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FliZ Acts as a Repressor of the *ydiV* Gene, Which Encodes an Anti-$FlhD_4C_2$ Factor of the Flagellar Regulon in *Salmonella enterica* Serovar Typhimurium▽

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YdiV acts as an anti-$FlhD_4C_2$ factor, which negatively regulates the class 2 flagellar operons in poor medium in *Salmonella enterica* serovar Typhimurium. On the other hand, one of the class 2 flagellar genes, *fliZ*, encodes a positive regulator of the class 2 operons. In this study, we found that the FliZ-dependent activation of class 2 operon expression was more profound in poor medium than in rich medium and not observed in the *ydiV* mutant background. Transcription of the *ydiV* gene was shown to increase in the *fliZ* mutant. Purified FliZ protein was shown *in vitro* to bind to the promoter region of the *nlpC* gene, which is located just upstream of the *ydiV* gene, and to repress its transcription. These results indicate that FliZ is a repressor of the *nlpC-ydiV* operon and activates the class 2 operons by repressing *ydiV* expression. Therefore, the *fliZ* and *ydiV* genes form a regulatory loop.

Salmonella enterica serovar Typhimurium cells swim by means of rotating flagella through their environment. The individual flagellum consists of three structural parts, a basal body, a hook, and a filament. More than 50 genes are specifically required for flagellar formation and function (1, 2, 4, 26). These genes are organized into at least 15 operons, and their transcriptional expression forms a highly organized three-tiered cascade called a flagellar regulon (22, 27). The *flhDC* operon is the sole one belonging to class 1 and encodes FlhD and FlhC, which assemble into an $FlhD_4C_2$ heterohexamer acting as an activator of class 2 operons (31, 42). Class 2 contains operons encoding component proteins for the hook-basal body structure and the flagellum-specific type III export apparatus as well as a gene encoding the flagellum-specific sigma factor σ^{28} (FliA), essential for class 3 expression (33). Class 3 operons encode proteins involved in filament assembly and flagellar function. σ^{28} activity is negatively controlled by an anti-σ^{28} factor, FlgM (15, 24, 34), which is excreted from the cell through the flagellum-specific type III export apparatus upon completion of hook-basal body assembly (11, 19).

Class 2 operons are transcribed by σ^{70} RNA polymerase in the presence of the $FlhD_4C_2$ complex, which binds to the DNA region upstream of the class 2 promoter (13, 30, 31). Two genes within the flagellar regulon, *fliT* and *fliZ*, have been shown to be involved in fine control of the class 2 operons (23). The *fliT* and *fliZ* genes belong to the *fliDST* and *fliAZ* operons, respectively, both of which are transcribed from both class 2 and class 3 promoters (12, 21, 46). FliT acts as an anti-$FlhD_4C_2$ factor, which binds to the $FlhD_4C_2$ complex through interaction with the FlhC subunit and inhibits its binding to the class 2 promoter, resulting in decreased expression of the class 2 operons (45). On the other hand, FliZ was shown to be a positive regulator of class 2 operons (23) and to participate in a positive-feedback loop that induces a kinetic switch in class 2 operon expression (38). Although class 2 operon expression is inhibited *in vivo* by the *fliZ* mutation (23), transcription from the class 2 promoter occurs efficiently *in vitro* in the absence of FliZ (13, 31). This suggests that FliZ affects indirectly the activation process of the class 2 operons. On the basis of the observation that the *fliZ* mutation decreased the amount of FlhC without having a significant effect on the transcription and translation of the *flhC* gene, Saini et al. (37) proposed that FliZ is a posttranslational activator of $FlhD_4C_2$. However, the molecular mechanism underlying FliZ control of the class 2 operons has remained obscure.

The *fliZ* mutation has also been shown to impact several cellular processes unrelated to flagellar biogenesis in *Salmonella*. For example, the *fliZ* mutation decreases the transcriptional expression of *Salmonella* pathogenicity island 1 (SPI1) genes (14) through affecting the activity of their positive regulator, HilD (5, 17). On the other hand, the *fliZ* mutation increases type 1 fimbrial gene expression through affecting posttranscriptionally the activity of the positive regulator FimZ (6, 39). Furthermore, in *Escherichia coli*, FliZ was shown to affect curli expression by interfering with RpoS, the stationary-phase-specific σ factor (35). However, the precise molecular mechanisms underlying these FliZ controls are also still unclear.

Recently, we found that a nonflagellar gene, *ydiV*, encodes another anti-$FlhD_4C_2$ factor (41). YdiV binds to $FlhD_4C_2$ by interacting with the FlhD subunit, resulting in inhibition of class 2 operon expression. The intracellular level of YdiV is higher in poor medium than in rich medium, which allows YdiV to act as a mediator of the nutritional level of the environment in the expression of the flagellar regulon. Nutritional control of YdiV expression was shown to be executed mainly at

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TABLE 1. *Salmonella enterica* strains used in this study

Strain	Relevant characteristic(s)	Source or reference
KK1004	LT2 Δ(*hin-fljBA*) ΔFels-2	25, 28
KK1004hDC	KK1004 *flhD2140*::Tn*10*	28
KK1004iA	KK1004 Δ*fliA*::FRT (in-frame)	This study
KK1004iZ	KK1004 Δ*fliZ*::FRT (in-frame)	This study
KK1004iAZ	KK1004 Δ*fliAZ*::FRT (in-frame)	This study
KK1004V	KK1004 Δ*ydiV*::FRT (in-frame)	41
KK1004ViA	KK1004V Δ*fliA*::FRT (in-frame)	This study
KK1004ViZ	KK1004V Δ*fliZ*::FRT (in-frame)	This study
KK1004ViAZ	KK1004V Δ*fliAZ*::FRT (in-frame)	This study
KK1004 *flhC-3F*	KK1004 *flhC*-3×FLAG	41
KK1004 *ydiV-lac*	KK1004 Δ*ydiV*::FRT::pKG137	41

TABLE 2. Plasmids used in this study

Plasmid	Description	Source or reference
pKD13	FRT-*kan*-FRT cassette template, *amp*	7
pKD46	Red expression plasmid, *amp*	7
pCP20	Flp expression plasmid, *amp cat*	7
pQE80L	Expression vector, His_6 tag, *amp*	Qiagen
pQE80L-*flhCD*	pQE80L *flhC flhD* (His_6-FlhC FlhD)	This study
pQE80L-*fliZ*	pQE80L *fliZ* (His_6-FliZ)	This study
pQE80L-*ydiV*	pQE80L *ydiV* (His_6-YdiV)	41
pQE80L-ΔP_{T5}-P_{nlpC}	pQE80L ΔP_{T5} P_{nlpC}-T_0	This study
pQE80K	pQE80L Δ*amp*::*kan*	Laboratory stock
pQE80K-*fliZ*	pQE80K *fliZ* (His_6-FliZ)	This study
pFZY1	Promoter probe vector, *amp*	18
pFZY1-P_{fliA}-*lacZ*	pFZY1 P_{fliA}-*lacZ*	This study
pFZY1-P_{nlpC}-*lacZ*	pFZY1 P_{nlpC}-*lacZ*	This study
pFZY1-ΔP_{nlpC}-*lacZ*	pFZY1-P_{nlpC}-*lacZ* ΔP_{nlpC}	This study

the translational level (41). On the other hand, Wozniak et al. (43) reported that *ydiV* transcription is under the negative regulation of the *flhDC* genes, suggesting that some gene(s) within the flagellar regulon may act as a negative regulator of the *ydiV* gene.

In this study, we showed that FliZ-dependent activation of class 2 operon expression was more profound in poor medium than in rich medium and not observed in the *ydiV* mutant background. This suggested the possibility that FliZ control of class 2 operon expression is exerted via the regulatory pathway of *ydiV* expression. Gel mobility shift assay and *in vivo* and *in vitro* transcription analyses revealed that FliZ binds to the promoter region of the *nlpC-ydiV* operon and acts as a repressor of its transcription. Therefore, FliZ-dependent activation of class 2 operon expression is achieved by repression of the *ydiV* gene by FliZ.

MATERIALS AND METHODS

Bacterial strains, plasmids, chemicals, and culture media. The *Salmonella* strains and plasmids used in this study are listed in Tables 1 and 2, respectively. All the *Salmonella* strains used were derivatives of a standard laboratory strain, LT2. Their construction procedures are described below. P22-mediated transduction was performed as described previously (25, 28). Unless otherwise specified, all the chemicals and DNA enzymes used were purchased from Nacalai Tesque (Kyoto, Japan) and Toyobo (Osaka, Japan), respectively. Oligonucleotide primers used were purchased from Life Technologies (Tokyo, Japan) and are summarized in Table 3. The rich and poor media used were Luria broth (LB) and M9 minimal medium (32) supplemented with 0.2% glycerol and 0.2% Casamino Acids (MGC), respectively (41). Hard-agar and motility agar plates were prepared by adding 1.2% and 0.25% agar (Shoei, Tokyo, Japan), respectively, to LB or MGC. Ampicillin, kanamycin, and tetracycline were used at final concentrations of 50, 50, and 20 μg/ml, respectively. Where required, arabinose or isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture media at final concentrations of 0.2% or 0.1 mM, respectively.

Construction of chromosomal deletion mutants. The λ Red recombination system (7) was used for construction of chromosomal in-frame deletion mutants. Double deletion mutants were constructed by P22-mediated transduction.

An in-frame deletion mutant of the *fliA* gene (KK1004iA) was constructed as follows. The kanamycin resistance Flp recombination target (FRT) cassette was PCR amplified from pKD13 with primers fliAH1P1 and fliAH2P4, which possess sequences homologous to the 5′- and 3′-end-flanking regions of the *fliA* gene, respectively. The amplified product was introduced into KK1004 harboring pKD46 by electroporation, and kanamycin-resistant colonies were selected on LB agar plates containing kanamycin and arabinose. After the correct replacement of the *fliA* gene with the kanamycin resistance gene cassette was confirmed, this mutation was moved to fresh KK1004 via P22-mediated transduction. The *kan* gene was removed through site-specific recombination between the flanking FRT sequences by transient exposure of the mutant cells to pCP20.

Similarly, an in-frame deletion of the *fliZ* gene (resulting in strain KK1004iZ) was carried out using primers fliZH1P1 and fliZH2P4, which possess sequences homologous to the 5′- and 3′-end-flanking regions of the *fliZ* gene, respectively. Through the same procedure, an in-frame deletion of the *fliAZ* operon (resulting in strain KK1004iAZ) was carried out using primers fliAH1P1 and fliZH2P4.

Plasmid construction. The *fliZ* gene was PCR amplified with primers IZP1 and SalIfliZR using genomic DNA from KK1004 as a template. The amplified product was digested with BamHI and SalI and inserted into the corresponding site of pQE80L or pQE80K to obtain pQE80L-*fliZ* or pQE80K-*fliZ*, respectively. They encode an N-terminally hexahistidine-tagged FliZ protein (His_6-FliZ). Introduction of these plasmids restored full motility to the cells of KK1004iZ (Δ*fliZ*) (data not shown), indicating that the His_6-FliZ protein is functional.

The *flhC* and *flhD* genes were separately PCR amplified with primers HCf1B and HCr1S and HDf1S and HDr1P, respectively, using genomic DNA from KK1004 as a template. The amplified *flhC* DNA was digested with BamHI and SalI, while the amplified *flhD* DNA was digested with SalI and PstI. These two DNA fragments were inserted together into the BamHI/PstI site of pQE80L to obtain pQE80L-*flhCD*. This plasmid encodes an N-terminally hexahistidine-tagged FlhC protein (His_6-FlhC) and a nontagged FlhD protein. Introduction of this plasmid restored full motility to the cells of KK1004hDC (*flhD*::Tn*10*) (data not shown), indicating that both the His_6-FlhC and FlhD proteins encoded by this plasmid are functional.

The promoter region of the *fliAZ* operon was PCR amplified with primers KpnIfliA0 and IAEIB using genomic DNA from KK1004 as a template. The amplified product was digested with KpnI and BamHI and inserted into the corresponding site of pFZY1 to obtain pFZY1-P_{fliA}-*lacZ*. In this plasmid, the *lacZ* gene is transcribed from the class 2 and class 3 promoters of the *fliAZ* operon (P_{fliA}).

The promoter region of the *nlpC* gene was PCR amplified with primers PnlpCf1E and PnlpCr1B using the genomic DNA from KK1004 as a template. The amplified product was digested with EcoRI and BamHI and inserted into the corresponding site of pQE80L. In order to delete the T5 promoter on the vector sequence, the resulting plasmid was digested with XhoI and EcoRI, blunt ended with the Klenow fragment, and then self-ligated. In the resulting plasmid, pQE80L-ΔP_{T5}-P_{nlpC}, transcription initiates at the *nlpC* promoter (P_{nlpC}) and terminates at the T_0 terminator on the vector sequence, yielding an RNA product of 184 or 183 bases.

Similarly, the same region was PCR amplified with primers PnlpCf2E and PnlpCr2B or NLPCF and PnlpCr2B, digested with EcoRI and BamHI, and inserted into the corresponding site of pFZY1 to obtain pFZY1-P_{nlpC}-*lacZ* or pFZY1-ΔP_{nlpC}-*lacZ*, respectively. In pFZY1-P_{nlpC}-*lacZ*, the *lacZ* gene is transcriptionally fused to P_{nlpC}. pFZY1-ΔP_{nlpC}-*lacZ* has the same structure as pFZY1-P_{nlpC}-*lacZ* except that it lacks the P_{nlpC} region.

β-Galactosidase enzyme assay. β-Galactosidase activity was assayed as described previously (20) using cells grown aerobically to exponential phase at 37°C in LB or MGC containing appropriate antibiotics. Each sample was assayed in triplicate.

Protein analysis. Cells were grown to exponential phase at 37°C in LB or MGC, and the cultures were directly subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). SDS-PAGE and Western blotting

TABLE 3. Oligonucleotide primers used in this study

Use and primer name	Nucleotide sequence (5′ to 3′)[a]
Construction of chromosomal deletion mutants	
fliAH1P1	TAGAAACGGATAATCATGCCGATAACTCATTTAAC GCAGGGCTGTTTATCGTGTAGGCTGGAGCTGCTTC
fliAH2P4	GGTAGTCTATACGTTGTGCGGCACTTTTCGGGTGC GATCATGCGCGACCTATTCCGGGGATCCGTCGACC
fliZH1P1	TCGCACCCGAAAAGTGCCGCACAACGTATAGACT ACCAGGAGTTCTCATGGTGTAGGCTGGAGCTGCTTC
fliZH2P4	AGGTTTGCCACGTTTCACCAACACGACTCTGCTA CATCTTATGCTTTTTTATTCCGGGGATCCGTCGACC
Construction of plasmids	
IAEIB	GGG*GGATCC*TCTGACGATACTCCGC
KpnIfliA0	GGG*GGTACC*GCTACAGGTTACATAA
IZP1	GG*GGATCC*ACGGTGCAGCAACCTAAAAG
SalIfliZR	GGG*GTCGAC*TTAATATATATCAGAA
HCf1B	GG*GGATCC*AGTGAAAAAAGCATTGTTCAGG
HCr1S	GGG*GTCGAC*TTAAACAGCCTGTTCGATCTG
HDf1S	GGG*GTCGAC*ACATCACGGGGTGCGGCTA
HDr1P	GGG*CTGCAG*TCATGCCCTTTTCTTACGCGC
PnlpCf1E	GGG*GAATTC*ATTTATGTCAGCCAGGAATTG
PnlpCf2E	GGG*GAATTC*CTACGGTTTGCGCTTTCGACG
NLPCF	ACCA*GAATTC*AAACAGAGGATTGTTGC
PnlpCr1B	GGG*GGATCC*AAAAACGCATGCCGCAACAAT
PnlpCr2B	GGG*GGATCC*CTGCAACTGATCGTTCAGACC

[a] Nucleotides corresponding to the created restriction enzyme cleavage sites are shown in italics.

of proteins were performed according to the method described previously (24). FliC (flagellin) was detected with an anti-FliC polyclonal antibody and an anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (Santa Cruz Biotechnology, CA) using the ECL Plus Western blotting detection system (GE Healthcare, NJ). FLAG-tagged proteins were detected with an anti-FLAG M2 monoclonal antibody (Sigma, MO) and an anti-mouse HRP-conjugated antibody (Santa Cruz Biotechnology).

Affinity purification of His_6-FliZ. His_6-FliZ protein synthesized in *E. coli* strain BL21 Δ*slyD* (laboratory stock) harboring pQE80L-*fliZ* was affinity purified according to the method described previously (44). The purified protein exhibited a single band at a position corresponding to approximately 21 kDa in an SDS-PAGE gel (data not shown).

***In vitro* transcription.** *In vitro* RNA synthesis was performed using *E. coli* RNA polymerase holoenzyme (Epicenter, WI) according to the method described previously (41, 44). The reaction mixture contained 40 mM Tris-HCl, pH 7.5, 150 mM KCl, 1 mM dithiothreitol (DTT), 10 mM $MgCl_2$, 50 U of RNase inhibitor, 100 μM (each) ATP, GTP, and CTP, 50 μM UTP with 4×10^5 Bq [α-^{32}P]UTP (Institute of Isotopes, Budapest, Hungary), 1 μg of template DNA, 1 U of RNA polymerase, and various concentrations (0 to 1,250 nM) of His_6-FliZ. Mixtures without substrates were prepared in a 45-μl volume and incubated at 37°C for 10 min. The reaction was initiated by addition of 5 μl prewarmed substrate mixture. After incubation at 37°C for 10 min, the reaction was terminated by adding 50 μl of a stop solution (0.6 M sodium acetate [pH 5.5], 20 mM EDTA, 200 μg tRNA/ml). The transcripts were extracted with phenol and then precipitated with ethanol. The precipitated materials were electrophoretically separated in an 8% polyacrylamide gel containing 6 M urea. The labeled transcripts were visualized by autoradiography.

Gel mobility shift assay. A 287-bp DNA fragment containing P_{nlpC} was PCR amplified from the KK1004 genomic DNA using primers PnlpCf2E and PnlpCr2B. The amplified product was gel purified and then labeled at the 5′ end with [γ-^{32}P]ATP (Institute of Isotopes) by T4 polynucleotide kinase and used as a probe. The DNA-binding activity of His_6-FliZ was assayed according to a method described previously (41). The binding reaction mixture (20 μl) contained a 0.2 nM concentration of the labeled probe DNA, 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin, 5% glycerol, and various concentrations (0 to 250 nM) of His_6-FliZ. The reaction mixture was incubated at 37°C for 30 min and subjected to electrophoresis on a native 5% polyacrylamide gel in 0.5× TBE buffer (25 mM Tris, 25 mM boric acid, 1 mM EDTA). Labeled DNAs were detected by autoradiography.

RESULTS

FliZ is required for efficient flagellar production in poor medium. We showed previously that a disruption of the *fliZ* gene reduced class 2 operon expression more than 5-fold (23), whereas Saini et al. (37) reported that the *fliZ* mutation had only a moderate effect on class 2 operon expression. We noted that the culture media used were different between these two studies; that is, Kutsukake et al. used MGC, whereas Saini et al. used LB. This suggested the possibility that the effect of the *fliZ* mutation on flagellar production is influenced by the nutritional condition. In order to test this possibility, we examined first more carefully the effect of the *fliZ* mutation on class 2 transcription with the cells grown in LB and MGC. For this purpose, we used the P_{fliA}-*lacZ* transcriptional fusion gene on the single-copy plasmid pFZY1-P_{fliA}-*lacZ*. The P_{fliA} DNA contains both class 2 and class 3 promoters of the *fliAZ* operon. Therefore, in order to avoid transcription from the class 3 promoter, all the host strains that we used for this study possessed an in-frame deletion of the *fliA* gene. As shown in Table 4, the effect of the *fliZ* mutation on β-galactosidase activity was more pronounced in MGC (more than 5-fold) than in LB (less than 2-fold).

Next, we examined motility and flagellin (FliC) production in LB and MGC. As shown in Fig. 1, the effect of the *fliZ* mutation on motility and flagellin level was also more pronounced in MGC than in LB. These results indicate that *Salmonella* cells require FliZ for efficient flagellar synthesis more strictly in poor medium than in rich medium.

FliZ acts in a regulatory pathway upstream of YdiV. Saini et al. (37) reported that the *fliZ* deletion caused an approximately 50% decrease in the FlhC-3×FLAG protein level in rich medium. Therefore, there is a possibility that inhibition of class 2

TABLE 4. Effects of growth media and *ydiV* mutation on FliZ control of class 2 transcription[a]

Medium	Genotype[b]			Plasmid	β-Galactosidase activity (Miller units)
	ydiV	*fliA*	*fliZ*		
LB	+	−	+	None	236 ± 8
	+	−	−	None	123 ± 21
	−	−	+	None	226 ± 60
	−	−	−	None	220 ± 26
MGC	+	−	+	None	31 ± 6
	+	−	−	None	6 ± 1
	−	−	+	None	248 ± 33
	−	−	−	None	251 ± 48
	+	−	−	pQE80K	76 ± 23
	+	−	−	pQE80K-*fliZ*	386 ± 60
	−	−	−	pQE80K	502 ± 59
	−	−	−	pQE80K-*fliZ*	519 ± 56

[a] β-Galactosidase activity was assayed with strains harboring pFZY1-P_{fliA}-*lacZ*.
[b] Strains used were *ydiV*$^+$ *fliA fliZ*$^+$ strain KK1004iA, *ydiV*$^+$ *fliA fliZ* strain KK1004iAZ, *ydiV fliA fliZ*$^+$ strain KK1004ViA, and *ydiV fliA fliZ* mutant KK1004ViAZ. −, the gene is deleted.

TABLE 5. Effects of the *flhD* or *fliZ* mutation on the *ydiV-lac* expression[a]

Medium	Plasmid	β-Galactosidase activity (Miller units) with mutation[b]		
		None	*flhD*::Tn*10*	*fliZ*
LB	None	173 ± 61	404 ± 15	376 ± 34
MGC	None	156 ± 13	381 ± 10	290 ± 37
MGC	pQE80L	212 ± 19	336 ± 9	341 ± 9
MGC	pQE80L-*flhCD*	198 ± 17	191 ± 44	323 ± 20
MGC	pQE80L-*fliZ*	159 ± 10	188 ± 13	180 ± 22
MGC	pQE80L-*ydiV*	380 ± 6	385 ± 20	379 ± 35

[a] All strains used carried the *ydiV-lacZ* fusion gene on the chromosome, which was transduced from KK1004 *ydiV-lac*.
[b] Strains used were wild-type KK1004, *flhD*::Tn*10* strain KK1004hDC, and *fliZ* strain KK1004iZ.

operon expression by the *fliZ* mutation in poor medium might be due to the decreased level of the FlhC protein. However, we could not observe any significant effect of the *fliZ* mutation on the FlhC-3×FLAG protein level in MGC (data not shown). Therefore, FliZ-dependent activation of class 2 operon expression is unlikely to be mediated by modulation of the intracellular $FlhD_4C_2$ level, at least in the strains used here.

Since YdiV was shown to be a mediator of the nutritional cue to flagellar synthesis (41), the above-mentioned results raised the possibility that YdiV is involved in the *fliZ*-mediated regulation of the flagellar regulon. In order to test this, the effect of the *fliZ* mutation on flagellar gene expression was examined in the absence of YdiV. As shown in Fig. 1 and Table 4, the negative effects of the *fliZ* mutation on motility, flagellin production, and class 2 gene transcription in MGC were all relieved in the *ydiV* mutant background. These results suggest the hypothesis that FliZ acts upstream of YdiV on the common regulatory pathway.

In order to further confirm this, the effect of FliZ overexpression from pQE80K-*fliZ* on class 2 operon transcription was examined with the strains harboring pFZY1-P_{fliA}-*lacZ* (Table 4). Introduction of the empty vector pQE80K enhanced P_{fliA}-*lacZ* expression. This is consistent with a previous report showing that the *lacI* gene on the plasmid upregulates at least some flagellar genes (8). In the *ydiV*$^+$ background, FliZ overexpression enhanced P_{fliA}-*lacZ* expression 5-fold. In contrast, FliZ overexpression had no significant effect on P_{fliA}-*lacZ* expression in the absence of YdiV. These results conform to the hypothesis mentioned above. Taken together, we conclude that the FliZ protein is a direct or indirect antagonist of the anti-$FlhD_4C_2$ factor YdiV in transcriptional control of the class 2 flagellar operons. Interestingly, overexpression of FliZ in the *ydiV*$^+$ strain did not attain a level of expression of P_{fliA}-*lacZ* equivalent to its expression level in the *ydiV* mutant. This is discussed below.

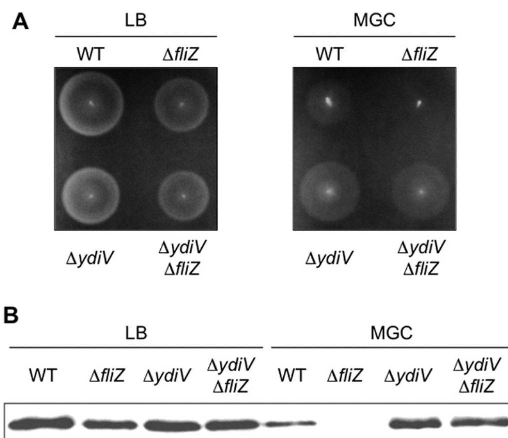

FIG. 1. Effect of the *fliZ* mutation on motility (A) and the steady-state level of flagellin (B). (A) Single colonies were stabbed onto an LB motility agar plate (left panel) or an MGC motility agar plate (right panel) and incubated for 4 h at 30°C. Strains used include KK1004 (wild type [WT]), KK1004iZ (Δ*fliZ*), KK1004V (Δ*ydiV*), and KK1004ViZ (Δ*ydiV* Δ*fliZ*). (B) When the bacterial growth reached logarithmic phase, the cells were sampled and separated by SDS-PAGE. Flagellin was detected by Western blotting with an anti-FliC polyclonal antibody. Strains used were the same as those used for panel A.

FliZ negatively regulates transcription of the *ydiV* gene. As described in the introduction, Wozniak et al. (43) reported that *ydiV* transcription is under the negative regulation of the *flhDC* genes. Since their experiment was carried out in rich medium, we examined the effect of the *flhD* mutation on *ydiV* transcription in poor medium. As shown in Table 5, when the cells of the strain carrying the *ydiV-lacZ* transcriptional fusion gene on the chromosome were grown in MGC, the *flhD*::Tn*10* mutation increased β-galactosidase activity 2.4-fold.

Since the *fliZ* gene is under the positive control of $FlhD_4C_2$ (12, 23), FliZ is one of the most plausible candidates of the regulator involved in $FlhD_4C_2$ control of the *ydiV* gene. In order to test this, the effect of the *fliZ* mutation on *ydiV-lacZ* expression was examined (Table 5). As expected, the *fliZ* mutation increased β-galactosidase activity about 2-fold. Ectopic expression of FliZ from pQE80L-*fliZ* in the *flhD*::Tn*10* mutant resulted in decreased β-galactosidase activity, whereas that of FlhC and FlhD from pQE80L-*flhCD* in the *fliZ* mutant had no significant effect on enzyme activity (Table 5). These results indicate that $FlhD_4C_2$ affects *ydiV* expression through its pos-

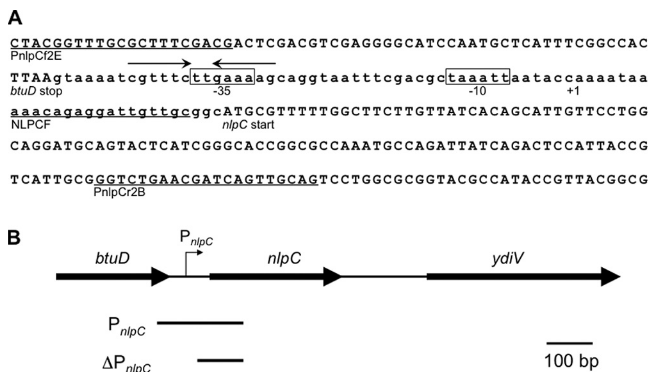

FIG. 2. Sequence of the *nlpC* promoter region (A) and the structure of the *nlpC-ydiV* operon (B). (A) The coding sequences of the *btuD* and *nlpC* genes are written in uppercase, whereas the sequence of their intergenic region is written in lowercase letters. The putative −35 and −10 sequences of P_{nlpC} are boxed. The predicted transcriptional start point of the *nlpC* promoter is labeled +1. The convergent arrows indicate the inverted repetitious sequences, which may act as a FliZ-binding site. Nucleotide sequences corresponding to the primers used for PCR amplification of the *nlpC* promoter region are underlined. (B) The *btuD*, *nlpC*, and *ydiV* genes are indicated by horizontal arrows. The chromosomal regions cloned on the recombinant reporter plasmids are shown by horizontal lines. The putative promoter sequence shown in panel A is present in the P_{nlpC} fragment but absent from the ΔP_{nlpC} fragment.

itive control of *fliZ* expression. It should be noted that the chromosomal *ydiV-lacZ* fusion gene was not completely turned off by FliZ expression from pQE80L-*fliZ* (Table 5). This is consistent with the result shown in Table 4. These results suggest that both FliZ-sensitive and FliZ-insensitive transcriptions are responsible for *ydiV* expression. This is discussed further below.

Because YdiV inhibits *fliZ* expression by acting as an anti-$FlhD_4C_2$ factor (41), the above-described result suggests that the *ydiV* gene should be autogenously activated. As expected, YdiV overexpression from pQE80L-*ydiV* enhanced *ydiV-lacZ* expression, and the *flhD* or *fliZ* mutation did not enhance further its expression (Table 5).

FliZ represses *ydiV* transcription from the *nlpC* promoter. The *ydiV* gene is located just downstream of the *nlpC* gene (Fig. 2). Jonas et al. (16) reported results suggesting that two promoters located within the *nlpC-ydiV* intergenic region may be responsible for *ydiV* transcription. Recently, however, we showed that these promoters are inactive in our strains and that the *ydiV* gene is transcribed mainly from a promoter, P_{nlpC}, located upstream of the *nlpC* gene (41). Therefore, in this study, we examined the structure and function of P_{nlpC} further. A bioinformatics study using the software Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html) (36) suggested one σ^{70}-type promoter sequence, TTGAAA-N_{18}-TAAATT, whose predicted transcriptional start site is nucleotide C or A at bp position 30 or 29 upstream of the start codon of the *nlpC* gene (Fig. 2A). Unfortunately, our attempts to identify the transcriptional start site of the *nlpC* gene by primer extension analysis of mRNA or by the RNA ligation-mediated reverse transcription-PCR method were unsuccessful for unknown reasons. So, we analyzed the activity of P_{nlpC} using a single-copy promoter-probe vector, pFZY1.

DNA fragments containing the upstream region of the *nlpC* gene with and without this promoter-like sequence were inserted into pFZY1 to obtain pFZY1-P_{nlpC}-*lacZ* and pFZY1-ΔP_{nlpC}-*lacZ*, respectively (Fig. 2B). In the wild-type cells grown in MGC, β-galactosidase activity was more than 20-fold higher from pFZY1-P_{nlpC}-*lacZ* (310 ± 37 Miller units) than from pFZY1-ΔP_{nlpC}-*lacZ* (15 ± 5 Miller units), suggesting that this promoter-like sequence is functional in *Salmonella*.

Next, we analyzed the effect of the *fliZ* mutation on β-galactosidase activity from pFZY1-P_{nlpC}-*lacZ*. In order to avoid any possible effect of YdiV on transcription from P_{nlpC} through the *ydiV-fliZ* regulatory loop, β-galactosidase activity was assayed in the *ydiV* mutant background. As a result, the enzyme activity was higher in the *ydiV fliZ* deletion strain (272 ± 51 Miller units) than in the *ydiV fliZ*$^+$ strain (38 ± 9 Miller units), indicating that this promoter is responsive to negative regulation by FliZ. Note that, in the presence of FliZ, the enzyme activity was much lower in the *ydiV* mutant than in the wild-type strain. This indicates that YdiV activates its own expression by inhibiting FliZ expression. Furthermore, in the *ydiV fliZ*$^+$ background, the enzyme activity from the chromosomal *ydiV-lacZ* fusion gene (Table 5) was higher than that from pFZY1-P_{nlpC}-*lacZ*, which suggests an additional promoter for *ydiV* expression on the chromosome. This promoter may be responsible for the FliZ-insensitive transcription of the *ydiV* gene described above.

FliZ is a repressor for the *nlpC* promoter. P_{nlpC} activity was analyzed further by an *in vitro* transcription experiment using plasmid pQE80L-ΔP_{T5}-P_{nlpC} as a template. If the above prediction about the transcriptional start site is correct, a 184- or 183-nucleotide transcript is expected to be produced by transcription from P_{nlpC} in this plasmid. In the presence of σ^{70} RNA polymerase, an RNA product of about 190 nucleotides was produced in addition to a 107-nucleotide RNA I derived from the vector sequence (Fig. 3, lane 2). This result indicates the validity of the above-mentioned prediction for P_{nlpC}.

In order to analyze the function of the FliZ protein *in vitro*, we purified the His_6-FliZ protein from *E. coli* cells harboring pQE80L-*fliZ* and examined its effect on *in vitro* transcription from P_{nlpC}. An empty vector, pQE80L, was also included in the reaction mixture as a control template, which produces a 230-nucleotide RNA from the T5 promoter. Addition of the purified His_6-FliZ protein into the reaction mixture reduced the amount of the *nlpC* transcript in a concentration-dependent manner, whereas that of the RNA product from the T5 promoter was not affected (Fig. 3, lanes 3 to 6). This result indicates that FliZ specifically represses transcription from P_{nlpC}. Interestingly, at the highest concentration of His_6-FliZ used in this study, the amount of RNA I was also somewhat reduced (Fig. 3, lane 6). This is discussed below.

FliZ is known to contain a SAM (sterile alpha motif)-like phage integrase domain (37), suggesting the possibility that FliZ has a DNA-binding ability. In order to test this, gel mobility shift assay of the ^{32}P-labeled DNA fragment containing P_{nlpC} was performed in the presence of the purified His_6-FliZ protein. A shifted band was observed in the presence of His_6-FliZ, and its amount increased in a concentration-dependent manner (Fig. 4, lanes 2 to 5). Band shift was not observed with

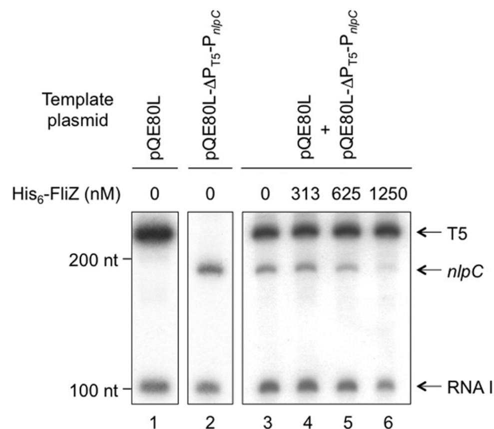

FIG. 3. Effects of FliZ on *nlpC* transcription *in vitro*. A plasmid carrying the T5 or *nlpC* promoter was transcribed *in vitro* with *E. coli* RNA polymerase holoenzyme with or without His$_6$-FliZ in the presence of [α-^{32}P]UTP. His$_6$-FliZ was added to the reaction mixture at the final concentrations indicated above the lanes. Synthesized RNAs were separated on an 8% polyacrylamide gel containing 6 M urea and detected by autoradiography. Positions of the T5, *nlpC*, and RNA I transcripts are indicated by arrows. nt, nucleotides.

the ^{32}P-labeled DNA fragment lacking P_{nlpC} (data not shown), indicating that FliZ binds specifically to the DNA region containing P_{nlpC}. Binding specificity was further examined by a competition experiment using unlabeled DNAs as competitors. An unlabeled DNA fragment containing P_{nlpC} behaved as an effective competitor (Fig. 4, lane 7), whereas an unlabeled DNA fragment without P_{nlpC} did not (Fig. 4, lane 8), indicating the high specificity of FliZ binding to the DNA region containing P_{nlpC}.

DISCUSSION

We showed that FliZ is required for efficient expression of the class 2 flagellar operons in poor medium in *Salmonella* (Table 4). Although Saini et al. (37) reported data suggesting that FliZ functions at the posttranslational level of $FlhD_4C_2$ by regulating its stability, we could not observe any significant effect of the *fliZ* mutation on the FlhC protein level (data not shown). Instead, we showed that FliZ binds to the promoter region of the *nlpC-ydiV* operon and represses its transcription (Fig. 3 and 4), which results in the decreased expression of the *ydiV* gene, encoding an anti-$FlhD_4C_2$ factor.

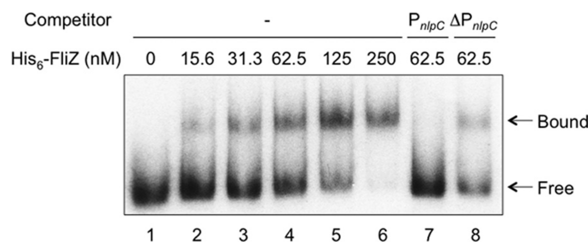

FIG. 4. Gel mobility shift assay of the *nlpC* promoter DNA by FliZ. A 287-bp P_{nlpC} DNA fragment containing the *nlpC* promoter was 5′-end labeled with ^{32}P and used as a probe. Each mixture contained 0.2 nM labeled DNA. After incubation with His$_6$-FliZ at the final concentrations indicated above the lanes, the DNA-protein mixtures were separated on a native 5% polyacrylamide gel, and labeled DNA was detected by autoradiography. In the competition experiments (lanes 7 and 8), 2 nM concentrations of the unlabeled P_{nlpC} and ΔP_{nlpC} fragments shown in Fig. 2B were used. Positions of free and bound DNAs are indicated by arrows.

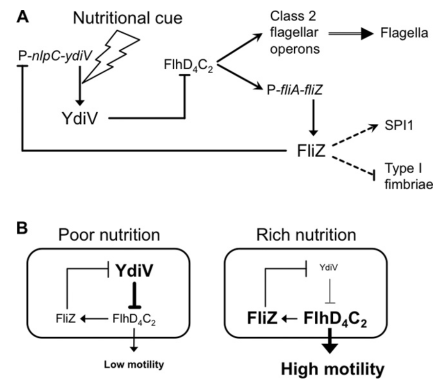

FIG. 5. Models illustrating the FliZ-dependent activation of the flagellar class 2 operons via control of the expression of the *ydiV* gene (A) and the interplay between YdiV and FliZ in motility control in response to nutritional conditions (B). Details are provided in the text.

Our current model depicting a regulatory pathway involving FliZ and YdiV is summarized in Fig. 5A. According to this model, the *fliZ* and *ydiV* genes form a regulatory loop and are differentially expressed in response to nutritional cues. Since *ydiV* expression is enhanced in poor medium at the translational step (41), an increased level of the YdiV protein would activate its own transcription by inhibiting the $FlhD_4C_2$-dependent expression of the *fliZ* gene. Under this condition, the flagellar operons stay at a low expression level. On the other hand, in a rich nutritional environment where the amount of the YdiV protein is low, the $FlhD_4C_2$ complex is free from inhibition by YdiV, resulting in high expression of the FliZ protein, which in turn represses *ydiV* expression. This leads the flagellar operons to the actively expressed state. Thus, the FliZ-YdiV regulatory loop has a stable bipartite expression profile in switching between highly and weakly motile lifestyles in responding to nutrient availability (Fig. 5B).

In this study, we analyzed *ydiV* expression by focusing on the P_{nlpC} promoter, which is susceptible to FliZ repression. However, as described above, we suggest that an additional promoter insensitive to FliZ repression may also be involved in *ydiV* transcription. We anticipate that transcriptional readthrough from the *btuCED* operon upstream of the *nlpC* gene may be responsible for this transcription, because there is no apparent ρ-independent terminator-like sequence in the intergenic region between the *btuD* and *nlpC* genes (Fig. 2A).

FliZ is known to regulate the SPI1 genes positively (5, 14, 17) and the type 1 fimbrial genes negatively (6, 39). FliZ control of these genes was observed in rich medium, in which YdiV expression must be low. Therefore, it is reasonable to predict that this FliZ control may not be affected by the *ydiV* mutation. This prediction is consistent with the previous observation that the expression of one of the SPI1 genes, *sipC*, was not affected by the *ydiV* mutation (10). Therefore, we believe that FliZ control of these genes is not mediated by YdiV. FliZ may regulate these genes through a mechanism different from that of FliZ control of the flagellar genes.

FliZ from *Xenorhabdus nematophila* was shown to specifically bind to the promoter region of the *flhDC* operon and to function as an activator (29). Although *X. nematophila* FliZ shows 56% identity with *Salmonella* FliZ, they differ significantly from each other in their C-terminal sequences (data not shown). Since FliZ is not involved in the regulation of the *flhDC* operon in *Salmonella* (23), there is a possibility that the C-terminal portion of FliZ determines its DNA-binding specificity. This possibility is supported by our preliminary experiment showing that a C-terminally truncated FliZ protein does not retain DNA-binding ability (our unpublished result).

The C-terminal portion of FliZ contains a SAM-like phage integrase domain (37), which is also included in Cre, Flp, and XerD recombinases (9). These recombinases are known to recognize palindromic sequences (3, 40). Interestingly, a 14-bp palindromic sequence (CGTTTCTTGAAAAG, where underlining indicates complementary nucleotides) was found also at the *nlpC* promoter region (Fig. 2A). This sequence overlaps the putative −35 element of the *nlpC* promoter and thus may be a plausible target site of FliZ binding. A similar palindromic sequence (GAGTTCTTGAAGTG) exists around the −35 element of the RNA I promoter, whereas such a sequence is not observed at the T5 promoter region of pQE80L. This structural difference conforms to the results of Fig. 3 showing that the *nlpC* and RNA I promoters were susceptible to FliZ repression but that the T5 promoter was not. Identification of a recognition sequence of FliZ will help us to understand a molecular mechanism underlying FliZ control of the *ydiV* gene and reveal additional targets of FliZ on the bacterial genome.

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