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FleQ DNA Binding Consensus Sequence Revealed by Studies of FleQ-Dependent Regulation of Biofilm Gene Expression in *Pseudomonas aeruginosa*

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ABSTRACT

The transcription factor FleQ from *Pseudomonas aeruginosa* derepresses expression of genes involved in biofilm formation when intracellular levels of the second messenger cyclic diguanosine monophosphate (c-di-GMP) are high. FleQ also activates transcription of flagellar genes, and the expression of these genes is highest at low intracellular c-di-GMP. FleQ thus plays a central role in mediating the transition between planktonic and biofilm lifestyles of *P. aeruginosa*. Previous work showed that FleQ controls expression of the *pel* operon for Pel exopolysaccharide biosynthesis by converting from a repressor to an activator upon binding c-di-GMP. To explore the activity of FleQ further, we carried out DNase I footprinting at three additional biofilm gene promoters, those of *psl*, *cdrAB*, and PA2440. The expression of *cdrAB*, encoding a cell surface adhesin, was sufficiently responsive to FleQ to allow us to carry out *in vivo* promoter assays. The results showed that, similarly to our observations with the *pel* operon, FleQ switches from a repressor to an activator of *cdrAB* gene expression in response to c-di-GMP. From the footprinting data, we identified a FleQ DNA binding consensus sequence. A search for this conserved sequence in bacterial genome sequences led to the identification of FleQ binding sites in the promoters of the *siaABCD* operon, important for cell aggregation, and the *bdlA* gene, important for biofilm dispersal, in *P. aeruginosa*. We also identified FleQ binding sites upstream of *lapA*-like adhesin genes in other *Pseudomonas* species.

IMPORTANCE

The transcription factor FleQ is widely distributed in *Pseudomonas* species. In all species examined, it is a master regulator of flagellar gene expression. It also regulates diverse genes involved in biofilm formation in *P. aeruginosa* when intracellular levels of the second messenger c-di-GMP are high. Unlike flagellar genes, biofilm-associated genes are not always easy to recognize in genome sequences. Here, we identified a consensus DNA binding sequence for FleQ. This allowed us to survey *Pseudomonas* strains and find new genes that are likely regulated by FleQ and possibly involved in biofilm formation.

Cyclic diguanosine monophosphate (c-di-GMP) is an intracellular second messenger that is produced by bacteria and has diverse effects on bacterial physiology. c-di-GMP binds to riboswitches and effector proteins to modulate transcription, translation, and protein activities (1, 2). In the opportunistic pathogen *Pseudomonas aeruginosa*, the transcription factor FleQ binds c-di-GMP to derepress expression of genes for biofilm components (3, 4). Biofilms are surface-attached communities of bacteria embedded in a matrix made of exopolysaccharides, proteins, and DNA. Biofilm infections are particularly difficult to treat because bacteria are protected by the biofilm matrix, making them resistant to antimicrobial treatment compared to planktonic cells (5). Genes controlled by FleQ include *pel* genes, coding for Pel exopolysaccharide; *psl* genes, coding for Psl exopolysaccharide; *cdrAB*, coding for an adhesin; and PA2440, coding for a polysaccharide deacetylase (4, 6–10). In a previous study of *pel* operon expression, we found that FleQ undergoes a conformational change when it binds c-di-GMP to switch it from a repressor to an activator of *pel* gene expression (3). FleQ also activates flagellar gene expression. In conjunction with σ^{54}, FleQ activates the expression of the two-component regulatory genes *fleSR*, as well as genes required for flagellar export apparatus assembly and flagellar basal body assembly (6, 11). c-di-GMP inhibits FleQ ATPase activity (12), which explains the modest (1.5- to 2-fold) downregulation of flagellar gene expression that is seen in the presence of c-di-GMP (4, 8). By inversely regulating biofilm and flagellar gene expression in response to c-di-GMP, FleQ helps to control the planktonic-to-biofilm lifestyle transition of *P. aeruginosa*.

To extend our understanding of FleQ as a transcriptional regulator of *psl*, *cdrAB*, and PA2440 genes involved in biofilm formation, we carried out DNA footprinting at high and low c-di-GMP concentrations. We also looked at the effects of FleQ and c-di-GMP on the *in vivo* promoter activities of the *cdrAB* operon, which encodes an adhesin. We determined that FleQ binds at two to three sites near the *psl*, *cdrAB*, and PA2440 operon promoters. This allowed us to define a common FleQ consensus binding sequence motif. A search for this conserved sequence in the genome sequence of strain PAO1 and several other *Pseudomonas* species

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led to the identification of FleQ binding sites in the promoter regions of a number of new genes.

MATERIALS AND METHODS

Strains and growth media. *P. aeruginosa* strain PAO1 and *Escherichia coli* strains were routinely grown on LB medium at 37°C. Antibiotics were added at the following concentrations: 60 μg/ml gentamicin or tetracycline for *P. aeruginosa* and 10 μg/ml gentamicin or tetracycline for *E. coli*. c-di-GMP was purchased from Biolog Life Science Institute (Bremen, Germany).

For all primer sequences, lowercase letters refer to restriction endonuclease recognition sequences. A *cdrAB* promoter deletion was constructed by sequence overlap extension PCR with primers cdrA-del-E1 (gaagaattc TCCTGGCAACTGACCGAGCAGCAC), cdrA-del-I1 (GCTGGCTGAT CTTGATCTCGTTGGGTTTTCCTCGACCATGCCTCCCTC), cdrA-del-I2 (GAGGGAGGCATGGTCGAGGAAAACCCAACGAGATCAA GATCAGCCAGC), and cdrA-del-E2 (gaaggatccGCATCCTGCGGCT TGCGGGTAACC). The *cdrAB* promoter deletion starts 341 bases upstream and ends 151 bases downstream of the translation start site. Upstream and downstream flanking regions of about 500 bases were cloned into the EcoRI and BamHI sites of suicide vector pEX19Gm and introduced into PAO1 Δ*pel* Δ*psl* or PAO1 Δ*pel* Δ*psl* Δ*fleQ* by conjugation with *E. coli* S17-1 (13). A similar procedure was followed to construct a PAO1 strain with *rpoN* deleted. Mutations in the *fleQ* gene ($fleQ_{D245A}$ and $fleQ_{T224S}$) were generated by overlapping PCR with oligonucleotides containing the mutations and flanking primers with the following sequences: FleQmutup, gacgaattcAGGCCGGCAACTGGGATGCCATCG, and FleQmutdown, gacaagcttAGGGAAATCTCGTGACCGTCATCC. The EcoRI-HindIII fragments were then cloned into pEX19-Gm and introduced into PAO1 Δ*fleQ* by conjugation with *E. coli* S17-1.

Construction of chromosomal *cdrA-lacZ* fusions and measurement of promoter activities *in vivo*. Chromosomal *cdrA-lacZ* reporter fusions were constructed using the mini-CTX-*lacZ* vector (14). A 441-bp DNA fragment starting 331 bases upstream of the translation start site and ending 110 bases downstream of the translation start site was amplified by PCR using primers PcdrA-up (gaaggatccATGCTGGCGATGGCGAACA TGAGG) and PcdrA-down (gaagaattcGCATGGCTCATGGAAGGTTCC TTG). The fragment was cloned between the BamHI and EcoRI sites of mini-CTX-*lacZ* vector. Overlapping PCR was used to generate mutations in the *cdrA* promoter. Mini-CTX-*lacZ*-cdrAmut1 was constructed by replacing the ACTGAC sequence with CAGTCA centered at position −206 relative to the *cdrA* translation start site. Mini-CTX-*lacZ*-cdrAmut2 was constructed by replacing the ACTGAC sequence with CAGTCA centered at position −54 relative to the *cdrAB* translation start site. Mini-CTX-*lacZ*-cdrAmut3 was constructed by replacing the TTTGAC sequence with GGGTCA centered at position −30 relative to the *cdrA* translation start site. The plasmids mini-CTX-*lacZ*-cdrAWT, -mut1, -mut2, and -mut3 were transformed into the *E. coli* S17-1 strain and then introduced at the *attB* site of the PAO1 Δ*pel* Δ*psl* Δ*cdrAprom* or PAO1 Δ*pel* Δ*psl* Δ*fleQ* Δ*cdrAprom* chromosome by mating. Tetracycline-resistant colonies were selected and confirmed by PCR analysis. Plasmids pJN105 (vector control, gentamicin resistant) and pJN1120 (allowing the overexpression of the diguanylate cyclase encoded by PA1120) were introduced by electroporation (4). Strains were grown aerobically overnight in LB with gentamicin (60 μg/ml) with shaking. β-Galactosidase activities of whole cells were measured by the Miller method (15). Results are the averages from three independent experiments.

Protein purification. FleQ and FleN were purified from *E. coli* strains with plasmid pFleQ-Int1 or pFleN-Int1, as previously described (4).

DNase I footprinting. DNase I footprinting experiments were performed using a nonradiochemical capillary electrophoresis method as previously described (3, 16). The fragments of DNA containing the *cdrAB*, *psl*, or PA2440 promoter were generated by PCR using 6-FAM (6-carboxyfluorescein phosphoramidate) primers labeled at the 5′ end with *P. aeruginosa* PAO1 chromosomal DNA as the template or mini-CTX-*lacZ*-*cdrAB* mutated versions when needed. The *cdrAB* promoter DNA fragment was generated using primers CdrA-FAM-up (5′-FAM-CTGGCGA TGGCGAACATGAG), which paired 107 bases downstream of the *cdrA* translation start site, and PcdrA-down (gaagaattcGCATGGCTCATGGA AGGTTCCTTG), which paired 331 bases upstream of the *cdrAB* translational start site, leading to a DNA fragment of 438 bases. The *psl* fragment was obtained with primer Psl-FAM-down (5′-FAM-TCGATGAATCCA GCCCGTGTCAG), annealing 53 bases downstream of the *pslA* translational start site, and primer TR0 (cggcgaattcGGCTGGTTCTGGTAGAG CCGTCCG), annealing 541 bases upstream of the *pslA* translational start site. The PA2440 DNA fragment was generated by PCR using primers PA2440-FAM-down (5′-FAM-TTCAGGAACGTCATCCCGATCAG), which paired 32 bases downstream of the PA2440 translational start site, and PA2440-up (ACCACCGGACATCCGTGCAAAC), which paired 490 bases upstream of the PA2440 translational start site. The DNA fragments (0.45 pmol) were mixed with purified FleQ and/or FleN in 50-μl reaction mixtures containing ATP (10 μM), c-di-GMP (500 μM), and binding buffer (10 mM Tris-HCl, pH 7.8, 50 mM KCl, 8 mM magnesium acetate, 50 ng/μl bovine serum albumin [BSA], and 5% glycerol). After incubation for 30 min at room temperature, DNase I (0.3 units; Promega) was added for 2 min. The samples were phenol extracted and ethanol precipitated. Fragments were analyzed (Genewiz, Inc.), and fragment sizes were determined using ABI Peak Scanner software.

Gel shift assays. To assess FleQ binding in gel shift assays, the probe DNA fragments (0.30 pmol) were mixed with 4 pmol of FleQ and/or 4 pmol of FleN in the presence of 50 μM ATP and in the presence or absence of 100 or 500 μM c-di-GMP in a 15-μl reaction mixture containing the same binding buffer as that used in footprinting experiments. After incubation for 25 min at room temperature, the samples were separated by electrophoresis on a 5% nondenaturing acrylamide Tris-glycine-EDTA (10 mM Tris [pH 8.0], 380 mM glycine, and 1 mM EDTA) gel in Tris-glycine-EDTA buffer at 4°C. The gel was soaked in 10,000-fold-diluted SYBR green I nucleic acid stain (Lonza, Walkersville, MD), and DNA was visualized under UV light. The DNA fragments containing the *cdrAB* or the PA2440 promoter were the same as the *cdrAB* or PA2440 fragment used for footprinting experiments (438 bases and 522 bases, respectively). The DNA fragment containing the *psl* operon promoter was generated by PCR using primer Psl-FAM-up (5′-FAM-CCCAGGCCAGAGCGCTCG CGG), which paired 297 bases upstream of the *pslA* translational start site, and primer DN1 (gatcggatccCTCGATGGAATCCACCCGTGTCA), which paired 54 bases downstream of the translation start site of *pslA* and led to a fragment of 361 bp.

RNA extraction, RT-PCR, and primer extensions. Cell growth and RNA extraction were performed as previously described (3). Reverse transcription was performed using SuperScript II reverse transcriptase (Invitrogen) for reverse transcription-PCR (RT-PCR) analysis or SuperScript III reverse transcriptase for primer extension experiments. RT-PCRs were performed using a Sybr Green master mix with 1 ng of cDNA as the template and 200 nM each set of primers (pelA-F, CCTTCAGCCATCCG TTCTTCT, and pelA-R, TCGCGTACGAAGTCGACCTT, or PA4625-F, CCAGGTGATCATCTACAACTCCAA, and PA4625-R, GGAAGTGTTC GTCGCTGATGT). Cycling parameters were previously described (4). Genomic DNA was used as a standard, and the transcript levels of all genes were normalized to total cDNA. Data presented are the averages from at least two biological replicates. Error bars represent the standard deviation between samples. Primer extension analysis was performed on 15 μg of total RNA using the primer CdrA-FAM-up (also used for footprinting experiments) and following a preestablished protocol (3).

Bioinformatics. MEME (Multiple Em for Motif Elicitation) software was used to identify a putative FleQ binding site motif (17). The 13 FleQ binding sites identified by footprinting were used as input into the MEME algorithm, and one occurrence per sequence was searched. MEME builds a position-specific scoring matrix wherein there is a probability associated with the occurrence of each base at each position. The MEME output also

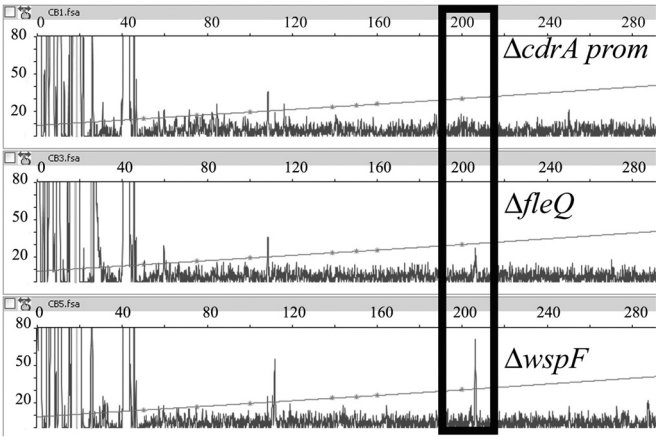

FIG 1 Identification of the *cdrAB* transcription start site. Primer extension analysis of the *cdrAB* transcript was done using a 6-FAM primer, which annealed to the mRNA 84 to 104 nucleotides downstream from the *cdrAB* translation start site. Total RNA was extracted from strains PAO1 Δ*pel* Δ*psl* Δ*cdrAprom* (Δ*cdrAprom*), PAO1 Δ*pel* Δ*psl* Δ*wspF* (Δ*wspF*), and PAO1 Δ*pel* Δ*psl* Δ*fleQ* (Δ*fleQ*). The fluorescence intensity is plotted against the sequence length of the DNA fragment. The horizontal lines designate an internal size ladder. The major transcript with a size of 206 nucleotides is boxed.

contains a sequence logo of the conserved motif as well as an E value, which is the probability of finding an equally well conserved pattern in random sequences. Position-dependent scoring matrices, as defined by MEME, were used as input to the MAST algorithm for searching in genome databases (18). Multiple alignments were performed using ClustalW from the EMBL-EBI website. The *Pseudomonas* Genome Database was used to retrieve *pel*, *psl*, PA2440, and *cdrA* orthologs (19).

RESULTS

Identification of the *cdrAB* operon transcription start site. The *cdrAB* operon encodes an adhesin essential for Psl exopolysaccharide structure (9). To determine the *cdrAB* transcription start site, we isolated RNA from *P. aeruginosa* strains PAO1 Δ*pel* Δ*psl* Δ*fleQ* and PAO1 Δ*pel* Δ*psl* Δ*wspF* and performed primer extension analysis. The expression of *cdrA* (PA4625) has been shown to be high in both *fleQ* and *wspF* mutants (4). The *wspF* mutation causes an increase in the intracellular concentration of c-di-GMP (7), and FleQ is an apparent transcriptional repressor of *cdrA* expression (4). As a control, we isolated RNA from a strain (PAO1 Δ*pel* Δ*psl* Δ*cdrAprom*) with a *cdrAB* promoter deletion. The deletion starts 341 bases upstream, and ends 151 bases downstream, of the *cdrA* translation start site. One major transcript was detected with RNA isolated from the Δ*wspF* and the Δ*fleQ* strains but not detected with RNA isolated from the strain with the *cdrAB* promoter deletion (Fig. 1). This fragment was 206 bp long, allowing us to map the *cdrAB* transcription start site to a location 99 bp upstream of the ATG start codon. FleQ has been shown to work with σ^{54} at flagellar promoters (6). These promoters are characterized by a GC doublet located between 11 and 14 bases upstream of the transcription start site (−12 site) and a conserved GG doublet that lies 10 bases farther upstream (−24 site) (20). No typical −24/−12 σ^{54} RNA polymerase binding site was found upstream of the transcription start site of *cdrAB* (Fig. 2). Instead, there are putative −10 (CATATT) and −35 (TTAAAA) boxes indicative of control by σ^{70} RNA polymerase in the promoter region of *cdrAB*.

We attempted but failed to identify the transcription start site of PA2440 using either an S1 nuclease protection assay or 5′ primer extension analysis. Our own transcriptome data and published transcriptome data (21) also failed to reveal a transcription start site for PA2440, possibly because this gene is typically expressed at very low levels. Thus, for footprinting and gel shift assays, we used a DNA fragment of 522 bp that spanned most of the intergenic region between PA2440 and its neighboring divergently transcribed gene, starting 32 bp downstream of the PA2440 translational start site.

Identification of FleQ binding sites in *cdrAB*, *psl*, and PA2440 promoter regions. We performed gel shift assays to test if FleQ binds directly to the promoter regions of *cdrAB*, PA2440, and the *psl* operon. As shown in Fig. 3, FleQ caused a modest mobility shift of *psl* and *cdrAB* promoter DNA fragments when added alone to reaction mixtures. Addition of FleQ alone did not convincingly shift the mobility of the PA2440 DNA fragment. However, as described below in more detail, there was a strong shift in the mobility of all the DNA fragments tested, including the PA2440 fragment, when both FleQ and the protein FleN were present (Fig. 3).

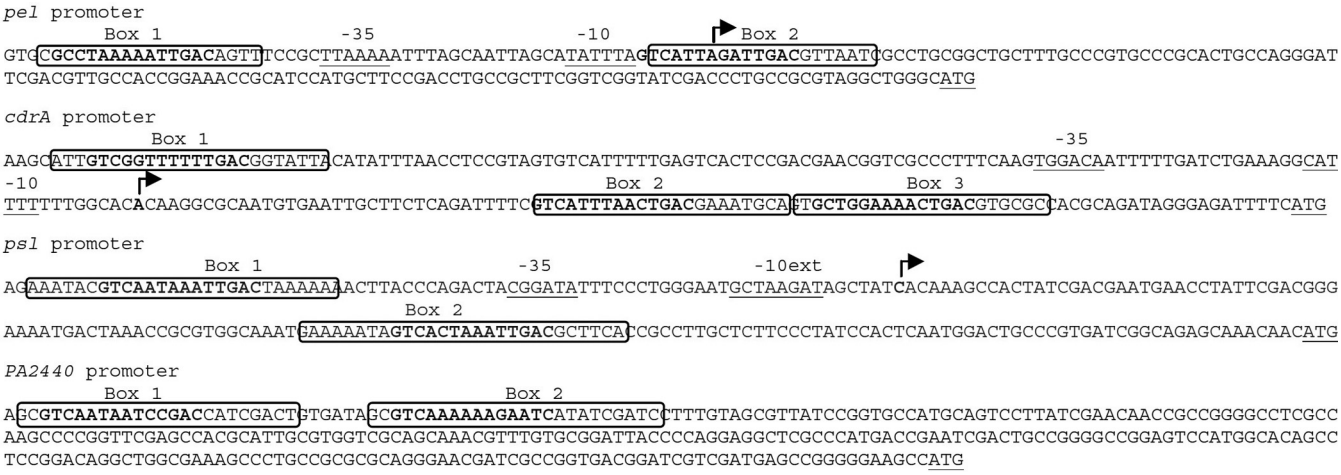

FIG 2 Nucleotide sequences of the *pel*, *cdrAB*, *psl*, and PA2440 promoter regions. The FleQ binding sites are boxed, and repeat sequences are in bold. The arrows indicating the transcription start sites were determined in this paper or previously. The translation start sites and the putative −10 and −35 elements are underlined.

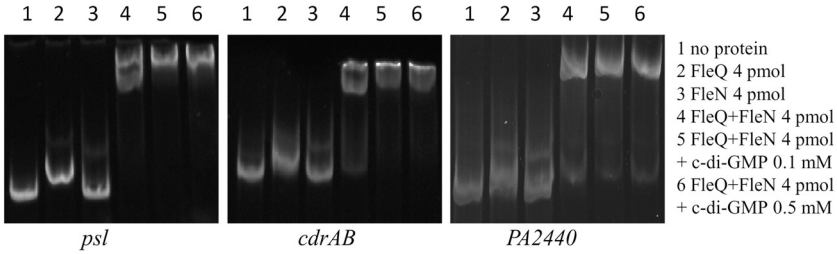

FIG 3 FleQ binds to the *psl*, *cdrA*, and PA2440 promoters. Fragments of DNA containing the *psl* (361-bp), *cdrAB* (438-bp), and PA2440 (522-bp) promoters (0.3 pmol) were incubated with or without FleQ (4 pmol), FleN (4 pmol), or c-di-GMP (0.1 or 0.5 mM). All reaction mixtures contained 50 μM ATP.

FleN has been shown to bind to FleQ but does not bind to DNA on its own (3, 22). To map the location of FleQ binding sites on these promoters, we performed footprinting experiments using 5′-FAM-labeled probes (Fig. 4). The assays shown in Fig. 4 were performed in the presence of 10 μM ATP and in the presence or absence of 500 μM c-di-GMP to allow for comparison to previously published results (3). In other experiments, we found that the properties of binding of FleQ to the DNA fragments shown in Fig. 4 were unchanged regardless of the presence or absence of ATP. ATP addition to a final concentration of 500 μM also had no effect on the DNA binding characteristics of FleQ. We tested c-di-GMP concentrations ranging from 1 μM to 1 mM in FleQ footprinting assays and found that the DNA binding properties of FleQ did not differ over this c-di-GMP concentration range. Footprinting of the *psl* operon promoter revealed two FleQ binding sites of 26 and 28 bases, centered at positions −62 and +76 relative to the previously determined transcription start site of the *psl* operon (Fig. 4A, rows 1 and 2, and Fig. 2) (23). Two FleQ binding sites were also identified upstream of the PA2440 coding region. These were centered at −294 and −263 relative to the translation start site of this gene (Fig. 4B, rows 1 and 2, and Fig. 2). DNase I footprinting with a fragment of DNA containing the *cdrAB* promoter revealed two protected regions separated by 131 bases: one of these is 24 bases and the second is 44 bases in length (Fig. 4C, rows 1 and 2, and Fig. 2). A closer look at the 44-base region revealed a repeated motif, ACTGAC, similar to the ATTGAC motif found in the FleQ binding sites in the *pel* promoter (3). Independent mutation of each of these motifs suggested that the 44-base box actually includes two FleQ binding sites, each about 22 bases in size (Fig. 4D, compare rows 1 with 2, 3 with 4, and 5 with 6). The *cdrAB* promoter thus contains three FleQ boxes centered at positions −109, +45, and +67 relative to its transcription start site (Fig. 2). The DNase I digestion profile observed between the boxes differed depending on the presence or absence of FleQ. Hyper- and hyposensitive sites appeared between FleQ boxes 1 and 2/3 when FleQ was bound. The positions of these sites are indicated by circles and triangles in the first row of Fig. 4C. This result suggests that FleQ binding induces a rearrangement of DNA, possibly DNA bending or wrapping of DNA around the FleQ protein.

FleQ stays bound to the *cdrAB*, *psl* operon, and PA2440 promoters in the presence of c-di-GMP or FleN. FleQ works in concert with another protein, FleN, which is an ATPase with a deviant Walker A motif (24, 25). FleN is required for full expression of biofilm-related genes in the presence of c-di-GMP (4), but its exact biological role in biofilm gene expression is not clear. In studies of *pel* operon transcriptional control, we found that FleN interacts with FleQ but does not bind to DNA (3). We previously showed that FleN, in conjunction with FleQ, induced a bending of *pel* operon promoter DNA (3). We hypothesized that the bending increases repression of *pel* transcription. To test if a similar phenomenon occurs at the *psl*, *cdrAB*, and PA2440 promoter regions, we performed footprinting experiments and gel shift assays with FleQ and FleN. Consistent with previous results, FleN alone did not bind to the promoter DNA (Fig. 3). When FleQ and FleN were both present, a band of much lower mobility than that observed with FleQ alone was observed in gel shifts (Fig. 3), suggesting that FleN binds to FleQ to give higher-molecular-weight complexes. When we footprinted the different promoter regions, the electropherograms were not modified when FleQ and FleN were incubated together prior to DNase I treatment (Fig. 4, compare rows 2 and 4). From this, we conclude that the FleQ-FleN complex binds to the same DNA sequences as does FleQ alone. No DNA rearrangements were observed to result from the presence of FleN at the *psl*, *cdrAB*, or PA2440 promoter region DNA. This is in contrast to what we observed at the *pel* operon promoter (3).

Addition of c-di-GMP did not dramatically change FleQ footprinting patterns at the *cdrAB*, *psl* operon, or PA2440 promoters in the presence or absence of FleN. c-di-GMP also did not change the promoter DNA mobility shifts observed in the presence of FleQ and FleN (Fig. 3 and 4, compare rows 2 with 3 and 4 with 5). From this, it appears that the FleQ-FleN complex remains on the promoters even in the presence of c-di-GMP, similarly to what we have observed at the *pelA* promoter (3). It is noteworthy that the DNA rearrangement observed when FleQ is bound to the *cdrAB* promoter is relieved in the presence of c-di-GMP, irrespective of the presence of FleN (Fig. 4C, compare rows 2 with 3 and 4 with 5).

FleQ both activates and represses *cdrAB* expression. To determine if c-di-GMP changes FleQ from a repressor to an activator of *cdrAB* expression in a manner similar to that observed for *pel* operon expression, we analyzed the *cdrAB* promoter region in more detail. We constructed transcriptional fusions of *lacZ* to *cdrAB* promoter fragments carrying wild-type or mutated versions of the FleQ boxes. We then introduced these constructs into the wild-type or *fleQ*-deletion strains with high (strains carrying pJN1120, expressing the diguanylate cyclase PA1120) or low (strains carrying pJN105, vector control) intracellular concentrations of c-di-GMP. The wild-type strain carrying a wild-type *cdrA* promoter (PcdrA-wt) had low β-galactosidase activity when c-di-GMP was low, while β-galactosidase activity was much higher when c-di-GMP was high (Fig. 5). In a strain with *fleQ* deleted, the same PcdrA-wt fusion had an intermediate level of expression in the presence or absence of c-di-GMP. These results suggest that

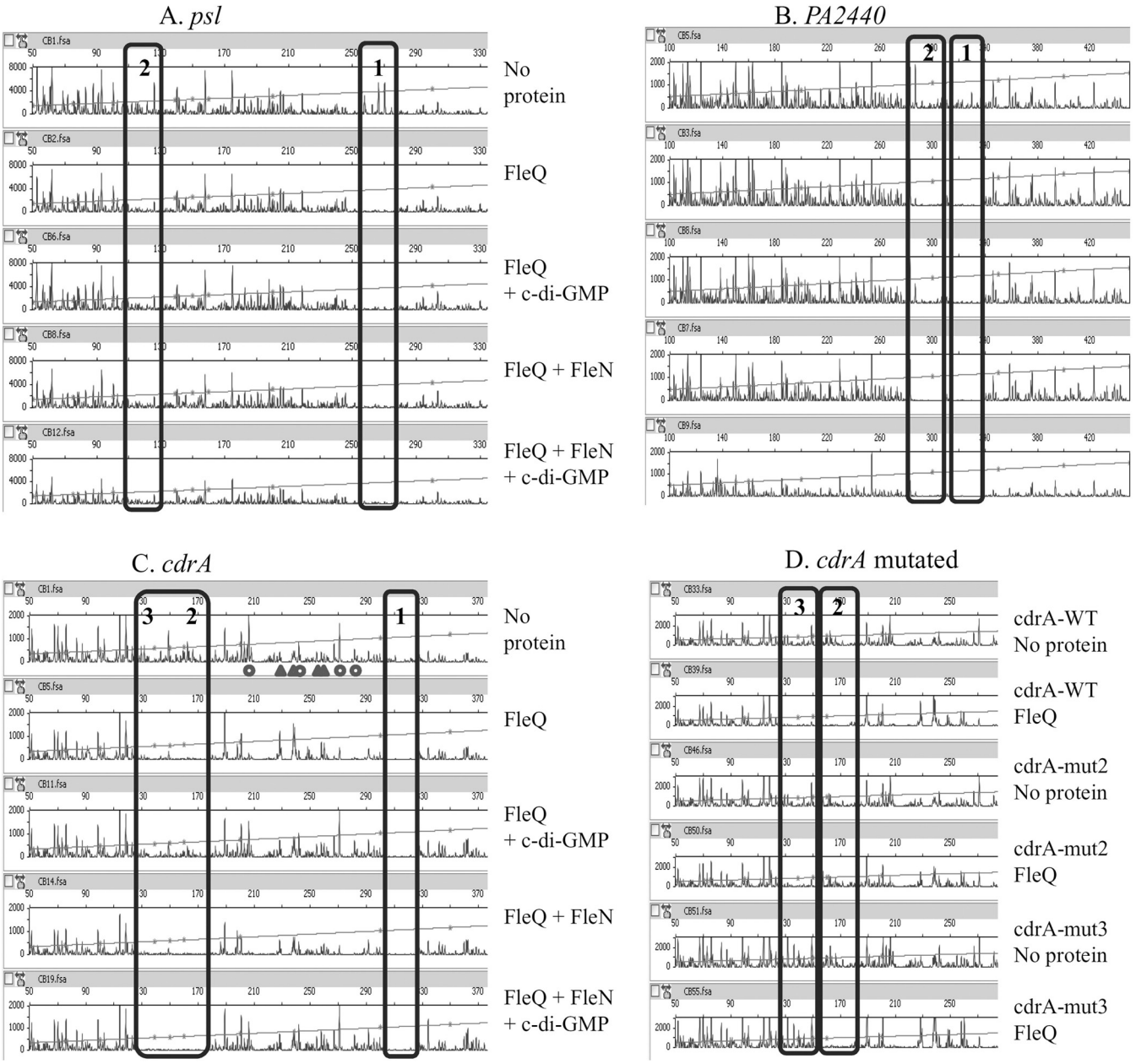

FIG 4 FleQ binding does not require FleN or c-di-GMP. FAM-labeled DNA (0.45 pmol) was incubated with or without FleQ or FleN in the presence of ATP (10 μM) and in the presence or absence of c-di-GMP (500 μM) and then submitted to DNase I treatment (0.3 U) and analyzed by capillary electrophoresis. The fluorescence intensity (arbitrary units, ordinate) is plotted against the sequence length (bases, abscissa) of the fragment relative to the first base of the FAM-labeled primer. The horizontal line indicates the GS-500 internal size standard. The FleQ binding sites are boxed. (A) *psl* DNA (594 bp) was tested with 10 pmol of FleQ or FleN. (B) PA2440 DNA (522 bp) was tested with 50 pmol of FleQ or FleN. (C) *cdrA* fragment DNA (438 bp) was tested with 50 pmol of FleQ or FleN, and the locations of major putative DNA rearrangements are indicated by triangles (hypersensitive sites) or circles (hyposensitive sites). (D) The wild-type (WT) *cdrA* DNA fragment or *cdrA* fragments mutated in box 2 or 3 were tested with 50 pmol of FleQ.

FleQ represses *cdrAB* expression in the absence of c-di-GMP and activates *cdrAB* expression in the presence of c-di-GMP. Promoter fusions carrying mutations inside FleQ box 2 or 3 had low levels of expression with or without c-di-GMP, indicating that intact box 2 or 3 is not required for repression but that the binding of FleQ to both boxes 2 and 3 is required for *cdrAB* activation. The activity of PcdrA-mut1 was high whatever the concentration of c-di-GMP, suggesting that the binding of FleQ to this box is required for *cdrAB* repression.

ATPase activity and RpoN are not required for biofilm gene expression. FleQ is an enhancer binding protein (EBP). Typically, EBPs interact with σ^{54} (RpoN) and open complex formation is driven by ATP hydrolysis catalyzed by the AAA ATPase domain of this class of protein. Expression of the *pel* or *cdrAB* operon was not affected in a strain with *rpoN* deleted (6). Also, the *cdrAB* and *pel* operon promoters contain −10 and −35 boxes instead of the −12/−24 boxes required for binding of σ^{54}. We found that the level of *pel* or *cdrAB* transcripts in wild-type or *rpoN* strains was

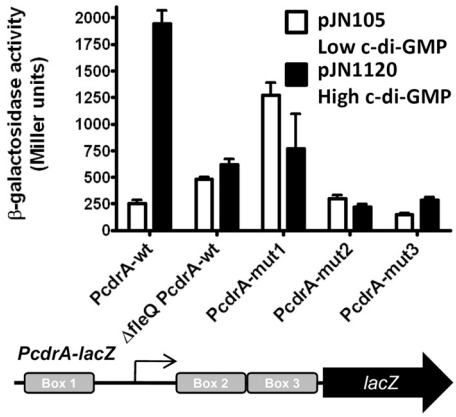

FIG 5 FleQ acts as a repressor in the absence of c-di-GMP and as an activator in the presence of c-di-GMP at the *cdrAB* promoter. β-Galactosidase activities of wild-type (wt) or mutated P*cdrA-lacZ* fusions were measured in PAO1 Δ*pel* Δ*psl* Δ*cdrAprom* (except when Δ*fleQ* is indicated, which corresponds to the PAO1 Δ*pel* Δ*psl* Δ*cdrAprom* Δ*fleQ* strain) carrying pJN105 (vector control) or pJN1120 (allowing the overexpression of the diguanylate cyclase encoded by PA1120). PcdrA-mut1 contains a mutation in FleQ box 1, PcdrA-mut2 contains a mutation in FleQ box 2, and PcdrA-mut3 contains a mutation in FleQ box 3, as indicated below the graph. The arrow indicates the position of the transcription start site of *cdrA*. Transcription of PcdrA-wt is significantly different in the *fleQ*-deleted strain than in the wild type under low c-di-GMP (*t* test, *P* value of 0.01).

B

Sequence name	P-value	Site
psl-1	4.06e-08	AAATAC GTCAATAAATTGAC TAAAAA
psl-2	2.10e-07	GAAAAATA GTCACTAAATTGAC GCTTCA
cdrA-1	5.89e-07	ATT GTCGGTTTTTTGAC GGTATTA
PA2440-1	1.37e-06	GC GTCAATAATCCGAC CATCGACT
cdrA-2	3.03e-06	. GTCATTTAACTGAC GAAATGCAG
fleS	4.69e-06	T GTCGGATGATTGAC AC
pel-2	5.85e-06	. GTCATTAGATTGAC GTTAATC
fliL	2.32e-05	CCCTTCATTT GACGCTATAGCGAC GGATTTTTGG
pel-1	3.45e-05	C GCCTAAAAATTGAC AGTT
cdrA-3	1.66e-04	T GCTGGAAAACTGAC GTGCGCC
fliE	1.87e-04	. GTCAAAAAGCCGC GGCTGTT
flhA	3.25e-04	CCGGC GTCAAAAGTTTGTT
PA2440-2	5.61e-04	AGC GTCAAAAAAGAATC ATATCGATC

FIG 7 FleQ binding consensus sequence. (A) MEME logo of the conserved sequence of FleQ binding sites. According to the MEME software, the E value of this motif is 7.9e−018. (B) Alignment and *P* values of the different FleQ boxes. The consensus sequences are centered. Gray bases were not initially identified as part of FleQ binding sites.

identical regardless of the concentration of c-di-GMP (Fig. 6). A mutation in the σ^{54} binding domain of FleQ ($FleQ_{T224S}$) did not induce a change in *pel* expression, confirming that the regulation of biofilm gene expression does not depend on σ^{54} RNA polymerase (Fig. 6) (6).

We also assessed the role of the ATPase activity of FleQ at the *pel* promoter, by introducing at the native location a mutation in the Walker B site of *fleQ* ($fleQ_{D245A}$). This mutation has previously been shown to abolish FleQ ATPase activity (12). *pel* expression was low in this mutant strain in the absence of c-di-GMP and high in the presence of c-di-GMP (Fig. 6). This result indicates that FleQ ATPase activity is not required for either *pel* repression or *pel* activation. Thus, FleQ is an unusual EBP able to both repress and activate gene expression independently of σ^{54} and ATPase activity.

Identification of a FleQ binding motif. From the FleQ footprinting data at different promoters, we were able to identify a conserved FleQ binding motif of 14 bases, GTCAATaaATTGAC (Fig. 7). We also found this motif, although in less conserved form, in previously identified FleQ binding sites in the promoter regions of several FleQ-controlled flagellar genes (Fig. 7) (11).

Conservation of the FleQ binding sites in other *Pseudomonas* species and identification of new FleQ binding sites. We examined the promoter regions of *pel*, *psl*, *cdrAB*, and PA2440 genes in other *P. aeruginosa* strains and other *Pseudomonas* species for the presence of FleQ consensus binding sequences. We found that the promoter sequences upstream of these genes are well conserved in all *P. aeruginosa* strains examined, with particularly high conservation in the FleQ consensus binding sequences (see Fig. S1A in the supplemental material). In looking at other species, we found that the region upstream of *pelA* in *Pseudomonas protegens* strains CHA0 and Pf-5 does not contain FleQ boxes. However, both strains have a gene coding for a putative epimerase (PFL_2971 and PFLCHAO_c30140) upstream of the *pelA* ortholog (see Fig. S1B). There are two putative FleQ boxes spaced 36 bases apart upstream of these genes, suggesting that the epimerase genes are the first genes in the *pel* operons in strains CHA0 and Pf-5 (see Fig. S1C). *Pseudomonas putida*, *Pseudomonas syringae*, and *Pseudomonas stutzeri* have orthologs of *psl* genes, but their promoter regions are not conserved and they do not have FleQ binding sites (data not shown).

From these observations, we can conclude that the FleQ binding sites that we determined experimentally are highly conserved in *P. aeruginosa* and can also be found in other *Pseudomonas* species, suggesting a common mechanism of regulation. We next looked for possible new FleQ target genes. Position-dependent scoring matrices, as defined by MEME, were used as input to the MAST algorithm for searching the FleQ binding sites in the upstream regions of genes. First, we examined the PAO1 genome. We found two FleQ binding consensus sequences in the promoter region of *bdlA* (PA1423) centered at positions −173 and −223 bases upstream of the *bdlA* translation start site (Fig. 8). *bdlA* codes for a chemosensory protein involved in bacterial dispersal. The phosphodiesterase DipA is activated where BldA is proteolytically processed. This then results in a net reduction of c-di-GMP

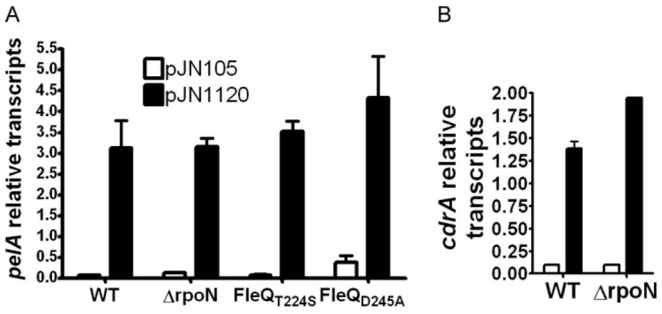

FIG 6 Neither FleQ ATPase activity nor σ^{54} is required for *pel* or *cdrAB* expression. Relative transcript levels of *pel* (A) or *cdrAB* (B) were measured by RT-PCR in the wild-type (WT) strain, a strain deleted for *rpoN*, or strains with *fleQ* mutations at the native chromosomal location. $fleQ_{T224S}$ has a mutation in the σ^{54} binding domain of FleQ. $fleQ_{D245A}$ has a mutation in the Walker B site of FleQ required for ATPase activity.

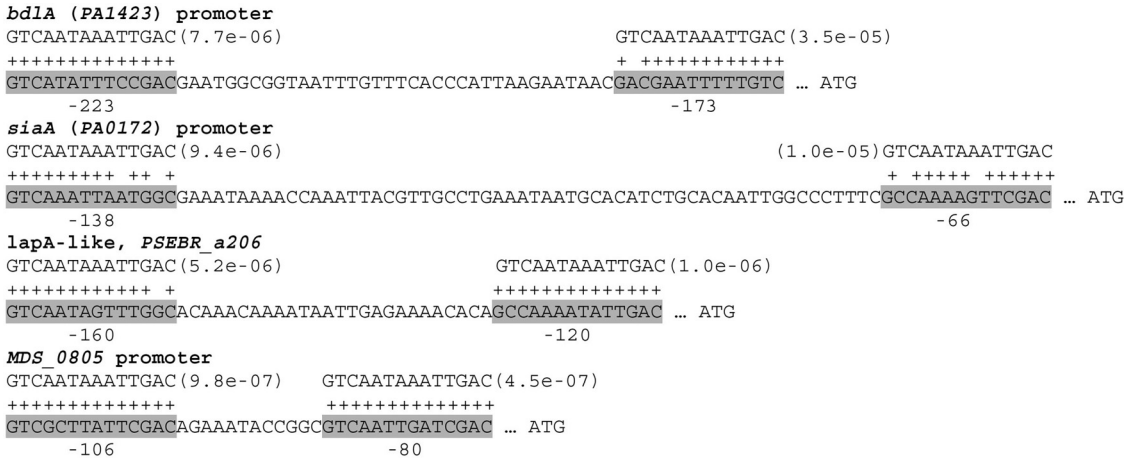

FIG 8 Promoter regions of putative FleQ-regulated genes. For each promoter region, the best possible match to the FleQ consensus binding motif and the position *P* value from the MAST algorithm are shown. Bases whose match to the motif has a positive score are indicated by a plus sign. The putative match sequences are indicated in gray, and the location of the sequences is indicated below the sequence and is relative to the translation start site.

and biofilm dispersal (26). We also identified two FleQ consensus sequences in the promoter region of *siaA* (PA0172), which is the first gene of the *siaABCD* operon involved in cell aggregation (27). The two FleQ boxes are spaced 58 bases apart and centered at positions −66 and −138 bases upstream of *siaA* ATG (Fig. 8).

When we looked for other potential FleQ binding sites among other *Pseudomonas* species, a common class of genes that emerged was *lapA*-like genes (Fig. 8). LapA is an outer membrane adhesin protein responsible for keeping cells attached to surfaces. When c-di-GMP is low, the LapD protein, which is a c-di-GMP receptor, allows LapG, a periplasmic protease, to interact with LapA and cleave the LapA N-terminal domain, inducing the release of LapA from the cell surface and promoting biofilm detachment (28–30). To illustrate the presence of putative FleQ binding sites in the promoter regions of a few *lapA*-like adhesins, an alignment of the promoter regions of these genes is shown in Fig. S2 in the supplemental material. In *Pseudomonas mendocina* NK01, the promoter region of *MDS_0805* contains two putative FleQ binding sites located at positions centered at −80 and −106 bases upstream of the translation start site (Fig. 8). *MDS_0805* codes for a response regulator-GGDEF domain protein, homologous to GcbA, which was shown to facilitate *P. aeruginosa* biofilm dispersion by activating BdlA (31). Finally, in *Pseudomonas brassicacearum* and *Pseudomonas fluorescens* F113 strains, we found homologous operons that have in their promoter regions (upstream of PSEBR_a3747 and PSF113_1970) two sequences (GTCAAGGACTTGAT and GTCA TTTTGCTGAC) that resemble the FleQ consensus binding motif (see Fig. S3 in the supplemental material). These operons appear to encode an exopolysaccharide that is distinct from Pel or Psl.

DISCUSSION

Here, we identified FleQ binding sites in the promoter regions of *cdrAB*, PA2440, and the *psl* operon. We found that FleQ and FleN bind as a complex at these sites irrespective of the presence of c-di-GMP. We also found that FleQ both represses and activates *cdrAB* expression in response to c-di-GMP, similar to our results describing *pel* operon transcription in response to FleQ (3). However, the two promoters are organized differently. In the case of *cdrAB*, FleQ binds to three sites, one site located upstream of the transcription start site and two sites located side by side downstream of the transcription start site. The upstream box is required for FleQ-mediated *cdrAB* repression in the absence of c-di-GMP, whereas the downstream boxes are required for FleQ-mediated *cdrAB* activation in the presence of c-di-GMP. In the *pel* operon, the box upstream of the transcription start site is required for activation and the FleQ box that overlaps the transcription start site is required for repression. Our model for *cdrAB* expression is that in the absence of c-di-GMP, FleQ binds to the *cdrAB* promoter and induces a distortion of DNA. This could impair RNA polymerase binding and repress *cdrAB* expression. We know that binding of c-di-GMP induces a conformational change in FleQ (3). This serves to relieve the DNA distortion even though FleQ remains bound to the *cdrAB* promoter. This may improve RNA polymerase binding, leading to activation of *cdrAB* expression. FleQ may also both repress and activate *psl* and PA2440 gene expression. However, we did not analyze the expression of these genes in response to c-di-GMP in detail. *lacZ* transcriptional fusions that we prepared to the promoter regions of these genes showed a 3-fold or less induction of expression in response to c-di-GMP.

It is unusual for transcription activator binding sites to be located downstream of transcription start sites in bacteria. A few transcription activator proteins have been shown to bind downstream of the transcription start site, but little is known concerning their mechanism of activation (32–34). In the case of regulation of bacteriophage lambda pR promoter activity by DnaA, the location of the binding site downstream of the transcription start site allows a direct interaction between DnaA and the RNA polymerase β subunit (32). Weak binding of DnaA to DNA may contribute to removal of this activator after transcription initiation (32).

FleQ interacts with σ^{54} RNA polymerase to activate flagellar gene expression. In contrast, neither σ^{54} nor the FleQ σ^{54} binding domain was required for biofilm gene expression (Fig. 6). This is in good agreement with the presence of −10/−35 boxes and the absence of −12/−24 boxes in the promoter regions of biofilm-related genes. Together, these data indicate that biofilm-related genes are probably regulated by σ^{70}. We also note, however, that *P.*

aeruginosa PAO1 encodes 24 putative RNA polymerase sigma factors, 19 of which are classified as σ^{70}-like extracytoplasmic function sigma factors (35). We cannot exclude that one of these other sigma factors participates in FleQ-mediated regulation of biofilm-related genes. A few EBPs regulate gene expression in conjunction with σ^{70}. However, in these proteins, the σ^{54} binding domain is often mutated, as in HupR and NtrC from *Rhodobacter capsulatus*, or deleted, as is the case for VpsR from *Vibrio cholerae* and TyrR from *E. coli* (36–41). As with other EBPs, FleQ has an ATPase activity (12), and this activity is required for flagellar gene expression. Here, we determined that ATP hydrolysis was not required for either FleQ-mediated repression or activation of *pel* operon expression (Fig. 6). We do not know if a mechanism exists to inhibit FleQ ATPase activity at biofilm gene promoters.

At this point, FleQ is probably the best-characterized c-di-GMP-responsive transcription factor in terms of its mechanism of action. Its homologue FlrA from *Vibrio cholerae* is also a master regulator of flagellar synthesis, and c-di-GMP inhibits its ability to activate transcription (42). FlrA and FleQ may respond to c-di-GMP similarly at flagellar gene promoters. However, FlrA does not appear to directly regulate biofilm biosynthesis genes. Transcription factors belonging to several other protein families bind c-di-GMP as an effector, and it will be interesting to explore their mechanisms of action in depth. These include VpsT from *Vibrio cholerae*, which inversely regulates biofilm matrix production and motility in response to c-di-GMP and is a member of the LuxR family of regulators (43, 44). In *Xanthomonas campestris*, c-di-GMP acts as a negative regulator of the protein Clp, impairing the binding of Clp to DNA and the induction of genes important for virulence (45). A homologous protein, Bcam1349, from *Burkholderia cenocepacia* regulates cellulose synthase (46). Both Clp and Bcam1349 are homologous to the well-studied cyclic AMP (c-AMP)-responsive regulator Crp. LtmA, an apparently new class of regulator from *Mycobacterium smegmatis*, binds c-di-GMP to positively regulate the expression of lipid transport and metabolism genes (47). Finally, BrlR, a MerR family member from *P. aeruginosa*, was recently shown to bind c-di-GMP to control gene expression (48).

The data presented here allowed the identification of a consensus sequence among the 13 FleQ binding sites characterized in this study as well as in previous studies (3, 11). This motif contains the inverted repeat GTCAAT and ATTGAC separated by two nucleotides that are mostly A or T rich (Fig. 7). The promoter regions of both biofilm- and flagellum-related genes contain this consensus sequence. Biofilm-related gene promoters contain two to three FleQ binding sites, whereas flagellar genes contain only one poorly conserved FleQ binding consensus sequence, explaining why this motif was not discovered earlier. A search of genomes for FleQ consensus binding sequences led to the identification of several possible new FleQ-regulated genes, including *bdlA*, *siaA*, and *lapA*-like genes, as well as a homologue of *gcbA* in *P. mendocina* and a new exopolysaccharide synthesis operon in *P. brassicacearum* and *P. fluorescens* F113. The promoter regions of *bdlA* and *siaA* contain two putative FleQ binding sites. It has been shown that the expression of *bdlA* and the *siaABCD* operon was induced in a *wspF* mutant (high c-di-GMP) by a factor of 2 or 2 to 5, respectively (7, 8). Expression of *bdlA* was also shown to be low in planktonic cells compared to biofilm growth conditions (49). Two putative FleQ boxes were also found in the promoter regions of different *lapA*-like genes. Recent data showing that FleQ modulates *lapA* expression in response to c-di-GMP in *P. putida* make us more confident in our prediction (50). The promoter region of the *P. mendocina gcbA* homologue contains particularly well conserved putative FleQ binding sites; however, *gcbA* expression does not seem to be regulated by c-di-GMP or *fleQ* in *P. aeruginosa* strain PAO1.

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