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FlrA Represses Transcription of the Biofilm-Associated *bpfA* Operon in *Shewanella putrefaciens*

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ABSTRACT Manipulation of biofilm formation in *Shewanella* is beneficial for application to industrial and environmental biotechnology. BpfA is an adhesin largely responsible for biofilm formation in many *Shewanella* species. However, the mechanism underlying BpfA production and the resulting biofilm remains vaguely understood. We previously described the finding that BpfA expression is enhanced by DosD, an oxygen-stimulated diguanylate cyclase, under aerobic growth. In the present work, we identify FlrA as a critical transcription regulator of the *bpfA* operon in *Shewanella putrefaciens* CN32 by transposon mutagenesis. FlrA acted as a repressor of the operon promoter by binding to two boxes overlapping the −10 and −35 sites recognized by σ^{70}. DosD regulation of the expression of the *bpfA* operon was mediated by FlrA, and cyclic diguanylic acid (c-di-GMP) abolished FlrA binding to the operon promoter. We also demonstrate that FlhG, an accessory protein for flagellum synthesis, antagonized FlrA repression of the expression of the *bpfA* operon. Collectively, this work demonstrates that FlrA acts as a central mediator in the signaling pathway from c-di-GMP to BpfA-associated biofilm formation in *S. putrefaciens* CN32.

IMPORTANCE Motility and biofilm are mutually exclusive lifestyles, shifts between which are under the strict regulation of bacteria attempting to adapt to the fluctuation of diverse environmental conditions. The FlrA protein in many bacteria is known to control motility as a master regulator of flagellum synthesis. This work elucidates its effect on biofilm formation by controlling the expression of the adhesin BpfA in *S. putrefaciens* CN32 in response to c-di-GMP. Therefore, FlrA plays a dual role in controlling motility and biofilm formation in *S. putrefaciens* CN32. The cooccurrence of *flrA*, *bpfA*, and the FlrA box in the promoter region of the *bpfA* operon in diverse *Shewanella* strains suggests that *bpfA* is a common mechanism that controls biofilm formation in this bacterial species.

KEYWORDS FlrA, *Shewanella*, biofilm, cyclic di-GMP, transcriptional factor

Biofilm formed by bacteria is a multicell architecture for adaptation to diverse niches (1). *Shewanella* can form biofilm on a variety of surfaces, such as ferric oxides, electrodes, stainless steel, and glass (2–4). This genus is also renowned for an extracellular respiration ability to reduce iron oxide, electrodes, and other extracellular electron acceptors, including heavy metal ions. Such an ability has diverse potential applications in bioengineering and bioremediation (5). Biofilm formation in *Shewanella* benefits close contact with solid electron acceptors, thereby accelerating iron oxide reduction and improving current output, as well as inducing spatially stratified metabolic responses during contaminant exposure (4, 6, 7). Biofilm formed by *Shewanella* prevents microbially induced corrosion of steel and cast iron pipes (8, 9). On the other hand, biofilm formation of *Shewanella* also causes problems in some circumstances. For

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example, *Shewanella* forms biofilm on the surfaces of food processing equipment, causing contamination and spoilage of seafood, which can be rescued by inhibiting biofilm formation (10, 11). Unfortunately, the understanding of the mechanism underlying biofilm formation in *Shewanella* has been very limited, restricting the biological control and biotechnological manipulation of its biofilm.

Biofilm-promoting factor A (BpfA) is a key protein contributing to biofilm formation in some *Shewanella* species. BpfA belongs to a subfamily of the RTX (repeats in toxins) proteins typically containing 80- to 300-amino-acid repeats ordered in tandem (12). RTX proteins are extracellular proteins whose secretion depends on the type I secretion system (TISS) and that function as adhesins or toxins. The role of RTX proteins in biofilm formation has been investigated previously in several bacteria, such as *Pseudomonas fluorescens* and *Pseudomonas putida* (13, 14), *Enterococcus faecalis* (15), and *Psychrobacter arcticus* (16). *P. putida* encodes two RTX proteins, LapA and LapF, which play sequential roles in the initial stage and a later stage of biofilm development, respectively (17). In *P. fluorescens* Pfo-1, a low level of phosphate triggers the cleavage of LapA through the cyclic diguanylic acid (c-di-GMP) signaling pathway (18). In *Shewanella oneidensis* MR-1, BpfA contributes greatly to biofilm formation (19). In *Shewanella* sp. strain HRCR-1, BpfA exists predominantly in loosely associated extracellular polymeric substances of biofilm (20). The *bpfA* operon in *Shewanella* species such as *S. oneidensis* MR-1 and *S. putrefaciens* CN32 commonly contains seven genes, including a TISS for BpfA translocation. Disruption of the TISS blocks formation of pellicle, a type of biofilm formed at the air-liquid interface which is probably caused by interruption of BpfA translocation (21). A hyper-aggregating mutant of *S. oneidensis* MR-1 with enhanced biofilm formation exhibits increased transcription of a TISS component up to about 5-fold, and inactivation of the TISS results in the loss of the hyper-aggregation phenotype (22). High O_2 tension induces autoaggregation of *S. oneidensis* MR-1 in a chemostat, and the transcription of TISS is also upregulated (23). Although these reports emphasize the importance of BpfA for biofilm formation, it is unclear how *bpfA* expression is regulated in response to environmental stimuli in these *Shewanella* species. We previously demonstrated that transcription of the *bpfA* operon in *S. putrefaciens* CN32 is increased with an increase in c-di-GMP level by a diguanylate cyclase (DosD) upon exposure to oxygen, thus promoting biofilm formation under conditions of aerobic growth (24). However, the responsive regulator that mediates the c-di-GMP regulation of the expression of the *bpfA* operon is still unidentified.

In the present work, we screened a transposon mutant library for potential regulators of the *bpfA* operon. FlrA was identified as a transcriptional repressor by binding to two boxes in the promoter of the *bpfA* operon. c-di-GMP weakened FlrA's binding to the promoter, therefore derepressing the expression of the *bpfA* operon. Transcriptional repression of *bpfA* by FlrA was also antagonized by an accessory protein of flagella, FlhG. Our work clarifies a central role of FlrA in the signaling pathway from DosD-derived c-di-GMP to BpfA-associated biofilm formation in *S. putrefaciens* CN32. Elucidation of the regulatory pathway of the *bpfA* operon by the bacterial second messenger c-di-GMP will deepen understanding of the physiology of these species under aerobic conditions.

RESULTS

FlrA represses the expression of the *bpfA* operon. Our previous work identified the adhesin BpfA as an important factor contributing to biofilm formation in *S. putrefaciens* CN32 (24). To further disclose the regulation of *bpfA* transcription, we constructed a reporter strain, 3591Z, with a transcriptional fusion of *lacZ* into the *bpfA* operon in the genome of *S. putrefaciens* CN32 and performed transposon mutagenesis to screen mutants with altered β-galactosidase activity. A screening of approximately 9,000 mutants identified four white colonies and three dark-blue ones (data not shown), which suggested decreased and increased *bpfA* transcription, respectively. The β-galactosidase activity in these mutants was further verified during batch cultivation. Mutants that showed stable and dramatically changed activities were selected to

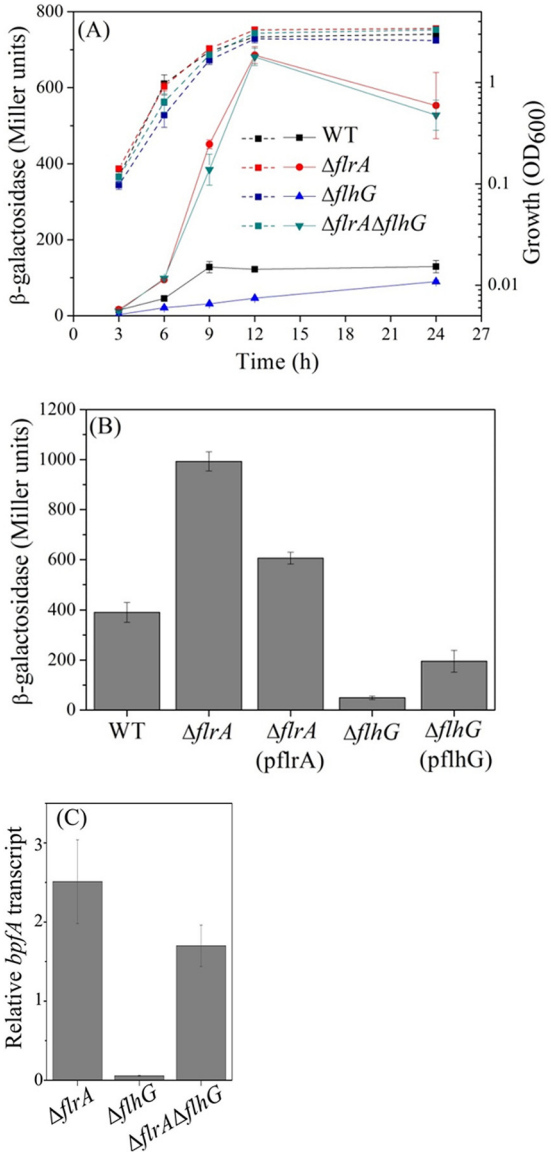


FIG 1 FlrA and FlhG regulate the expression of *bpfA*. (A) The expression of *bpfA* was reported by *lacZ* transcriptionally fused into the *bpfA* operon in the genome. The wild type (WT) (3591Z) and the 3591Z Δ*flrA*, 3591Z Δ*flhG*, and 3591Z Δ*flrA* Δ*flhG* mutants were cultured in LB aerobically. Their growth and β-galactosidase activities were measured at the indicated time points. (B) Levels of expression of *bpfA* in 3591Z Δ*flrA* and 3591Z Δ*flhG* complemented with plasmid-borne *flrA* (pflrA) and *flhG* (pflhG), respectively. The wild type and Δ*flrA* and Δ*flhG* mutants were transformed with the empty vector. To all samples, we added 0.5% L-arabinose. (C) The levels of transcription of *bpfA* in the wild type and the Δ*flrA*, Δ*flhG*, and Δ*flrA* Δ*flhG* mutants were examined and compared by qRT-PCR. RNA was isolated from cultures grown to an OD of 1.0. Error bars indicate standard deviations from three samples.

determine the site of transposon insertion. One of the insertion sites was mapped to Sputcn32_2580, which encodes FlrA, a master transcription regulator of flagellar biosynthesis in *S. oneidensis* MR-1 (25). To detect the effect of FlrA on *bpfA* transcription, we deleted *flrA* in 3591Z (3591Z Δ*flrA*). The β-galactosidase activity in the parental 3591Z strain reached maximum at late-log phase (Fig. 1A), indicating the maximum expression of *bpfA*. Deletion of *flrA* increased the activity by 4- to 7-fold, while no obvious growth defect was observed compared with the growth of the parental strain (Fig. 1A). These results suggested that FlrA acts as a repressor of the *bpfA* operon.

FlrA as a transcription factor has orthologs in *Vibrio* and *Pseudomonas* species. It can interact with an ATPase and FleN and be antagonized by the latter in *P. aeruginosa* but

not in *V. cholerae* (26, 27). Since *S. putrefaciens* CN32 encodes an FleN ortholog (FlhG), we tested whether FlhG interferes with the negative effect of FlrA on *bpfA* expression. Deletion of *flhG* (3591Z Δ*flhG*) decreased β-galactosidase activity compared with that of the parental strain. However, a deficiency of *flrA* along with an *flhG* mutation (3591Z Δ*flrA* Δ*flhG*) restored β-galactosidase activity to the high level seen with the Δ*flrA* single mutant (3591Z Δ*flrA*) (Fig. 1A). Complementation with plasmid-borne *flhG* and *flrA* largely restored the β-galactosidase activities in the corresponding mutants to the level in 3591Z (Fig. 1B). These results indicated that FlhG regulation of the expression of the *bpfA* operon was dependent on FlrA. Interestingly, β-galactosidase activity in 3591Z Δ*flrA* was fully recovered to the level in 3591Z by integrating a single copy of *flrA* into a neutral site in the genome of 3591Z Δ*flrA* (see Fig. S1 in the supplemental material). A possible explanation is that FlrA also regulates other genes but not the *bpfA* operon or flagellum cassette. Expression of FlrA in excess might alter the cross talk between the products of these genes, thereby affecting *bpfA* transcription. For example, FlrA positively regulates *flhG* transcription (Fig. S2), and hence overexpression of FlrA likely changes the ratio between FlrA and FlhG and the consequent expression of *bpfA*.

To obtain direct evidence for transcription, we used quantitative reverse transcription-PCR (qRT-PCR) to compare levels of *bpfA* transcripts in different strains. The *flrA* and *flhG* genes were deleted from the wild-type strain of *S. putrefaciens* CN32. Consistently with results based on β-galactosidase activity, the *bpfA* transcript level increased in the Δ*flrA* and the Δ*flrA* Δ*flhG* mutant but decreased in the Δ*flhG* mutant (Fig. 1C). With consideration of the above results together, FlrA and FlhG act as negative and positive regulators, respectively, for the transcription of *bpfA* in *S. putrefaciens* CN32.

FlrA and FlhG regulation of biofilm depends on BpfA. Since FlrA and FlhG are associated with *bpfA* transcription and since BpfA is required for biofilm development, we evaluated the role of FlrA and FlhG in biofilm formation. Compared to that of the wild-type strain, the biofilm formation of a strain with a deficiency of *flrA* increased and that of a strain with a deficiency of *flhG* was undermined (Fig. 2A), demonstrating the genes' opposite effects on biofilm formation. Deletion of *bpfA* along with an *flrA* mutation reduced the biofilm level to that of the Δ*bpfA* single mutant, indicating that FlrA regulation of biofilm was dependent on BpfA. Similar biofilm levels were observed for the Δ*flhG*, Δ*bpfA*, and Δ*flhG* Δ*bpfA* strains. Taken together, these results indicated that the effects of both FlrA and FlhG on biofilm formation were dependent on BpfA. The increased and reduced biofilm levels of the Δ*flrA* and Δ*flhG* single mutants, respectively, could be restored to the wild-type level by introducing plasmid-borne *flrA* or *flhG* (Fig. 2B).

FlrA functions as a repressor of the *bpfA* operon. After confirming FlrA as a regulator of biofilm formation via BpfA, we asked whether FlrA could function as a direct regulator to control the transcription of the *bpfA* operon. In enzyme-linked immunosorbent assays (EMSAs), purified recombinant FlrA (Fig. S3) was incubated with a 6-carboxyfluorescein (FAM)-labeled DNA (577 bp) spanning from bp −480 to +97, relative to the translational start site of the *bpfA* operon. Binding of FlrA to the promoter of the *bpfA* operon was evidenced by the shifts of DNA bands (Fig. 3A). The binding was outcompeted by DNA with the same sequence but without FAM labeling (Fig. 3A). The *in vitro* assay supported the idea that FlrA can directly bind to the promoter of the *bpfA* operon. Further, we tested the *in vivo* binding of FlrA to the promoter of the *bpfA* operon by chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) analysis. In a strain of CN32FlrA-FLAG expressing FlrA with an N-terminal FLAG epitope from its native site in the genome, FlrA demonstrated binding to the promoter of the *bpfA* operon (Fig. 3B). The FlrA also showed binding to the promoters of *flrA*, *fliE*, and *flrB* (Fig. 3B), all of which belong to the flagellum cassette and are under the direct regulation of FlrA. The *in vitro* and *in vivo* assays supported the finding that FlrA can directly bind to the promoter of the *bpfA* operon, confirming its simultaneous regulation of the *bpfA* operon and flagellum cassette through direct binding.

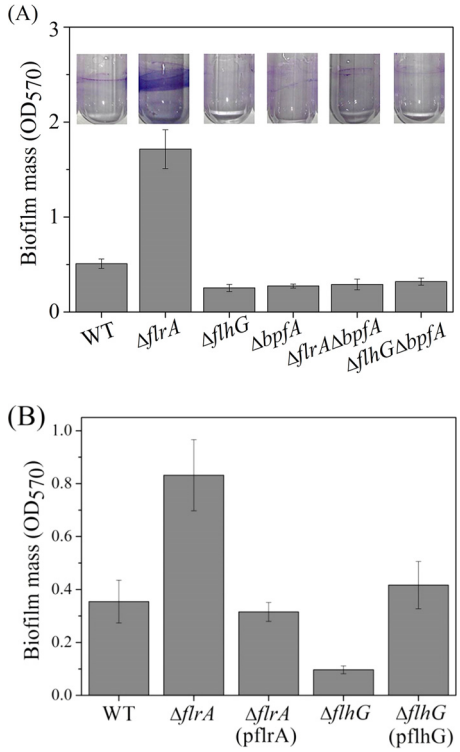


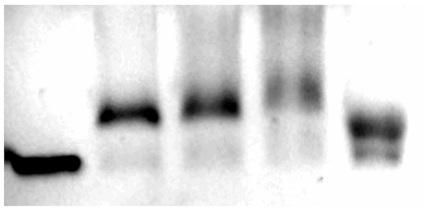
FIG 2 FlrA and FlhG regulate biofilm formation in a BpfA-dependent manner. (A) Biofilms formed by the wild type and mutants on the walls of glass tubes were quantified using a crystal violet assay after cells were grown in LB medium for 18 h. Images represent biofilms formed on glass walls. (B) Biofilms formed by the Δ*flrA* mutant and Δ*flhG* mutant complemented with *flrA* (pflrA) and *flhG* (pflhG), respectively. The wild type and Δ*flrA* and Δ*flhG* mutants were transformed with the empty vector. To all the samples, we added 0.5% L-arabinose. Error bars indicate standard deviations of results from three samples.

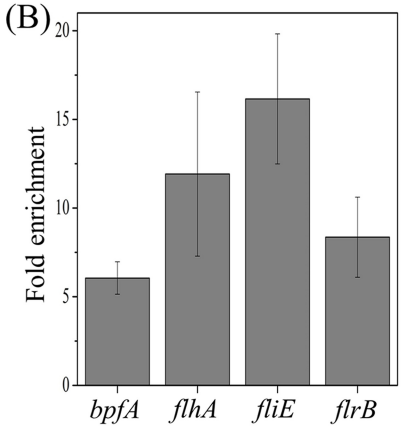
We next determined the precise site of FlrA binding to the promoter of the *bpfA* operon by performing both DNase I footprinting and primer extension analysis. When the same DNA probe as that used in an EMSA was preincubated with FlrA and then digested by DNase I, two DNA regions (box 1, GTGGAATTTTA; box 2, TCAATTTTTTGA CGC) were protected by FlrA in a concentration-dependent manner (Fig. 3C). The transcription start site (TSS) of the *bpfA* operon was determined by the primer extension assay to be bp −76 upstream of the start codon (ATG), and this was supported by two independent assays using two primers (P_{bpfA}-R1-FAM and P_{bpfA}-R2-FAM) (Fig. 3D and S4). Therefore, FlrA-binding box 1 and box 2 were located at bp −79 and −109 upstream of the TSS, respectively. Furthermore, the same region recognized by σ^{70} was independently predicted by two online software programs, BPROM and Virtual Footprint (28, 29). Predicted −35 and −10 regions (TTGAT/AGCTAAAAT) were located at bp −84 and −107, respectively, upstream of the start codon, ATG. Thus, box 1 and box 2 overlapped the −10 and −35 regions, respectively (Fig. 3D). These results supported the hypothesis that FlrA acts as a transcriptional repressor of the *bpfA* operon, presumably by competing binding sites with σ^{70}.

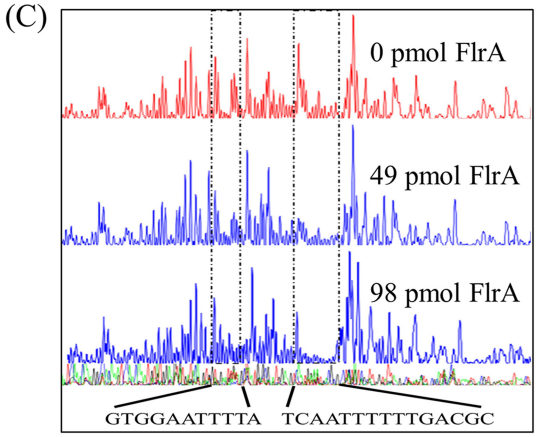
To evaluate *in vivo* the role of the two FlrA-binding boxes in the transcription of the *bpfA* operon, we constructed several reporter constructs carrying the *lacZ* gene, which is transcriptionally fused to the promoter with or without a mutation in the two boxes. The mutation sites are outside the −10 and −35 regions but still inside box 1 and box 2, avoiding interference with the DNA binding of σ^{70} for transcription initiation (Fig. 3D). The activity of β-galactosidase was measured to evaluate the strengths of promoters with a mutation in these boxes. Mutations in both box 1 and box 2 increased the transcription activity of the promoter compared to that of the wild-type promoter (Fig. 4). Thus, a full activity of FlrA to repress the transcription of *bpfA* involves both of the binding sites of box 1 and box 2.

(A)

FlrA (μM)	0	5	10	15	10
labeled probe (nM)	5	5	5	5	5
unlabeled probe (μM)	0	0	0	0	5







(D)

GCACGCATTCAAGAGCAATTAGGTGAACAGGGTTACGCCAACCAAAGCTATCGTCAAGCTGCCAATCTGTAATACCAATAACG

P_{bpfA}-F

ACATCGATAACGCCATAATACCCAAATTATGGCGTTCCCTAGCTCCTTATCCTTCCTCATATCCGTAGTTAAAGCCCCAAGTATTT

TGCATTCATAAAAATTTAATCCTCAGTTAAAGATCTATTCGCTATATAAAAGCTTACATTTATAAAACAAACACACTAAATGCAG

TTGTTTTTACAAATAAACATACAACATCTAGTTTTATACAAAACCAGCGTCAATTTTACAGGTAATGCGCAAGAAAAAGCCTTA

BOX 2 BOX 1

ATACCAAGCGATAGAGGCGCGTCAAAAAA*TTGACT*TCCATTCGCTCATT*AGCTAAAAT*TCCACCACTTGTATCACACTTCCATAC

TATGAC GAACA

ATTTGTTGGATTTGTGGGCCTTTGTTTGGATTAACACTTGGGTGTTGGGAGCTAATT**ATG**GGATCGGTCATTACATCAAAAAA

AGGTTTGCTAAAGTTAGTTAAAGGGCAAATCAATATCGAAGTTGATGGTACAAATCAACCCGCTAAGGATGGCGAGCAACTCC

P_{bpfA}-R1-FAM

CGAAAGGTGCAGTCCTCCAT

P_{bpfA}-R2-FAM

FIG 3 FlrA binds to two boxes in the promoter region of the *bpfA* operon. (A) FlrA binds to DNA fragments of the operon promoter in EMSAs. The 5′-FAM-labeled DNA was incubated with recombinant FlrA in increasing concentrations. Unlabeled DNA with the same sequence was used for competition. (B) ChIP-qPCR analysis of FlrA binding to different promoters. DNA fragments of promoters of *bpfA*, *flhA*, and *fliE* pulled down by FlrA were quantified using qRT-PCR. Fold enrichment was calculated as the *CT* of the ChIP samples relative to the *CT* of nonantibody samples using the formula $2^{-(CT\text{ChIP} - CT\text{Negative})}$. Error bars indicate standard deviations from three samples. (C) Determination of FlrA binding sites in the DNase I footprinting assay. FAM-labeled DNA that was the same as that used in EMSAs was incubated with FlrA in various amounts. Electropherograms indicate the protection pattern of DNA in the absence and presence of FlrA in the indicated concentrations. The protected DNA determined by sequencing is also shown. (D) Nucleotide sequence of the promoter region of the *bpfA* operon. The transcriptional start site, indicated by an asterisk, was determined by the primer extension assay using two primers (PE-bpfA-R1, PE-bpfA-R2) in independent assays. The sequences of two FlrA boxes are shown shaded. Sequences of the putative −10 and −35 regions are shown in italics. The translation start site, ATG, is in bold. Mutations introduced into FlrA boxes are underscored, and nucleotides after the substitutions are also indicated by arrows.

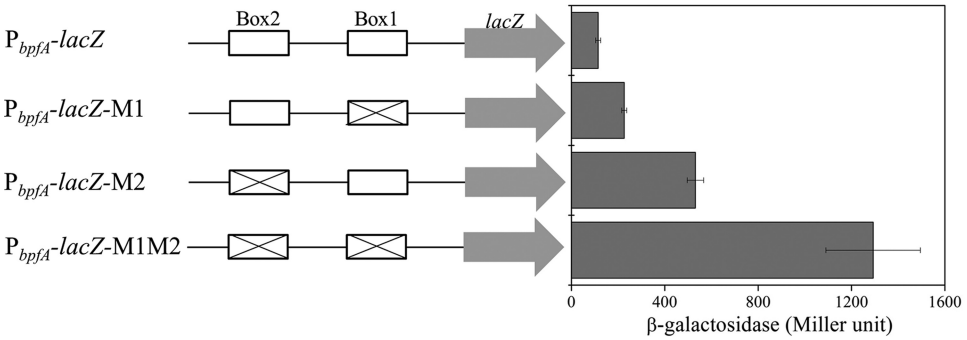


FIG 4 The two FlrA boxes are essential for FlrA repression of *bpfA* expression. Plasmids containing the promoter of the wild type (P_{bpfA}-*lacZ*) or promoters with a mutation in FlrA box 1 (P_{bpfA}-*lacZ*-M1), box 2 (P_{bpfA}-*lacZ*-M2), or both (P_{bpfA}-*lacZ*-M1M2) were introduced into the wild-type strain. The β-galactosidase activities of overnight cultures were measured to evaluate promoter strength. Error bars indicate standard deviations of results from three samples.

c-di-GMP interferes with the binding of FlrA to the promoter of *bpfA*. FleQ, an FlrA ortholog in *Pseudomonas aeruginosa*, functions as a c-di-GMP-responsive regulator, and its residues involved in the biding of c-di-GMP have been characterized (30). FlrA also possesses these conserved residues (Fig. S5) (30), which suggests that the activity of FlrA may be tuned by c-di-GMP, a second messenger found in many bacteria (31). We first tested the effect of c-di-GMP on the binding of FlrA to the FlrA box ib *bpfA* promoter region *in vitro* via fluorescence polarization (FP). When the FAM-labeled DNA of the promoter containing box 2 was incubated with FlrA, fluorescence anisotropy of the DNA increased with the increase of FlrA's concentration (Fig. 5A), indicating the binding of FlrA to the labeled DNA. The binding was outcompeted by an unlabeled DNA with the same sequence but was almost not affected by a DNA with a mutation in box 2, as observed before (Fig. 5B). Moreover, binding of FlrA to the labeled DNA was almost completely abolished in the presence of c-di-GMP (Fig. 5B). This *in vitro* data supported the hypothesis that a small molecule of c-di-GMP inactivated the binding of FlrA to the promoter of the *bpfA* operon.

We have identified DosD as a diguanylate cyclase for c-di-GMP synthesis in *S. putrefaciens* CN32. Deletion of DosD results in a level of intracellular c-di-GMP that is decreased by approximately 50% under aerobic growth conditions (24). A decreased level of intracellular c-di-GMP is expected to increase the transcriptional repression of the *bpfA* operon by FlrA, thus reducing the expression of the *bpfA* operon. Indeed, deletion of *dosD* to a lower intracellular c-di-GMP level significantly reduced the transcriptional activity of the *bpfA* promoter, as indicated by expression of *lacZ* fused to the promoter (Fig. 6). Deletion of *flrA* along with deletion of *dosD* increased the promoter activity to a level comparable to that in the Δ*flrA* mutant (Fig. 6). These results indicated that the effect of c-di-GMP derived from DosD on *bpfA* transcription depends on FlrA. Taking these results together, we concluded that the regulation of FlrA on the transcription of the *bpfA* operon is modulated by c-di-GMP.

DISCUSSION

The present work reveals FlrA as a c-di-GMP-responsive regulator of *S. putrefaciens* CN32 that controls the expression of an operon encoding the adhesin BpfA and its accessory system, thereby regulating biofilm formation (Fig. 7). FlrA acts as a repressor of the *bpfA* operon by binding to two boxes in the promoter region of the *bpfA* operon, and the binding is abolished by c-di-GMP. c-di-GMP is a key messenger controlling biofilm formation in many bacteria, including *Shewanella* (32). Increasing the c-di-GMP level in *S. oneidensis* MR-1 by deletion of *pdeB*, encoding a c-di-GMP phosphodiesterase, decreases cell motility and increases biofilm formation (33). Many *Shewanella* species, such as *S. oneidensis* MR-1, encode a large number of putative proteins with conserved GGEDF and/or EAL domains, presumably involved in c-di-GMP synthesis, hydrolysis, or

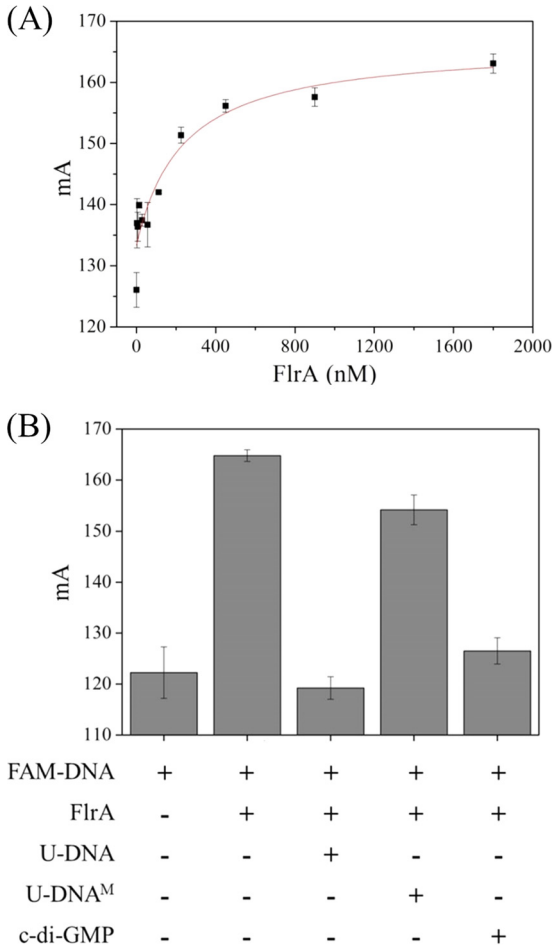


FIG 5 c-di-GMP represses the binding of FlrA to the promoter of the *bpfA* operon as determined by fluorescence polarization. (A) The binding of FlrA to FAM-labeled DNA covering box 2 of the promoter region was determined by fluorescence polarization. FAM-labeled DNA (15 nM) was incubated with FlrA at various concentrations. mA, mili-anisotropy value. (B) The binding of FlrA (450 nM) to this FAM-labeled DNA (FAM-DNA; 15 nM) was abolished by 25 μM c-di-GMP. The binding was outcompeted by unlabeled DNA with the same sequence (U-DNA) but not by unlabeled DNA with a mutation in box 2 (U-DNA^M). Error bars indicate standard deviations from three samples.

binding as effectors (34), suggesting that c-di-GMP might play a role in the physiology and niche adaptation of these species. However, the activities of most of these putative proteins have not been examined. We previously revealed that BpfA plays an essential role in the aerobic biofilm formation of *S. putrefaciens* CN32, which is regulated by the diguanylate cyclase DosD, whose activity is controlled by oxygen (24). However, there had been a missing component in this c-di-GMP signaling pathway which regulates BpfA-dependent biofilm formation, that is, the factor that mediates c-di-GMP's regulation of the transcription of the *bpfA* operon. In the present work, we identify FlrA as a responsive regulator connecting DosD with the *bpfA* operon.

Shewanellae are phylogenetically closer to *Vibrio* than to *Pseudomonas*. However, the regulatory mode of FlrA in *S. putrefaciens* CN32 is similar to that of its ortholog in *P. aeruginosa*, FleQ, but different from that of its ortholog in *V. cholerae*, FlrA. In *V. cholerae*, the FlrA regulon has been limited in flagellum-coding genes to date, and the FlrA regulation of flagellum synthesis seems unaffected by FlhG (26). The deficiency of FlrA in *Vibrio* results in the altered expression of some virulence genes and binding to epithelial cells, while it is still unclear whether FlrA directly regulates the transcription of these genes (35, 36). In contrast, FleQ in *P. aeruginosa* regulates many biofilm-related genes, such as *pel* and *psl* for exopolysaccharide synthesis and

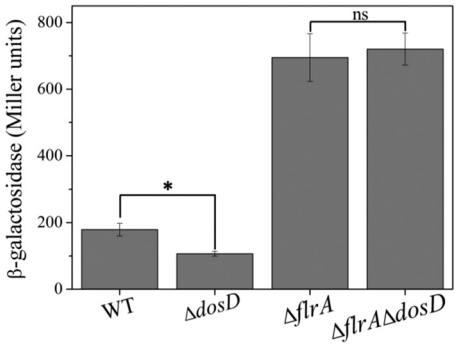


FIG 6 The regulation of DosD on the expression of the *bpfA* operon was dependent on FlrA. The β-galactosidase activities in overnight cultures of the wild type (3591Z), 3591Z Δ*dosD*, 3591Z Δ*flrA*, and 3591Z Δ*flrA* Δ*dosD* were measured to evaluate the expression of the *bpfA* operon. Error bars indicate standard deviations from three samples. *, significantly different [$P < 0.05$]; ns, not significantly different.

cdrAB, coding for an adhesin. Moreover, FleQ's regulation of the *pel* operon's transcription is antagonized by FleN, an FlhG ortholog (37). As with these characteristics of FleQ, FlrA in *S. putrefaciens* CN32 controls biofilm formation through regulating transcription of the *bpfA* operon, which is also antagonized by FlhG (Fig. 7). Our present results suggest a potential interaction between FlrA and FlhG to control the transcription of the *bpfA* operon; however, we still cannot exclude the possibility that other mechanisms may be involved. It has been reported that FlhG affects the number and location of flagella not as a result of FlrA but through direct interaction with flagellar C-ring proteins (38).

Despite its similarity to FleQ in *Pseudomonas*, FlrA of *S. putrefaciens* has special features in its regulatory mode. FleQ functions both as a repressor and as an activator in the expression of several biofilm-related genes in *P. aeruginosa* in response to the c-di-GMP level. It represses the transcription of the *pel* and *cdr* operons by binding to two or three boxes around −35/−10 regions under conditions of low c-di-GMP levels, while activating their transcription with an increase of the intracellular c-di-GMP level (39). In contrast, FlrA of *S. putrefaciens* CN32 binds to two boxes overlapping the −10 and −35 regions in the promoter of *bpfA*, presumably by repressing the binding of σ^{70} and subsequent initiation of the transcription of the *bpfA* operon. Therefore, the binding site of FlrA to the *bpfA* promoter indicates that FlrA functions as a repressor only of the *bpfA* operon. Moreover, the activity of FlrA as a transcriptional repressor can be tuned by c-di-GMP, since c-di-GMP abolished the binding of FlrA to the promoter of the *bpfA* operon.

Orthologs of FlrA in *Pseudomonas* and *Vibrio* species are master regulators of

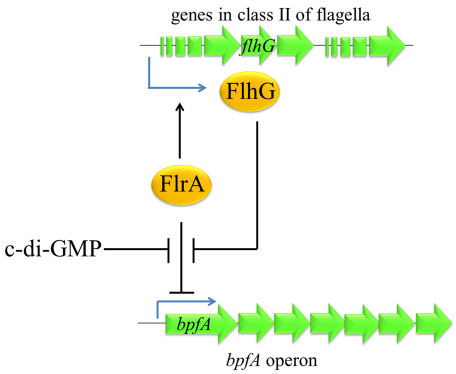


FIG 7 Schematic of the proposed regulation of FlrA on the expression of the *bpfA* operon. The repression of FlrA on the transcription of the *bpfA* operon is inhibited by c-di-GMP and FlhG. FlrA is also the master regulator of flagellum synthesis.

flagellum synthesis (35, 40). Thus, FlrA is supposed to be involved in flagellum synthesis in *S. putrefaciens* CN32. Indeed, the transcription of several flagellar genes and the phenotype of cell motility decreased in the *flrA*-deficient mutant (see Fig. S2 in the supplemental material). Biofilm and motility, representing sessile and planktonic states, respectively, are mutually exclusive lifestyles of bacteria in response to the fluctuation of diverse environmental conditions, such as nutrients and stress (1). This work reveals a coordination of biofilm and motility by FlrA in *S. putrefaciens* CN32. Interestingly, *flrA* and *bpfA* cooccur in the genomes of many *Shewanella* species (Fig. S6A and B). Moreover, two FlrA boxes are also found in the promoter regions of the genes coding for BpfA orthologs. Sequences of one box are highly conserved, and those of another box are relatively varied (Fig. S6C). These results suggest that FlrA regulation of *bpfA*-dependent biofilms is a common mechanism in these bacteria. However, the distribution of *dosD* is not as wide as those of FlrA and BpfA (24). In *Shewanella* species containing FlrA and BpfA but not DosD, other genes coding enzymes for c-di-GMP synthesis or hydrolysis are expected to be involved in tuning FlrA activity and consequent *bpfA* transcription.

The *bpfA* operon is the first target of FlrA identified to date in *Shewanella* species, except for genes involved in flagellum synthesis. Further investigation into the FlrA regulon is under way to comprehensively understand its regulatory mechanism and consequent physiological role in this bacterial species.

MATERIALS AND METHODS

Strains and growth conditions. *S. putrefaciens* CN32 and *Escherichia coli* strains (Table 1) were cultured in Luria broth (LB) medium at 30°C and 37°C, respectively. Chemicals were added when needed at the following concentrations: 100 μg ml^{-1} diaminopimelic acid, 50 μg ml^{-1} kanamycin, 34 μg ml^{-1} chloramphenicol, and 20 μg ml^{-1} gentamicin for *E. coli* strains and 10 μg ml^{-1} chloramphenicol and gentamicin for *S. putrefaciens* strains.

Strain construction. Mutants with an in-frame deletion of the desired genes were constructed as described previously (24). Briefly, two flanking regions of a target gene were amplified, ligated, and inserted into pRE112. The generated plasmids were introduced into strains of *S. putrefaciens* CN32 by conjugation with *E. coli* WM3064. After a two-step selection, mutants with a deletion of a target gene were obtained, with the DNA sequence at mutation sites being verified by sequencing.

The *S. putrefaciens* CN32 derivative of 3591Z containing *lacZ* transcriptionally fused to the *bpfA* operon in the genome (P_{bpfA}-*lacZ* fusion) was constructed to report the transcription from the *bpfA* operon promoter *in vivo*. The open reading frame of *lacZ* from *E. coli* MG1655 was cloned into the pRE112 vector, flanked by the promoter and the coding region of *bpfA*. *S. putrefaciens* was transformed with the generated plasmid. After a two-step selection, we obtained the 3591Z strain with a *lacZ* insertion into the genome, and it was under the control of the *bpfA* operon promoter. The *flrA* and *flhG* genes were deleted from 3591Z, generating 3591Z Δ*flrA*, 3591Z Δ*flhG*, and 3591Z Δ*flrA* Δ*flhG*.

To construct a strain of CN32FLAG-FlrA expressing FlrA with an N-terminal tag of FLAG, the suicide vector pRE-flag-flrA was introduced into the wild-type strain *S. putrefaciens* CN32 by conjugation. After a two-step selection and sequencing procedure, we obtained CN32FLAG-FlrA expressing FLAG-FlrA from its native transcription and translation initiation signals. All strains and vectors used in this study are listed in Table 1.

Transposon mutagenesis and screening. To identify factors that regulate *bpfA* expression, transposon mutagenesis was performed using 3591Z as a parental strain. The suicide plasmid pRL27, carrying a transposase, mini-Tn*5* transposon, and an RK6 origin (41), was introduced into 3591Z by conjugation. The resultant cells were then plated on LB agar plates containing kanamycin and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) to select mutants containing the mini-Tn*5* transposon. After incubation at 30°C for 24 to 48 h, white or dark-blue colonies were inoculated into LB for analysis of bacterial growth and β-galactosidase activity according to the method developed by Miller (42).

Mutants with increased β-galactosidase activity, compared with that of the parental strain 3591Z, were selected to map the insertion site of a transposon according to a modified nested semiarbitrary PCR method (43). Briefly, during the first round of PCR, genomic DNA was amplified using two primers, with one (pRL27Etdx or pRL27Extsx) hybridizing to one end of mini-Tn*5* and the other (a mixture of five degenerate primers, arb1, arb2, arb3, arb4, and arb5) hybridizing to an arbitrary sequence with a 5′-GC clamp. One microliter of the product was subjected to a second round of PCR using the pRL27Intdx/pRL27Intsx primer pair and arb6, with the same sequence as the 5′-GC clamp. The second-round product was purified and sequenced to determine the insertion site of a transposon. Primers used are listed in Table 2.

RNA isolation and qRT-PCR. Total RNA was isolated from *S. putrefaciens* cultures grown to an optical density at 600 nm (OD_{600}) of 1.0 using RNAiso Plus (TaKaRa Co., China) according to the manufacturer's instructions. Total RNA was treated with DNase I to digest genomic DNA and subjected to PCR analysis to ensure the complete digestion of the DNA. DNA-free RNA was then used for the reverse transcription to synthesize cDNA using the PrimeScript RT reagent kit (TaKaRa Co., China). Quantitative reverse

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
Strains		
S. putrefaciens		
CN32	Wild type	ATCC
3591Z	CN32 derivative with *lacZ* transcriptionally fused into the *bpfA* operon	This study
CN32FLAG-FlrA	CN32 derivative with FLAG translationally fused into the 5′ end of *flrA*	This study
3591Z Δ*flrA*	3591Z derivative with deletion of Sputcn32_2580	This study
3591Z Δ*flhG*	3591Z derivative with deletion of Sputcn32_2560 and *flhG*	This study
3591Z Δ*flrA* Δ*flhG*	3591Z derivative with double deletion of *flrA* and *flhG*	This study
CN32 Δ*flrA*	CN32 derivative with deletion of *flrA*	This study
CN32 Δ*flhG*	CN32 derivative with deletion of *flhG*	This study
CN32 Δ*flrA* Δ*flhG*	CN32 derivative with double deletion of *flrA* and *flhG*	This study
CN32 Δ*bpfA*	CN32 derivative with deletion of Sputcn32_3591	24
CN32 Δ*flrA* Δ*bpfA*	CN32 derivative with double deletion of *flrA* and *bpfA*	This study
CN32 Δ*flhG* Δ*bpfA*	CN32 derivative with double deletion of *flhG* and *bpfA*	This study
E. coli		
JM109	General cloning host for plasmid manipulation	New England Biolabs
MG1655	Derivative of *E. coli* K-12	ATCC
WM3064	Auxotrophic to DAP; used for conjugation	48
BL21(DE3)	Expression host carrying the T7 RNA polymerase	Novagen
Plasmids		
pRL27	Tn*5* delivery vector, Km^r	41
pRE112	Suicide vector, Cm^r	49
pRE-flrA	pRE112 derivative to delete *flrA*	This study
pRE-flhG	pRE112 derivative to delete *flhG*	This study
pRE-P$_{bpfA}$-*lacZ*	pRE112 derivative to construct 3591Z	This study
pRE-flag-flrA	pRE112 derivative to construct CN32FLAG-FlrA	This study
pET-28a(+)	Bacterial expression vector	Novagen
pET-FlrA	pET-28a(+) derivative to express His-tagged FlrA	This study
pJN105	Broad-host-range vector carrying the *araBAD* promoter	50
pflrA	pJN105 derivative to express FlrA	This study
pflhG	pJN105 derivative to express FlhG	This study
pBBR1MCS-5	Broad-host-range plasmid	51
P$_{bpfA}$-*lacZ*	pBBR1MCS5 derivative bearing the wild-type *bpfA* promoter fused with *lacZ*	This study
P$_{bpfA}$-*lacZ*-M1	P$_{bpfA}$-*lacZ* derivative with a mutation in FlrA box 1	This study
P$_{bpfA}$-*lacZ*-M2	P$_{bpfA}$-*lacZ* derivative with a mutation in FlrA box 2	This study
P$_{bpfA}$-*lacZ*-M1M2	P$_{bpfA}$-*lacZ* derivative with a mutation in FlrA box 1 and box 2	This study

[a]DAP, diaminopimelic acid.

transcription-PCR (qRT-PCR) was performed with the StepOne real-time PCR system (Applied Biosystems, USA) using synthesized cDNA and the SYBR Premix *Ex Taq* kit (TaKaRa Co., China). Relative expression values of target genes were obtained from three determinations, with normalization against 16S rRNA using the method of $2^{-\Delta\Delta CT}$, where *CT* is the threshold cycle.

TABLE 2 Primers used in this study

Application(s) and primer	DNA sequence
Transposon mutagenesis	
pRL27Etdx	CCAGAAAGTGAGGGAGCCA
pRL27Extsx	GACAACAAGCCAGGGATG
pRL27Intdx	GAGTCGACCTGCAGGCATGC
pRL27Intsx	CGCACTGAGAAGCCCTTAGAGC
arb1	GGCCACGCGTCGACTAGTACNNNNNNNNNNAGAG
arb2	GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCC
arb3	GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT
arb4	GGCCACGCGTCGACTAGTACNNNNNNNNNNAACGC
arb5	GGCCACGCGTCGACTAGTACNNNNNNNNNNTCGAC
arb6	GGCCACGCGTCGACTAGTAC
EMSA, footprinting, and primer extension	
P$_{bpfA}$-F	GCACGCATTCAAGAGCAATTAG
P$_{bpfA}$-R1-FAM	FAM-ACAAATCAACCCGCTAAGGATG
P$_{bpfA}$-R2-FAM	FAM-ATGGAGGACTGCACCTTTCG

Biofilm formation and quantification. The biomass of biofilm was quantified as described before (24). Overnight cultures were inoculated into fresh LB to an OD_{600} of 0.01 in glass tubes and incubated at 30°C for 18 h with shaking at 100 rpm. Biofilm formed on the tube walls was stained using 0.5% crystal violet for 15 min at room temperature, rinsed with 10 mM phosphate-buffered saline (PBS), pH 7.2, and air dried. Absorbed crystal violet was dissolved using 33% (vol/vol) glacial acetic acid. The absorption of dissolved crystal violet at 570 nm was determined for quantification of biofilm mass.

FlrA production and purification. FlrA was cloned into the pET-28a(+) vector (Novagen, USA) between NcoI and XhoI sites, and the resulting plasmid, pET-flrA, was sequenced to verify the *flrA* sequence. FlrA with an N-terminal 6×His tag was expressed in *E. coli* BL21(DE3) and purified. In detail, *E. coli* BL21(DE3) carrying pET-flrA was subcultured from an overnight culture into fresh LB and grown at 37°C with shaking at 200 rpm. After growth to an OD_{600} of 0.6 to 0.8, the culture was transferred to 16°C for 30 min before addition of 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) to induce the expression of FlrA. After induction for 12 h, cells were harvested, resuspended in a lysis buffer (50 mM HEPES [pH 8.0], 250 mM NaCl, 0.1% Triton X-100, and 10% glycerol), and lysed by sonication. The supernatant after centrifugation was loaded onto a 1.0-ml preequilibrated nitrilotriacetic acid (Ni-NTA; Qiagen, USA) and sequentially washed with wash buffer (50 mM Tris-HCl [pH 9.0], 300 mM KCl, and 10% glycerol) containing imidazole at concentrations of 10, 20, 50, and 100 mM. The FlrA was eluted with elution buffer (50 mM HEPES [pH 8.0], 250 mM NaCl, 0.1% Triton X-100, 250 mM imidazole, and 10% glycerol), dialyzed against storage buffer (50 mM HEPES [pH 8.0], 250 mM NaCl, 0.1% Triton X-100, 50% glycerol) at 4°C overnight, and stored at −70°C until use.

EMSA. Electrophoretic mobility shift assay (EMSA) was performed to test the binding of FlrA to DNA fragments of the *bpfA* operon promoter *in vitro*. The promoter probe of the *bpfA* operon was amplified using the P_{bpfA}-F and P_{bpfA}-R1-FAM primer pair with 6-carboxyfluorescein (FAM) labeling at its 5′ end (Table 2). This probe covered the intergenic region between *bpfA* and its upstream gene Sputcn_3590 from bp −97 to +480, relative to the *bpfA* translational start site. In detail, the probe of 0.1 pmol (40 ng) was incubated with various amounts of FlrA in a reaction buffer [50 mM Tris-HCl (pH 8.0), 100 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 0.01 mM ATP, 25 ng poly(dI-dC), and 10% glycerol] in a total volume of 20 μl. Besides poly(dI-dC), sheared salmon sperm DNA (100 ng μl^{-1}) was added as a nonspecific competitor. After incubation at 25°C for 30 min, the mixture was loaded into a 2% agarose gel for electrophoresis in a 1× TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA). Gels were scanned with an ImageQuant LAS 4000 mini (GE Healthcare, USA).

DNase I footprinting assay. To determine the binding sites of FlrA in the promoter of the *bpfA* operon, DNase I footprinting was performed as described previously, with modification (44, 45). In detail, 1.5 pmol (600 ng) of the same FAM-labeled probe as used in the EMSA was incubated with various amounts of FlrA in a total volume of 40 μl. The mixture was preincubated at 25°C for 30 min for the binding between DNA and FlrA. Then, 0.015 unit DNase I (Promega, USA) was added and the mixture was incubated for 1 min for DNA digestion; then the reaction was immediately stopped by addition of 140 μl of a DNase I stop solution (200 mM unbuffered sodium acetate, 30 mM EDTA, and 0.15% SDS). The DNA in the mixture was extracted with phenol-chloroform, precipitated with ethanol, and finally dissolved in 30 μl of MilliQ water. Preparation of a DNA ladder, electrophoresis, and data analysis were the same as described before, except that the GeneScan-LIZ500 size standard (Applied Biosystems, USA) was used.

Primer extension analysis. The transcriptional start site of the *bpfA* operon was determined using the fluorescence-based primer extension method described previously, with modification (46). Briefly, total RNA was isolated from cultures of the *S. putrefaciens* CN32 wild-type strain grown to an OD_{600} of 1.0. The cDNA was synthesized using the Primer Extension System (Promega, USA) according to the manufacturer's instructions. Total RNA of 10 μg was annealed with 5′-FAM-labeled primers (P_{bpfA}-R1-FAM or P_{bpfA}-R2-FAM) (Table 2). The resulting cDNA was precipitated with 2.5 volumes of 95% ethanol and 0.1 volume of 3 M sodium acetate, washed with 75% ethanol, air dried, and dissolved in 15 μl of MilliQ water. The size of the cDNA was determined by capillary electrophoresis using an Applied Biosystems 3730 DNA analyzer, with a GeneScan-LIZ500 size standard (Applied Biosystems, USA) as a molecular weight ladder. The transcription start site was then analyzed based on the sequences of the first-strand synthesis primers and the cDNA size mapping to the *bpfA* promoter.

ChIP and qRT-PCR analysis. Mid-exponential-phase culture (OD_{600} of 0.6) of 20 ml was treated with 1% formaldehyde in PBS, pH 7.4, at room temperature for 30 min. Cross-linking was quenched with 250 mM glycine at room temperature for 10 min. Cells were washed three times with PBS, resuspended in 500 μl of a lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 0.5% Triton X-100, 0.5 mM DTT, and protease inhibitor cocktail from Sigma), and lysed by sonication with the SCIENTZ08 ultrasonicator (Scientz, China). The lysate was centrifuged at 12,000 × *g* for 20 min at 4°C. The supernatant was diluted with chromatin immunoprecipitation (ChIP) dilution buffer (Tris-HCl [pH 8.0], 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, and protease inhibitor cocktail from Sigma). Diluted lysate was incubated with a 1:300 dilution of anti-FLAG antibody (Cell Signaling Technology [CST], USA) as the ChIP sample or of IgG (CST, USA) as the negative control. After incubation at 4°C overnight with rotation, protein G magnetic beads (CST, USA) were added, and the mixture was incubated at 4°C for 4 h with rotation. Beads were washed with low-salt buffer (50 mM HEPES [pH 7.5], 0.1% deoxycholate, 1% Triton X-100, 1 mM EDTA, 150 mM NaCl) and high-salt buffer (50 mM HEPES [pH 7.5], 0.1% deoxycholate, 1% Triton X-100, 1 mM EDTA, 500 mM NaCl). Cross-linked complex was eluted by incubation with 150 μl of a freshly prepared elution buffer (1% SDS, 0.1 M $NaHCO_3$) at 65°C with gentle vortexing for 30 min. Cross-links were reversed by the addition of 6 μl of 5 M NaCl and 2 μl protease K (20 mg/ml; TaKaRa, Japan) and incubation at 65°C for 4 h. DNA was purified using a PCR purification kit (Qiagen, USA).

The qPCR was performed as described above to quantitatively determine the amount by which a ChIP sample was enriched for individual loci. Fold enrichment was calculated using the formula $2^{-(CT\text{IP} - CT\text{Negative})}$, where *CT* is the fractional threshold cycle of the immunoprecipitation (IP) samples and negative controls.

Site-directed mutagenesis of the FlrA-binding DNA boxes. The same promoter region of the *bpfA* operon as tested in the EMSA was used to examine the functions of two FlrA boxes. Mutations in the FlrA-binding boxes were introduced by overlapping PCR using two primer pairs with expected substitutions at the 5′ ends of a joint primer. The resulting DNA fragments with expected mutations were ligated with *lacZ*, inserted into pBBR1MCS5, and then introduced into the wild-type strain of *S. putrefaciens* CN32. The activity of β-galactosidase was measured to evaluate the transcription from mutated promoters.

FP. The binding activity of FlrA to the promoter DNA of the *bpfA* operon was assayed by fluorescence polarization (47). The DNA of 30 bp (AGAGGCGCGTCAAAAAATTGACTTCCATTC) covering FlrA box 2 in the promoter region was chosen. For the competition assay, a site mutation of the same FlrA box 2 as used for the above-described experiment was introduced into the 30-bp DNA (AGAGGCTATGACA AAAATTGACTTCCATTC).The FAM-labeled and unlabeled synthetic oligonucleotides of 30 bases were synthesized by General Biosystems (China). The two strands of complementary oligonucleotides were annealed in annealing buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl) by heating them to 95°C for 10 min and slowly cooling them (30 min) to room temperature. FlrA was serially diluted in the same storage buffer as used for FlrA purification. To analyze the binding affinity, 15 nM DNA with 5′-labeled FAM was incubated with various concentrations of FlrA in a fluorescence polarization (FP) buffer (50 mM Tris-HCl [pH 8.0], 100 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM DTT, 0.01 mM ATP) at 25°C for 30 min before FP measurement. For competition assay, 15 nM FAM-labeled DNA and 450 nM FlrA were incubated with 1.5 μM unlabeled DNA containing wild-type or mutated FlrA box 2. In addition, 15 nM FAM-labeled DNA and 450 nM FlrA were incubated with 25 μM c-di-GMP to analyze c-di-GMP's effect on FlrA's binding to DNA. The fluorescence anisotropy was measured using SpectraMax M5 (Molecular Devices, USA), and the results are presented in mili-anisotropy values. The change in mili-anisotropy value as a function of FlrA concentration was used to determine the equilibrium dissociation constant (K_d) for the interaction between FlrA and FAM-labeled DNA. The data were fitted to the equation $mA = mA_f + [(mA_b - mA_f) \cdot x/(K_d + x)]$, where mA indicates the measured mili-anisotropy value, mA_f the mili-anisotropy value of the free FAM-labeled DNA, mA_b the mili-anisotropy value of the bound FAM-labeled DNA, and x the FlrA concentration.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02410-16.

TEXT S1, PDF file, 0.7 MB.

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