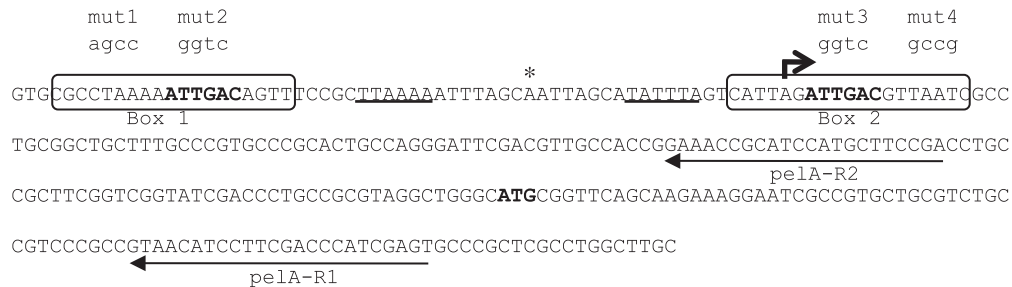
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      mut1    mut2                                                          mut3    mut4
      agcc    ggtc                                                          ggtc    gccg
                                                              *                 ┌──►
GTG CGCCTAAAAATTGACAGTT TCCGCTTAAAAATTTAGCAATTAGCATATTTAGT CATTAGATTGACGTTAATC GCC
      └─────Box 1──────┘                                     └──────Box 2──────┘
TGCGGCTGCTTTGCCCGTGCCCGCACTGCCAGGGATTCGACGTTGCCACCGGAAACCGCATCCATGCTTCCGACCTGC
                                                    ◄──────────────────────
                                                           pelA-R2
CGCTTCGGTCGGTATCGACCCTGCCGCGTAGGCTGGGCATGCGGTTCAGCAAGAAAGGAATCGCCGTGCTGCGTCTGC

CGTCCCGCCGTAACATCCTTCGACCCATCGAGTGCCCGCTCGCCTGGCTTGC
◄──────────────────────
           pelA-R1
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Figure 1. Nucleotide sequence of the *pel* promoter region. The two FleQ binding sites are boxed and the repeated sequences are in bold. The identity of various promoter mutations is shown above the sequence. The transcription start site is indicated by a thick arrow and the putative −10 and −35 elements are underlined. The translation start site ATG is in bold. The asterisk indicates the location of the hypersensitive site. The two thin arrows indicate the two primers pelA-R1 and pelA-R2 used for primer extension and DNAse I footprinting experiments.

was performed using SuperScript III reverse transcriptase (Invitrogen). Briefly, 0.4 nM of 6-FAM primers pelA-R1 or pelA-R2 (see the sequences above or Figure 1), were incubated with 15 µg of total RNA for 20 min at 58°C and then incubated at room temperature for 10 min, the SuperScript III reagents for cDNA synthesis were then added as specified by the manufacturer and the reaction mixtures were incubated for 1 h at 55°C. Synthesized cDNA was purified with a Qiagen PCR purification kit, concentrated by ethanol precipitation and then analyzed as described above. Primer extension analysis was performed at least two times with two different primers to confirm the transcription start site.

Protein purification and limited proteolysis

FleQ and FleN proteins were purified from *E. coli* strain ER2566 carrying pFleQ-Int1 or pFleN-Int1, as previously described (27). The limited proteolysis experiments were done on 8 µg of each purified protein with three different conditions: FleQ plus FleN, FleQ plus BSA and FleN plus BSA. Oligonucleotides (50 µM) corresponding to both strands of the FleQ binding sites (positions −61 to +20 relative to the transcription start site) were mixed together in 10 mM Tris–HCl (pH 7.3), 1 mM EDTA and 185 mM NaCl, and annealed by incubation at 95°C for 5 min followed by chilling on ice. When indicated, DNA fragments equimolar to FleQ were added. In a final volume of 45 µl, 16 µg of protein was incubated with or without ATP (10 µM) or c-di-GMP (1 mM) for 10 min before adding trypsin at a protein/enzyme mass ratio of 10/1 for FleQ digestion (1.6 µg of trypsin) or 4/1 for FleN digestion (4 µg of trypsin). The reactions were stopped by removing 11 µl from the proteolysis mix at 2, 10, 30 and 60 min intervals and then plunging each sample into a cold tubes containing 1.5 µl of 100 mM PMSF (phenylmethylsulfonyl fluoride), a protease inhibitor. SDS loading buffer was added and the samples were subsequently incubated for 10 min at 95°C. The samples were then analyzed on SDS–PAGE followed by transfer to Hybond™-ECL™ nitrocellulose membrane and detected with FleQ or FleN antibodies. Each experiment was repeated at least three times and gave the same profile of proteolysis.

Construction of chromosomal *pelA-lacZ* fusions and measurement of promoter activities *in vivo*

Chromosomal promoter-*lacZ* reporter fusions were constructed using the mini-CTX-*lacZ* vector (41). A 537-bp PCR product, encompassing the region between 29 bases downstream and 508 bases upstream of the translational start site of the *pelA* gene, was generated using primers pPelfullfor (5′-CGGCGAATTCCTGGTG CGGTTCCTCGCACGCAAC-3′) and pPelfullrev (5′-GA TCGGATCCACGGCGATTCCTTTCTTGCTG-3′). The fragment was then cloned into a mini-CTX-*lacZ* vector (Y. Irie and M. Parsek, unpublished data). Mutations in the *pelA* promoter (Figure 1) were generated by an overlapping PCR procedure with two sets of divergent and complementary primers, each containing the expected substitutions and the convergent primers pPelfullfor and pPelfullrev. Primer sequences are available upon request. The fragments were cloned into the mini-CTX-*lacZ* vector. The resulting plasmids (mini-CTX-*lacZ*-*pelA*wt, mut1, 2, 3 or 4), bearing the wild type or mutated *pelA* promoters, were used to transform *E. coli* S17-1, and then introduced at the *attB* site of the *P. aeruginosa* chromosome by mating. Transconjugants were selected on M63 medium with succinate and containing tetracycline. Strains were grown overnight aerobically in LB at 37°C and β-galactosidase activities of whole cells were measured by the method of Miller (42). Results are the average of three independent experiments.

RESULTS

Identification of the *pelA* promoter region

The *pel* operon encoding *pelABCDEFG* genes directs the synthesis of an exopolysaccharide important for biofilm formation by *P. aeruginosa* (43). To determine the *pelA* transcription start site we isolated RNA from strains PAO1Δ*fleQ*, PAO1Δ*wspF* and PAO1Δ*pel* and did primer extension analysis. Based on previous work, we expected to see *pel* transcripts in strain PAO1Δ*fleQ*, because in such a background *pel* genes are expressed at high levels (27). Similarly, a *wspF* deletion causes the DGC WspR to be activated, resulting in c-di-GMP accumulation, which induces *pel* expression (1,27). Strain PAO1Δ*pel* was used as negative control. The *pel* deletion is 3.2 kb in size. It starts

355 bases upstream of the translation start site of *pelA* and encompasses most of *pelA* (32). One specific *pelA* transcript was detected with RNA isolated from strains PAO1Δ*fleQ* and PAO1Δ*wspF* but not detected with RNA isolated from strain PAO1Δ*pel* (Figure 2). Other fragments were observed in all three conditions tested, and thus were not specific to the *pelA* transcript. The 6-FAM specific *pel* fragment identified with primer pelA-R1 (Figure 1) was 207 bp long, allowing us to map the *pelA* start site to a location 134-bp upstream of the ATG start codon (Figure 1). The same experiment performed with another primer, pelA-R2 (Figure 1), confirmed the location of the transcription start site. A putative −10 sequence (TATTTA), but no typical −24/−12 σ^{54}-RNA polymerase binding site was found upstream of the transcription start site (Figure 1). This observation is in good agreement with previous data showing that *pel* operon expression does not depend on σ^{54} (30). A putative −35 region (TTAAAA) close to the consensus (TTGACA) of σ^{70} promoters was found in the *pel* promoter region but the spacing between the −10 and −35 sequences is 15 bases instead of the conventional spacer of 17 bases.

Identification of two FleQ binding sites on *pel* DNA

Binding sites for FleQ on the flagella regulon *flhA*, *fliE*, *fliL* and *fleSR* promoters have been reported, but no sequence conservation among these binding sites was observed (31). To determine the location of FleQ binding on the *pelA* promoter *in vitro*, DNaseI footprinting experiments were performed with a 5′FAM labeled
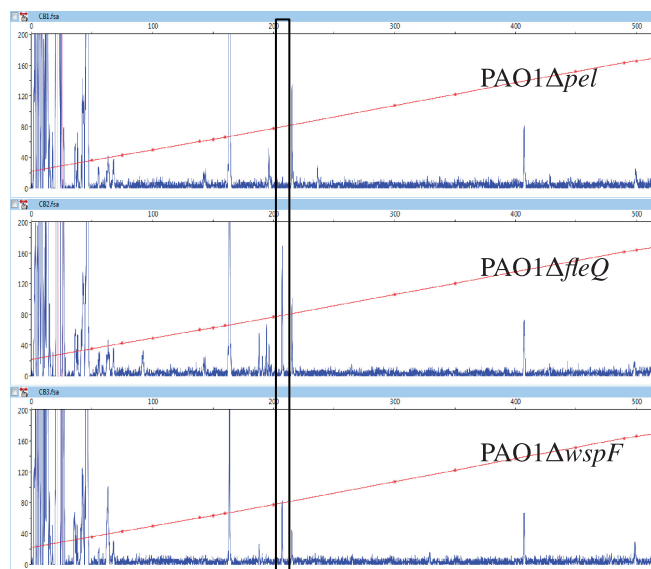

Figure 2. Identification of the transcription start site of *pelA*. Primer extension analysis of the *pelA* transcript was performed using a 6-FAM labeled primer (pelA-R1), which hybridizes to the RNA 49–73 bp downstream from the translation start site of *pelA*. Total RNA were isolated from PAO1Δ*pel*, PAO1Δ*fleQ* and PAO1Δ*wspF* strains. The fluorescence intensity of FAM-labeled cDNA fragments (ordinate) is plotted against the sequence length of the fragment (abscissa). The horizontal line indicates the GS-500 internal size standard. The major transcript detected in strains PAO1Δ*fleQ* and PAO1Δ*wspF* but not detected in strain PAO1Δ*pel* is boxed.

probe of 572 bases corresponding to positions +207 to −365 relative to the transcription start site of the *pelA* gene. As the concentration of FleQ was increased, two protected regions appeared (Figure 3). Whatever the concentration of FleQ, both regions seemed to be occupied simultaneously, without apparent hierarchy between the two. The use of a fragment of 456 bases corresponding to position +91 to −365, relative to the transcription start site of the *pelA* gene, confirmed these results. The two protected regions are 19 bases long and separated by 34 bases. They are centered at position −48 (FleQ box 1) and +6 (FleQ box 2) relative to the transcription start site (Figure 1). It is noteworthy that FleQ box 2 overlaps the *pelA* transcription start site. Since FleQ has a AAA$^+$ ATPase domain, we also tested the effect of ATP on FleQ binding to the *pelA* promoter and observed similar footprint patterns plus or minus ATP (data not shown). This result, together with previous gel shift assays results (27), confirms that the binding of FleQ to the *pel* promoter is independent of ATP.

Each FleQ box contains the sequence ATTGAC (Figure 1). To determine, *in vitro*, the functional role of the two FleQ boxes and the repeated sequences, we generated several mutated versions of the *pelA* promoter by substituting four bases inside the two FleQ boxes, and inside or outside the repeated sequence (Figure 1). DNase I footprinting experiments were performed on these variant DNA templates. Mutations, either outside (mut1) or inside (mut2) the ATTGAC motif in FleQ box 1, abolished the binding of FleQ to the FleQ box 1, but did not affect the binding of FleQ to FleQ box 2 (Figure 4). A mutation in the ATTGAC motif in FleQ box 2 (mut3) abolished the binding of FleQ to it but not to FleQ box 1. Mutation outside the repeated sequence of FleQ box 2 (mut4) did not abolish FleQ binding to either box. This result shows that the binding of FleQ to one binding site is independent of protein occupancy at the other binding site. The 19 bases of the FleQ boxes are not equally involved in the binding of FleQ but an intact repeated sequence seems to be required for the binding of FleQ to the *pelA* promoter.

FleN stimulates bending of *pelA* promoter DNA when FleQ and ATP or ADP are present

Previous gel shift data showed that FleN in conjunction with FleQ retarded the migration of a *pelA* promoter probe fragment more than did FleQ alone and we also know that FleN and FleQ interact with each other (27,35). In *in vitro* footprinting experiments with FleN and FleQ, we found that FleQ protected the same two regions on the *pelA* promoter, but in addition FleN induced the appearance of a DNase I hypersensitive site (Figure 5). The hypersensitive site was observed when ATP, ADP (data not shown) or ATPγS, a non-hydrolysable analogue of ATP, were included in the footprinting reaction mixtures. However, no hypersensitive site was observed in the presence of AMP or in the absence of any nucleotides. The DNase I hypersensitive site is located between the two FleQ binding boxes, centered at −20 relative to the *pelA* transcription start
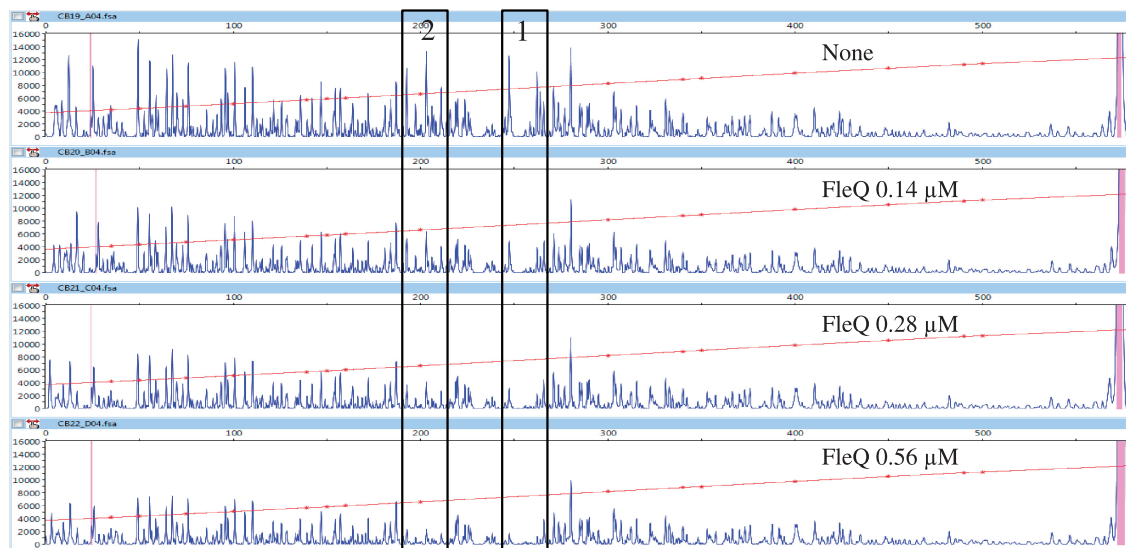
Figure 3. The *pel* promoter contains two FleQ boxes. 5′-FAM-labeled *pel* DNA (0.45 pmol) was incubated with or without FleQ (0.14, 0.28 or 0.56 μM, as indicated) and in the presence of 10 μM of ATP, then submitted to DNase I digestion (0.3 U) and analyzed by capillary electrophoresis. The fluorescence intensity of FAM-labeled DNA fragments (ordinate), in arbitrary units, is plotted against the sequence length of the fragment (abscissa), in bases, relative to the first base of the pelA-R1 primer. The whole electrophoregram is shown. The horizontal line indicates the GS-500 internal size standard. The two boxes labeled 1 and 2 indicate the two regions protected by FleQ.
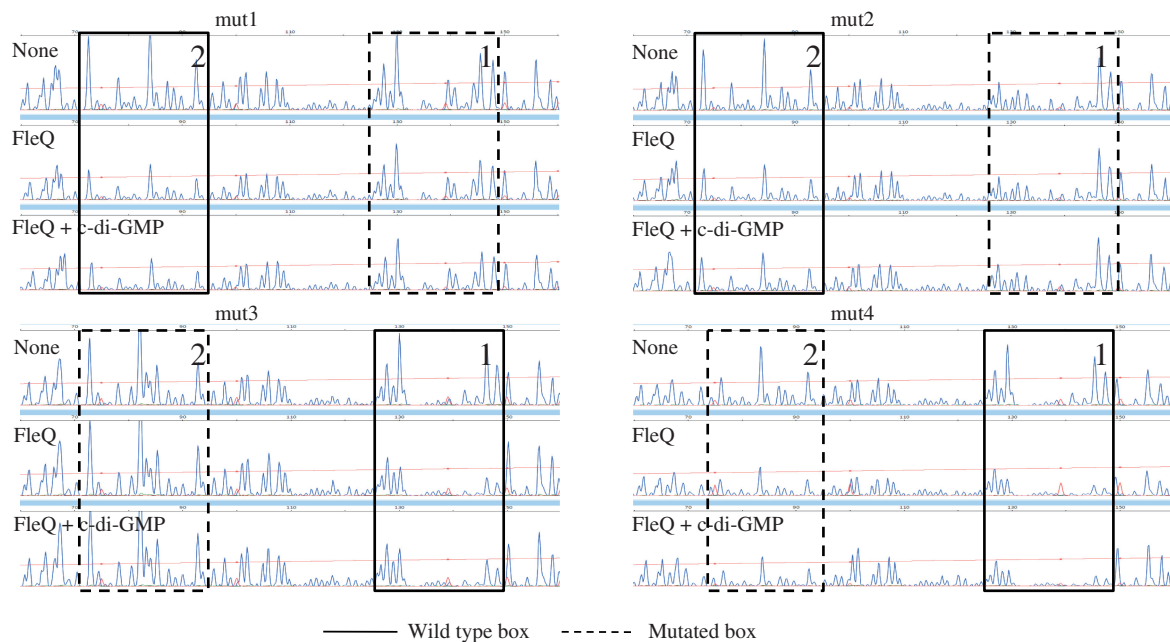

Figure 4. Effects of mutations in the *pelA* promoter on the binding of FleQ. 5′-FAM-labeled *pel* DNA (0.45 pmol) carrying different nucleotide substitutions as indicated in Figure 1 was incubated in the presence of ATP (10 μM), with or without FleQ (0.28 μM) and in the presence or absence of c-di-GMP (100 μM), as indicated. The sequence length of the fragment, in bases, is relative to the first base of the pelA-R2 primer. Part of the electrophoregram is shown. The two boxes labeled 1 and 2 indicate the two regions of FleQ binding, the dashed line of the box indicates which box is mutated.

site (Figure 1). Neither the protected regions nor hypersensitive sites were detected when DNase I footprinting experiments were carried out with FleN alone. DNase I hypersensitive sites are often indicative of flexible DNA regions, able to bend or to loop. FleN likely causes the *pelA* promoter to distort in the presence of FleQ and this distortion requires ATP but not its hydrolysis. DNase I

footprinting experiments were also performed with FleQ and FleN on the four variant *pel* promoters. The hypersensitive site was observed when mut4 was the template but not when mut1, 2 or 3 were the templates (data not shown). This suggests that the DNA bending mediated by FleN occurs only when FleQ is bound to both FleQ boxes.
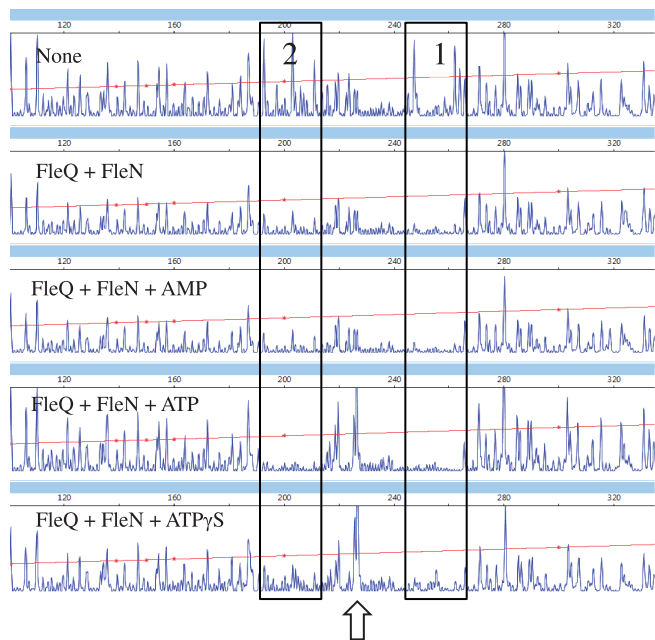
Figure 5. FleN causes the *pel* promoter to bend in conjunction with FleQ and ATP or ATPγS. Analysis of the 5′-FAM-labeled *pel* DNA (0.45 pmol) incubated with FleQ alone or with FleN at equimolar concentrations (0.28 μM) and in the presence and absence of ATP (10 μM), AMP (10 μM) or ATPγS (10 μM). The sequence length of the fragment (abscissa), in bases, is relative to the first base of the pelA-R1 primer. The two FleQ binding sites are boxed and the hypersensitive site is indicated by an arrow.

FleQ stays bound to the *pelA* promoter and the DNA bending mediated by FleN is relieved when c-di-GMP is added

It was previously suggested that the binding of c-di-GMP to FleQ induces a dissociation of FleQ from the *pelA* promoter (27). To test this hypothesis, we footprinted the *pelA* promoter in the presence of c-di-GMP. When FleQ was incubated with c-di-GMP prior to DNase I treatment, the two FleQ protected regions on the *pelA* promoter persisted on the electropherogram, indicating that FleQ remains bound to the *pelA* promoter (Figure 6). In addition, c-di-GMP did not influence the footprint patterns of FleQ on the mutated variant *pelA* promoters (Figure 4). When FleN, FleQ and c-di-GMP were incubated together prior to DNase I treatment, the two FleQ protected regions on the *pelA* promoter again persisted, but no hypersensitive site was detected (Figure 6). We therefore conclude that in the presence of c-di-GMP, FleQ remains on the *pelA* promoter but the bending mediated by FleN is relieved. It is noticeable that the footprint of FleQ on FleQ box 2 is slightly less pronounced in the presence of c-di-GMP, regardless of whether FleN is present. This may reflect a change in the affinity of FleQ for FleQ box 2 in the presence of c-di-GMP.

Previous gel shift assay results showed that when FleQ was present, FleN induced a supershift of a *pelA* promoter fragment and this shift was then abrogated in the presence of c-di-GMP. This was interpreted to suggest that

c-di-GMP binding to FleQ caused it to dissociate from the *pelA* promoter (27). An alternative explanation that is consistent with the footprinting data is that the supershift reflects bending of DNA induced by FleQ and FleN, and c-di-GMP alleviates this bending to abolish the supershift. Another possible explanation is that the gel shift assays measure complexes stable enough to be trapped upon migration on a gel; however, footprinting experiments identify even weakly bound protein/DNA complexes too unstable to be retained on a gel.

Analysis of FleQ and FleN interaction in the presence of ATP or c-di-GMP

The DNA bending stimulated by FleN occurs only when ATP is present and disappears when c-di-GMP is added (Figure 6). It could be that ATP is required for FleQ and FleN to interact and that c-di-GMP stimulates the dissociation of FleQ from FleN. Alternatively, conformational changes in FleQ or FleN due to the binding of ATP or c-di-GMP could be required to trigger DNA bending/unbending. To test these hypotheses, the interaction between FleQ and FleN was analyzed by limited proteolysis. Purified FleQ and FleN, together or separately (with BSA as control), were incubated with trypsin for different intervals of time in the presence or absence of ATP or c-di-GMP. The digestion products were then separated by SDS–PAGE, transferred to a membrane and revealed with FleQ or FleN antibodies. A fragment of 20 kDa was detected when FleQ was subjected to trypsin digestion for 30 min (Figure 7A and B, left panel). When c-di-GMP was also present in the incubation mixture, the 20 kDa FleQ fragment was not present, but a new proteolysis-resistant fragment of 30 kDa appeared (Figure 7A and B, right panel). This suggests that FleQ undergoes a conformational change when it binds c-di-GMP. In contrast, FleQ does not undergo an obvious conformational change upon ATP binding and ATP does not affect the conformational change of FleQ due to c-di-GMP binding. Inclusion of c-di-GMP did not alter the digestion profile of FleN (Figure 7D). When FleQ and FleN were incubated together with trypsin, FleQ is more resistant to proteolysis as exemplified by the persistence of the band corresponding to the intact FleQ protein (55 kDa) after 10 min of incubation (Figure 7C, all panels), whereas without FleN, the same band had disappeared by this time (Figure 7A). The same digestion profile of FleQ was observed with or without ATP (Figure 7C), indicating that FleQ and FleN interact irrespective of the presence of ATP. If c-di-GMP induces the dissociation of a FleQ/FleN complex, then the digestion profile of FleQ in the presence of FleN and c-di-GMP should be equivalent to the digestion profile of FleQ alone in the presence of c-di-GMP. This was not the case. Our results show that in the presence of c-di-GMP, the FleQ protein was still protected from trypsin digestion by FleN (Figure 7C). This result was confirmed when FleQ and FleN digests were revealed with FleN antibodies (compare Figure 7D and E). In order to get closer to *in vivo* conditions, we tested the effect of *pel* promoter DNA on FleQ and FleN interaction. FleN, plus or
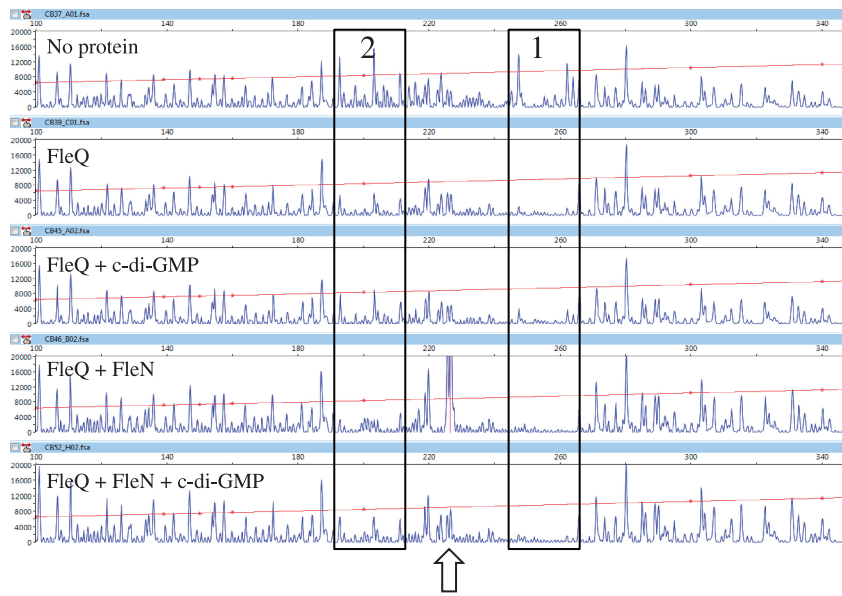
Figure 6. Effect of c-di-GMP on FleQ and FleN binding to the *pel* promoter. Analysis of the 5′-FAM-labeled *pel* DNA (0.45 pmol) incubated with FleQ alone or with FleN at equimolar concentrations (0.28 μM), in the presence of ATP (10 μM) and in the presence or absence of c-di-GMP (100 μM). The sequence length of the fragment (abscissa), in bases, is relative to the first base of the pelA-R1 primer. The two FleQ binding sites are boxed and the hypersensitive site is indicated by an arrow.
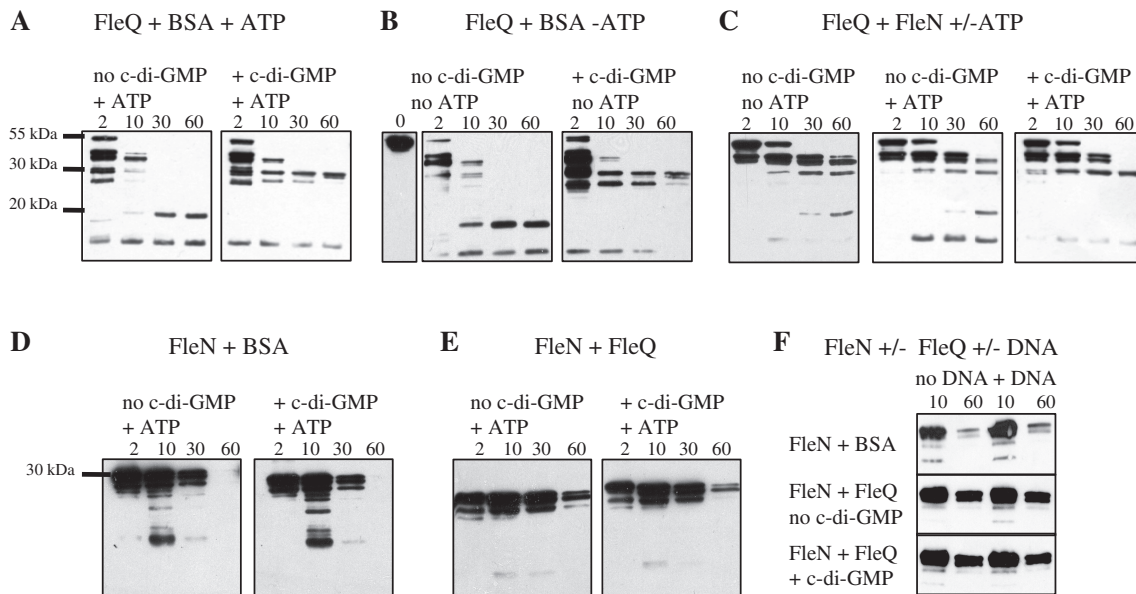

Figure 7. Effects of c-di-GMP and ATP on the kinetics of trypsin digestion of FleQ alone or FleQ and FleN. FleQ and BSA (**A** and **B**), FleN and BSA (**D**) or FleQ and FleN (**C**, **E** and **F**) were incubated in the presence or absence of c-di-GMP (1 mM), ATP (100 μM) or a DNA fragment encompassing the two FleQ binding sites (3.2 μM), as indicated and then subjected to proteolysis with trypsin. Times of incubation are indicated above the gel. Digestion products were separated by SDS–PAGE, transferred to a nitrocellulose membrane and detected with anti-FleQ antibodies (A, B and C) or with anti-FleN antibodies (D, E and F).

minus FleQ and c-di-GMP, was incubated with double stranded DNA encompassing the two FleQ binding sites prior to trypsin addition. The FleQ and FleN proteins interacted similarly with or without the DNA present (Figure 7F). After 60 min of incubation with trypsin, the FleN protein was almost entirely digested in the absence of FleQ whereas with FleQ, FleN was more resistant to proteolysis (Figure 7F).

These results indicate that FleQ and FleN form a complex in the presence as well as in the absence of c-di-GMP or ATP. However, the trypsin digestion profile of FleQ in the presence of c-di-GMP and FleN (Figure 7C, right panel) was different from the digestion profile obtained in the absence of c-di-GMP (Figure 7C, middle panel), suggesting that a conformational change of a FleQ/FleN complex occurs in the presence of c-di-GMP.

The distortion of DNA seen in footprints that depends on the presence of c-di-GMP or ATP is more likely to represent conformational changes related to their binding to FleQ and/or FleN rather than to effects related to the relief of interactions between the two proteins.

FleQ functions as a transcriptional repressor in the absence of c-di-GMP and as an activator when c-di-GMP is present

To study the *in vivo* participation of the two FleQ boxes in regulating *pelA* promoter activity, we constructed transcriptional fusions of *lacZ* to promoter fragments carrying mutations in the two FleQ boxes and introduced each of them at the *attB* site of strains PAO1Δ*pel*Δ*psl* (referred to here as the WT strain), PAO1Δ*pel*Δ*psl*Δ*fleQ* (referred to here as the *fleQ* strain), PAO1Δ*pel*Δ*psl*Δ*fleN* (referred as the *fleN* strain) and PAO1Δ*pel*Δ*psl*Δ*fleQ*Δ*fleN* (referred as the *fleQfleN* strain). The level of intracellular c-di-GMP was manipulated in these strains by expressing the DGC PA1120 from plasmid pJN1120 (27,44). The empty vector pJN105 was used as a control. The wild-type strain expressing the wild-type *pelA* promoter (*PpelA-wt*) had low β-galactosidase activity. As expected β-galactosidase activity was much higher in the strains deleted of *fleQ* or with a high intracellular level of c-di-GMP (Figure 8). These results confirm the previous report of FleQ-mediated repression of the *pel* genes at low intracellular c-di-GMP and derepression when c-di-GMP levels are high (27).

In the wild-type strain carrying the vector control plasmid, the promoter-fusions carrying mutations inside FleQ box 1 (*PpelA-mut1* and *PpelA-mut2*) had low, wild-type levels of β-galactosidase (Figure 8). Thus, in the absence of c-di-GMP, repression of *pel* genes does not require an intact FleQ box 1. The wild-type strain with the promoter carrying mutations outside of the repeated sequence of FleQ box 2 (*PpelA-mut4*) also had low β-galactosidase activity when c-di-GMP was low. In contrast, mutations in the repeated sequence of FleQ box 2 (*PpelA-mut3*) caused an increase of β-galactosidase activity (Figure 8). From the footprinting experiments, we know that a mutation inside the repeated sequence of FleQ box 2 (mut3) impairs the binding of FleQ to FleQ box 2, whereas a mutation in the FleQ box 2 but outside of the repeated sequence (mut4) does not (Figure 4). We can, therefore, conclude that the binding of FleQ to FleQ box 2 of the *pel* promoter is a necessary requirement for FleQ-mediated repression. When intracellular c-di-GMP was high (strains carrying pJN1120), an intact FleQ box 1 was required for *PpelA-lacZ* activation. Moreover, the activity of *PpelA-mut3* was increased by nearly a factor two compared to the activity of *PpelA-wt* in high c-di-GMP cells (Figure 8). This increased activity depended on the presence of FleQ. From this we conclude that the role of c-di-GMP is not simply to derepress *pelA* expression but rather to switch FleQ from a repressor to an activator.

Previous results using RT-PCR showed that FleN was required for full expression of *pel* genes when c-di-GMP is high (27). The data presented in Figure 8 indicate that FleN participates in the FleQ-mediated activation of *pel* genes in response to c-di-GMP, but the effects are not as pronounced as previously reported. For an unknown reason, the expression of the *PpelA-mut4* fusion did not seem to be affected by the deletion of *fleN*.

	WT (vector)	WT (pJN1120)	*fleQ* (vector)	*fleQ* (pJN1120)	*fleN* (vector)	*fleN* (pJN1120)	*fleQfleN* (vector)	*fleQfleN* (pJN1120)
	low c-di-GMP	high c-di-GMP	low c-di-GMP	high c-di-GMP	low c-di-GMP	high c-di-GMP	low c-di-GMP	high c-di-GMP
PpelA-wt	170±36	1195±262	715±71	605±164	205±28	885±180	700±39	695±135
PpelA-mut1	280±42	345±45	525±83	595±176	430±63	490±68	545±40	515±45
PpelA-mut2	236±33	230±18	315±19	370±47	320±42	305±66	360±26	335±45
PpelA-mut3	1010±186	2135±327	600±49	555±94	1045±106	1280±229	610±84	555±77
PpelA-mut4	205±35	970±65	660±40	610±113	230±26	975±163	660±57	600±89

Figure 8. FleQ acts as a repressor in the absence of c-di-GMP and as an activator in the presence of c-di-GMP. β-Galactosidase activities of wild type or mutated *PpelA-lacZ* fusions, as indicated on the left of the diagram, in PAO1Δ*pel*Δ*psl* (WT), PAO1Δ*pel*Δ*psl*Δ*fleQ* (*fleQ*), PAO1Δ*pel*Δ*psl*Δ*fleN* (*fleN*) or PAO1Δ*pel*Δ*psl*Δ*fleQ*Δ*fleN* (*fleQfleN*) strains carrying pJN105 (vector control) or pJN1120 (allowing the overexpression of the DGC encoded by PA1120). The white boxes indicate the FleQ boxes, the grey boxes, the repeated sequences observed in the FleQ boxes. Mutations in either boxes are indicated by an X and the transcription start site is indicated by an arrow. The β-galactosidase values are indicated on the right and expressed as means ± standard deviation.

DISCUSSION

This work suggests that FleQ controls gene expression by a novel mechanism that involves binding to two sites in the promoter of the operon it controls. At one site (box 2), FleQ functions to repress gene expression, and at the other site (box 1), FleQ functions to activate gene expression in response to c-di-GMP. A revised model for *pel* regulation by FleQ can be proposed based on the data presented here (Figure 9). In the absence of c-di-GMP, FleQ binds to its two FleQ binding sites on the *pel* promoter (Figure 9A). FleN is found bound to FleQ (Figure 9B). In the presence of ATP, we propose that FleN forms dimers inducing a bending of the *pelA* promoter by bridging the bound FleQ proteins (Figure 9C). We think that this conformation could either impair the binding of the RNA polymerase or prevent RNA polymerase bound to the *pelA* promoter from forming an open complex, leading to *pel* repression. In the presence of c-di-GMP, FleQ undergoes a conformational change that probably induces a cascade of conformational changes in the FleQ/FleN/DNA complex such that the bending is relaxed. FleQ is switched to an activator (Figure 9D). We propose that the relief of the bending may either induce RNA polymerase binding or remodel RNA polymerase binding and the FleQ bound to FleQ box 1 favors transcription initiation. This model is speculative and still incomplete, but it is consistent with the ability of FleQ to mediate both repression and activation in response to c-di-GMP while bound at the same sites on DNA. The intracellular concentration of c-di-GMP in *P. aeruginosa* is subject to both temporal
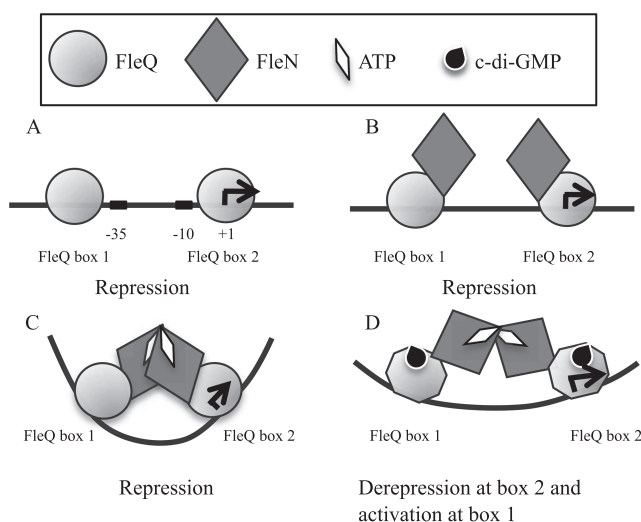
variations depending on the activity of DGCs and PDEs responding to different signals, to spatial variations due to specific subcellular localization of some DGCs (45,46) and the partitioning of c-di-GMP during cell division (47). Thus, having the necessary macromolecular components pre-assembled at the promoter could be a biological advantage in allowing cells to respond rapidly to c-di-GMP concentration changes to fine-tune gene expression.

Only a few bacterial transcription factors have been shown to both activate and repress gene expression from a single promoter and these have mechanisms of action that are distinct from that of FleQ. The well-described MerR protein both activates and represses expression of *mer* genes, coding for mercury resistance (48–50). MerR shares with FleQ that the binding of their effectors, mercury or c-di-GMP, switches the regulators from a repressor to an activator without changing the location or the occupancy of the binding sites. However, while MerR directs repression and activation from a single binding site by remodeling the spacing between the −10 and −35 region in response to mercury, FleQ has separate binding sites for each function and likely uses a different mechanism to stimulate RNA polymerase. Another example is the TyrR protein from *E. coli*. In the presence of tyrosine, TyrR, an EBP, forms hexamers and binds to adjacent high affinity and low affinity sites, leading to *tyrP* repression (51–54). In the presence of phenylalanine, TyrR forms dimers that bind only to the high affinity site, allowing interaction with the RNA polymerase, and activation of gene expression (52–55). Finally, LldR, a Gnt family member from *E. coli,* both activates and represses expression of the *lldPRD* operon, involved in lactate metabolism in response to lactate (56). Binding of LldR to two sites located on either side of the *lldPRD* transcription start site is required to repress transcription. Binding of a LldR/lactate complex to the site upstream of the transcription start site is required to activate gene expression (56). FleQ behaves somewhat differently in that it needs to bind to only one of its two binding sites (FleQ box 2) to repress *pel* expression (Figure 8). Also full activation of *pel* expression in response to c-di-GMP requires a second protein, FleN.

FleQ is an EBP that has the additional unusual property of regulating gene expression independently of RNA polymerase σ^{54} in some circumstances. Expression of biofilm-related genes, such as *pel* genes, occurs normally in a σ^{54} (*rpoN*) mutant (30). Also the *pelA* promoter encompasses putative −10 and −35 elements, suggesting that transcription is mediated by a σ^{70}-RNA polymerase complex (Figure 1). Transcriptional control by an EBP in combination with σ^{70}-RNA is uncommon but not unprecedented. TyrR from *E. coli* (52), PhhR from *Pseudomonas putida* (57), as well as NtrC (58) or HupR (59) from *Rhodobacter capsulatus* are such proteins. σ^{54} does mediate FleQ-dependant activation of flagella gene expression (30). Moreover, the σ^{54} binding domain and the catalytic ATP site of FleQ are perfectly well conserved whereas the abovementioned EBP-like proteins that work with σ^{70}-RNA polymerase either lack or have a poorly conserved σ^{54} binding domain and/or catalytic site

Figure 9. Model of *pel* regulation. FleQ binds to two FleQ boxes on the *pel* promoter (**A**). FleQ interacts with FleN in the absence of ATP (**B**) as well as in the presence of ATP, but in this case it induces a distortion of *pel* DNA (**C**). We propose that FleN forms a bridge between two FleQ bound to their binding sites. The binding of FleQ to FleQ box 2 is essential for repression. The binding of c-di-GMP to FleQ induces a conformational change of FleQ, probably propagated through FleN, which induces the relief of *pel* distortion and leads to *pel* expression (**D**). The binding of FleQ to FleQ box 1 is essential for activation.

(51,60). FleQ is apparently a versatile protein able to function with two different kinds of RNA polymerase holoenzyme. At this point, we are uncertain as to how c-di-GMP may influence FleQ mediated regulation of flagella genes. Based on microarray data, flagella gene expression is down regulated by 1.5- to 2-fold in response to high c-di-GMP in *P. aeruginosa*. We have been unable to obtain reproducible DNaseI footprints of FleQ at one flagella promoter (*fleSR*) that we tested and thus have not evaluated effects of c-di-GMP on this interaction.

Recent studies analyzed four other transcriptional regulators that respond to c-di-GMP (23–26). In *Xanthomonas campestris*, c-di-GMP acts as a negative regulator of Clp, impairing the binding of Clp to DNA and the induction of genes important for virulence (25,61,62). In *Vibrio cholerae*, c-di-GMP induces the oligomerization of VpsT necessary for its binding to *vpsL*, the first gene of a *Vibrio* polysaccharide operon. C-di-GMP enhances the binding of MrkH from *Klebsiella pneumoniae* and Bcam1349 from *Burkholderia cenocepacia* to the promoter region of type 3 fimbriae and cellulose synthase genes, respectively (23,26). VpsT belongs to the LuxR family of regulators, whereas Clp and Bcam1349 are homologous to Crp (24,25,27). MrkH contains a PilZ domain but does not contain an obvious DNA binding domain (26). C-di-GMP binds to the W[F/L/M][T/S]R sequence of VpsT (24) and to the conserved cyclic nucleotide monophosphate binding domain of Clp (25,61,62). The c-di-GMP binding site of FleQ remains unknown but we have determined that its N-terminal domain is not involved in the detection of c-di-GMP (27). Alignments of a variety of EBPs and FleQ homologues revealed that a region of about 30 amino acids at the C-terminal margin of the AAA$^+$-ATPase domain is conserved in FleQ homologues. A deletion of 20 amino acids that we made in this region abolished c-di-GMP binding but point mutations in that region did not (Baraquet and Harwood, unpublished data). Thus we cannot distinguish whether we removed the c-di-GMP binding site or whether the deletion caused a conformational change in FleQ that impaired the binding of c-di-GMP elsewhere in the protein.

A large number of activators and repressors induce distortion or bending of DNA upon binding (63), and the binding of effectors to such regulators can modulate DNA bending (64–70). Modulation of DNA bending by transcription factors can influence transcription by impairing or improving RNA polymerase binding. The region of the *pel* promoter located between the two FleQ boxes is distorted when FleQ, FleN and ATP are all present (Figure 5). Our data suggest that FleN, a non-DNA binding protein, induces the distortion probably by interacting with the DNA-binding protein FleQ. FleN is a putative ATPase with a deviant Walker A motif (33), belonging to a functionally diverse group including MinD and ParA (71), which have the common feature of undergoing ATP dependent dimerization (72,73). We hypothesize that FleN forms dimers upon ATP binding and then interacts with FleQ bound to DNA to form a bridge. In fact we observe bending only when FleQ can bind to its two FleQ binding sites simultaneously.

While DNA bending mediated by FleN appears prerequisite for *pel* expression the precise role of FleN is still unclear. FleN could simply have a structural role and the oligomerization of FleN upon ATP binding may be crucial to induce distortion of DNA via FleQ. It may also be that FleQ, FleN, ATP and DNA form a complex ready to sense c-di-GMP more efficiently than FleQ alone. Another hypothesis could be that FleN has a regulatory role, detecting ATP as an additional signal for *pel* expression. Indeed, continuous *pel* expression is not necessary for biofilm maintenance but for growing larger biofilms (74). This result implies that consumption of metabolic energy is required and ATP could signal the energetic status of cells.

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