JOURNAL OF BACTERIOLOGY, Feb. 2006, p. 958–967 Vol. 188, No. 3
0021-9193/06/$08.00+0 doi:10.1128/JB.188.3.958–967.2006

FljA-Mediated Posttranscriptional Control of Phase 1 Flagellin Expression in Flagellar Phase Variation of *Salmonella enterica* Serovar Typhimurium

Shouji Yamamoto[1] and Kazuhiro Kutsukake[1,2]*

Graduate School of Natural Science and Technology,[1] and Department of Biology, Faculty of Science,[2] Okayama University, Tsushima-Naka 3-1-1, Okayama 700-8530, Japan

Received 13 September 2005/Accepted 7 November 2005

Flagellar phase variation of *Salmonella* is a phenomenon where two flagellin genes, *fliC* (phase 1) and *fljB* (phase 2), are expressed alternately. This is controlled by the inversion of a DNA segment containing the promoter for the *fljB* gene. The *fljB* gene constitutes an operon with the *fljA* gene, which encodes a negative regulator for *fliC* expression. Previous biochemical analysis suggested that phase variation might depend on alternative synthesis of phase-specific flagellin mRNA (H. Suzuki and T. Iino, J. Mol. Biol. 81:57–70, 1973). However, recently reported results suggested that FljA-dependent inhibition might be mediated by a posttranscriptional control mechanism (H. R. Bonifield and K. T. Hughes, J. Bacteriol. 185:3567–3574, 2003). In this study, we reexamined the mechanism of FljA-mediated inhibition of *fliC* expression more carefully. Northern blotting analysis revealed that no *fliC* mRNA was detected in phase 2 cells. However, only a moderate decrease in β-galactosidase activity was observed from the *fliC-lacZ* transcriptional fusion gene in phase 2 cells compared with that in phase 1 cells. In contrast, the expression of the *fliC-lacZ* translational fusion gene was severely impaired in phase 2 cells. The half-life of *fliC* mRNA was shown to be much shorter in phase 2 cells than in phase 1 cells. Purified His-tagged FljA protein was shown to bind specifically to *fliC* mRNA and inhibit the translation from *fliC* mRNA in vitro. On the basis of these results, we propose that in phase 2 cells, FljA binds to *fliC* mRNA and inhibits its translation, which in turn facilitates its degradation.

Salmonella enterica serovar Typhimurium has 5 to 10 flagella per cell. The individual flagellum is composed of three substructures, a basal body, a hook, and a filament (33). The filament extends into the extracellular space and is up to 10 μm in length. Although the filament is the largest substructure in the flagellum, it is composed of a single species of protein, flagellin. Serovar Typhimurium has two nonallelic flagellin genes, *fliC* and *fljB*, which encode antigenically distinct proteins. Individual cells express only one of these two flagellin genes and alternate the expression between the two at a rate of 10^{-3} to 10^{-5} per cell generation. This phenomenon is known as flagellar phase variation, and the FliC-expressing cells are called phase 1, while FljB-expressing cells are called phase 2 (19).

Flagellar phase variation is controlled by the reversible inversion of a DNA segment, called the H segment, containing the promoter for the *fljB* gene (55, 56). The H segment is flanked by inverted repetitious sequences, *hixL* and *hixR*, between which site-specific recombination occurs, leading to H inversion (25). The cognate recombinase, called DNA invertase, is encoded by the *hin* gene, which is located within the H segment (28, 29, 47). The *fljB* gene constitutes an operon together with the *fljA* gene, which encodes a negative regulator for *fliC* expression (12, 13, 40, 48). Therefore, when the H segment is in the "on" orientation, both the *fljB* and *fljA* genes are transcribed, resulting in phase 2 flagellin being synthesized and the *fliC* gene being repressed. On the other hand, when the H segment turns to the "off" orientation, neither *fljB* nor *fljA* is expressed, resulting in phase 1 flagellin being synthesized.

A pioneering study to understand the molecular mechanism of FljA-mediated inhibition of the *fliC* expression in phase 2 cells was performed previously by Suzuki and Iino (50). They isolated mRNAs from phase 1 and phase 2 cells separately and used them as templates in an in vitro protein-synthesizing system. They found that the flagellin molecules synthesized were of the same phase as the cells from which the mRNAs were derived. This indicated that phase 2 cells did not contain *fliC* mRNA, suggesting that *fliC* repression should be accomplished at a transcriptional level. Inoue et al. (21) isolated mutants whose *fliC* expression became insensitive to FljA. Their mutation sites were mapped around the Shine-Dalgarno (SD) sequence of the *fliC* gene, which locates far downstream of the *fliC* promoter. This situation is unusual because the classical operator sequences are usually located close to or within the promoter regions (9). This suggested the possibility that FljA-mediated inhibition of *fliC* expression might occur at a translational level. Recently, Bonifield and Hughes (2) showed that FljA reduced β-galactosidase activity 200-fold from the *fliC-lacZ* translational fusion gene, while FljA reduced the enzyme activity from the *fliC-lacZ* transcriptional fusion gene by only fivefold and the steady-state level of *fliC* mRNA by only threefold. Based on these results, they proposed that FljA might act at both transcriptional and posttranscriptional levels.

This study was aimed at understanding the mechanism of *fliC* repression by FljA at a molecular level. To address this issue, we reexamined the mechanism of *fliC* repression by FljA more carefully. Northern blotting analysis revealed that no *fliC* mRNA was detected in phase 2 cells. However, only a marginal

* Corresponding author. Mailing address: Department of Biology, Faculty of Science, Okayama University, Tsushima-Naka 3-1-1, Okayama 700-8530, Japan. Phone: 81-86-251-7863. Fax: 81-86-251-7876. E-mail: ktkk@cc.okayama-u.ac.jp.

TABLE 1. *Salmonella* strains used

Strain	Relevant characteristic(s)	Source or reference
LT2	Wild type	Laboratory stock
KK1004	LT2 Δ(*hin-fljBA*)	34
KK1110	KK1004 *fliC-lac*	30
KK2604	KK1004 *fliC*::Tn*10*	30
KK211	LT2 Δ*hin* Δ*fin*::*kan* (fixed in phase 1)	32
KK212	LT2 Δ*hin* Δ*fin*::*kan* (fixed in phase 2)	32
KK211CL	KK211 *fliC-lac*	This study
KK212CL	KK212 *fliC-lac*	This study
KK211CT	KK211 *fliC*::Tn*10*	This study
KK212CT	KK212 *fliC*::Tn*10*	This study

or moderate decrease in β-galactosidase activity was observed from the *fliC-lacZ* transcriptional fusion gene in phase 2 cells. In contrast, the expression of the *fliC-lacZ* translational fusion gene was severely impaired in phase 2 cells. We further showed that the half-life of *fliC* mRNA was much shorter in phase 2 cells than in phase 1 cells. Using purified His-tagged FljA protein, FljA was shown to bind specifically to *fliC* mRNA and inhibit the translation in vitro from *fliC* mRNA. On the basis of these results, we propose the mechanism of posttranscriptional control of *fliC* expression by FljA in phase 2 cells.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Salmonella* strains used in this study are listed in Table 1. LT2 has another DNA invertase gene, *fin*, in addition to *hin* (32). KK211 and KK212 are phase-locked mutants of LT2 whose H segments are fixed in the "on" and "off" orientations, respectively, due to null mutations in both *hin* and *fin* genes (32). Therefore, KK211 is fixed in phase 1 and does not express FljA, whereas KK212 is fixed in phase 2 and expresses FljA constitutively. When mRNAs transcribed from plasmid-borne *fliC* genes were analyzed, KK211CT and KK212CT were used as host strains. These strains carry an *fliC*::Tn*10* mutation introduced from KK2604 by P22-mediated transduction. KK211CL and KK212CL carry a chromosomal *fliC-lacZ* transcriptional fusion gene introduced from KK1110 by P22-mediated transduction. The *Escherichia coli* strain used was JM109 (53). Plasmids used are listed in Table 2. Procedures for plasmid construction are described below.

β-Galactosidase enzyme assay. The activity of β-galactosidase was assayed as described previously (30, 31, 37) using cells grown to an optical density at 600 nm (OD_{600}) of ~0.4 in LB at 37°C. If necessary, tetracycline or ampicillin was added to the medium at a final concentration of 5 or 100 μg/ml, respectively. Each sample was assayed in triplicate.

Protein analysis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blotting analysis of proteins were performed according to methods described previously (31).

DNA procedures. DNA manipulation and transformation were performed as described previously (31). Unless otherwise specified, all the chemicals used were purchased from Nacalai Tesque (Kyoto, Japan). Restriction enzymes and T4 DNA ligase were purchased from Toyobo (Osaka, Japan) or Takara (Kyoto, Japan). PCR amplification was carried out with an iCycler (Bio-Rad, CA) as specified by the manufacturer by using *Thermococcus kodakaraensis* DNA polymerase (KOD Dash) purchased from Toyobo. Customized DNA primers were purchased from Hokkaido System Science (Sapporo, Japan). Their sequences are summarized in Table 3.

Construction of plasmids carrying *fliC-lacZ* translational and transcriptional fusions. Plasmid pKK1012 contains the entire *fliC* operon of serovar Typhimurium. Using this plasmid as a template, a DNA sequence encompassing the promoter through the last codon of the *fliC* gene was amplified by PCR with primers (fliC)SmaIS and (fliC)SmaIE. The amplified product was digested with SmaI and inserted into the corresponding site of pMC1871 to obtain pMCIC0. In this plasmid, the *lacZ* gene was translationally fused to the entire *fliC* open reading frame (ORF) and expressed under the transcriptional and translational control of the *fliC* gene. Using the same template, a DNA sequence encompassing the promoter through the termination codon of the *fliC* gene was PCR amplified with primers (fliC)S1(Sph)124 and (fliC)Eco3E. The amplified product was digested with SphI and EcoRI and inserted into the corresponding site of pRL124 to obtain pRLIC0. In this plasmid, the *lacZ* gene was transcriptionally fused to the *fliC* gene.

Construction of plasmids expressing *fliC* mRNAs of various structures. Plasmids expressing various versions of *fliC* mRNAs were constructed as follows.

TABLE 2. Plasmids used

Plasmid	Description	Source or reference
pBAD33	*araBADp* expression vector	15
pBAD1549	pBAD33 *fliC*(+1 to +1550)	This study
pBAD33*trc*UTR-*fliC*ORF	pBAD33 *trc*(+1 to +39) *fliC*(+63 to +1550)	This study
pKK1001	pBR322 *hin fljBA*	32
pKK1012	pBR322 *fliC*	21
pMC1871	*lacZ* translational fusion vector	5
pMCIC0	pMC1871 *fliC*(−35 to +1547)-*lacZ*	This study
pQE80L	*tac* expression vector	Qiagen
pQE*fljA*	pQE80L *fljA*	This study
pRL124	*lacZ* transcriptional fusion vector	36
pRLIC0	pRL124 *fliC*(−35 to +1550)-*lacZ*	This study
pSPT18	SP6/T7 expression vector	Roche
pIC18UTR	pSPT18 *fliC*(+1 to +300)	This study
pSPT18*fliC*UTR-ORF	pSPT18 *fliC*(+1 to +1550)	This study
pSPT18*trc*UTR-*fliC*ORF	pSPT18 *trc*(+1 to +39) *fliC*(+1 to +1550)	This study
pSPT19	SP6/T7 expression vector	Roche
pSPT19*fliC*	pSPT19 *fliC*(+590 to +1109)	This study
pSPT19*fljB*	pSPT19 *fljB*(+586 to +1138)	This study
pTrc97A	*trc* expression vector	1
pICP1	pTrc97A Δ*trcp fliC*(−35 to +102)	This study
pICP2	pTrc97A Δ*trcp fliC*(−35 to +662)	This study
pICP2M	pICP2 A-to-C substitution in the initiation codon of the *fliC* gene	This study
pTrc99A	*trc* expression vector	1
pIC495-1	pTrc99A *fliC*(+63 to +1550)	This study
pIC495-2	pTrc99A *fliC*(+1 to +1550)	This study

TABLE 3. DNA primers used

Primer name	Nucleotide sequence (5′ to 3′)
(fliC)Eco3E	GGGGAATTCTTAACGCAGTAAAGAGAGGA
(fliC)SmaIE	GGGCCCGGGACGCAGTAAAGAGAGGACGT
(fliC)SmaIS	GGGCCCGGGTAAAGTTCGAAATTCAGGTG
(fliC)S1(Sph)124	GGGGCATGCTAAAGTTCGAAATTCAGGTG
CifBiSa	GGGGGAGCTCACGGTGAGAAACCGTGGGCA
CirBi2Sp	GGGGGCATGCTTAACGCAGTAAAGAGAGGA
FLIC1	GGCCATGGCACAAGTCATTAATACAAAC
FLIC9	GGGGATCCTTAACGCAGTAAAGAGAGGA
ICE4B	GGGGGATCCCTTCAGTGGTCTGCGCAATGG
ICf1Bg	GGGAGATCTCTAAAGTTCGAAATTCAGGTG
ICiPr1Bm	GGGGGATCCACAAAAATATAAGGTCAGGGA
ICiPr2Ec	GGGGAATTCTTTCGTAGTAGTATTAATACTTAT
ICmt1	AATGACTTGTGCCAGGATCTTTTCC
ICmt2	GGAAAAGATCCTGGCACAAGTCATT
ICr1E	GGGGAATTCTCAACAGCGACAGGCTGTTTG
ICS3E	GGGGAATTCACGGTGAGAAACCGTGGGCAA
JAf2B	GGGGGATCCGAATGTATAGCTGTAAATGAT
JAr1S	GGGGTCGACTTATTCAGCGTAATCCGAAGA
JBPr1Bm	GGGGGATCCGAAAGCGTATGATGTGAAAGA
JBPr2Ec	GGGGAATTCACTTGTGGTTTTAGCTTTAAT
PTRCP7	GGGGAATTCGCAGCAGATCTGGTTTG
TRCf1Sa	GGGGAGCTCAATTGTGAGCGGATAACAATT

Structures of the hybrid *fliC* genes on the constructed plasmids are summarized in Fig. 1.

The nucleotide sequence between positions −35 and +102 (with reference to the transcription start site) of the *fliC* gene was PCR amplified using pKK1012 as a template and primers ICf1Bg and ICr1E. The amplified product was digested with BglII and EcoRI and inserted into the corresponding site of pTrc97A to obtain pICP1. In this plasmid, the first 102 nucleotides of *fliC* mRNA fused to a 313-nucleotide RNA derived from the vector sequence are expressed from the *fliC* promoter, and transcription terminates at the *rrnB* terminator.

The entire *fliC* coding sequence (positions +63 to +1550) was PCR amplified from pKK1012 with primers FLIC1 and FLIC9. The amplified product was digested with NcoI and BamHI and inserted into the corresponding site of pTrc99A to yield pIC495-1. In this plasmid, mRNA consisting of the 39-nucleotide 5′ untranslated region (5′-UTR) of *trc* fused to the entire *fliC* ORF is expressed from the *trc* promoter and terminated at the *rrnB* terminator.

A DNA fragment (positions +1 to +1550) containing the entire 5′-UTR and ORF of the *fliC* gene was PCR amplified from pKK1012 with primers CifBiSa and CirBi2Sp. The amplified product was digested with SalI and SphI and inserted into the corresponding site of pBAD33 to yield pBAD1549. A 1.5-kb SacI-HindIII fragment was excised from this plasmid and inserted into the corresponding site of pTrc99A to obtain pIC495-2. In this plasmid, mRNA consisting of the 39-nucleotide *trc* 5′-UTR, the 62-nucleotide *fliC* 5′-UTR, and the entire *fliC* ORF is expressed from the *trc* promoter and terminated at the *rrnB* terminator.

A DNA fragment (positions −35 to +662) containing the promoter, the entire *fliC* 5′-UTR, and the N-terminal region of the *fliC* ORF was PCR amplified from pKK1012 with primers ICf1Bg and ICE2E. The amplified product was digested with BglII and EcoRI and inserted into the corresponding site of pTrc97A to obtain pICP2. A plasmid, pICP2M, in which adenine of the initiation codon of the *fliC* gene on pICP2 was replaced with cytosine was constructed as follows. Using two primer pairs, ICf1Bg and ICmt1 and ICmt2 and ICE2E, two DNA fragments were PCR amplified from pKK1012. These two fragments were mixed and used as templates for a second-round PCR with primers ICf1Bg and ICE2E. The amplified product was inserted into pTrc97A as described above to obtain pICP2M.

Construction of plasmids for in vitro synthesis of *fliC* mRNA. A 1.5-kb SacI-HindIII fragment was excised from pBAD1549 and inserted into the corresponding site of pSPT18 to yield pSPT18*fliC*UTR-ORF. In this plasmid, the *fliC* mRNA containing its own 5′-UTR and ORF is transcribed from the T7 promoter.

A 1.5-kb DNA fragment was PCR amplified from pIC495-1 with primers TRCf1Sa and CirBi2Sp. The amplified product was digested with SalI and SphI and inserted into the corresponding site of pBAD33 to yield pBAD33*trc*UTR-*fliC*ORF. From this plasmid, a 1.5-kb SacI-HindIII fragment was excised and inserted into pSPT18 to obtain pSPT18*trc*UTR-*fliC*ORF. This plasmid directs the synthesis of mRNA consisting of the 39-nucleotide *trc* 5′-UTR and the entire *fliC* ORF from the T7 promoter.

Purification of His-tagged FljA protein. The *fljA* gene was PCR amplified with primers JAf2B and JAr1S using genomic DNA of LT2 as a template. After digestion with BamHI and SalI, the amplified product was inserted into the corresponding site of pQE80L to obtain pQE*fljA*. In this plasmid, His_6-tagged FljA (His-FljA) was expressed from the *tac* promoter. Strain JM109 harboring this plasmid was grown at 37°C with shaking in 40 ml of LB containing 100 μg of ampicillin/ml. When the cell growth reached an OD_{600} of 0.5, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 1 mM. After further cultivation for 5 h, cells were harvested by centrifugation and resuspended in 1 ml of NA buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0) containing 1 mg of lysozyme (Seikagaku Kogyo, Tokyo, Japan). After incubation on ice for 30 min, the cells were disrupted by sonication. The sonicated sample was centrifuged, and the resulting supernatant was mixed with 50 μl of Ni^+-nitrilotriacetic acid agarose (QIAGEN, Hilden, Germany). After the mixture was shaken gently at 4°C for 30 min, the Ni^+-nitrilotriacetic acid agarose was collected by centrifugation, washed three times with 1 ml of NB buffer (50 mM NaH_2PO_4, 300 mM NaCl, 40 mM imidazole, pH 8.0), and resuspended in 50 μl of NC buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). After centrifugation, the supernatant fractions containing His-FljA were pooled and analyzed by SDS-PAGE. The purified His-FljA exhibited a single band at a position corresponding to approximately 21 kDa (data not shown).

Preparation of DIG-labeled RNA probes. The RNA probe complementary to the 5′ portion of *fliC* mRNA (probe 1) was prepared as follows. A DNA fragment corresponding to the first 300 nucleotides of *fliC* mRNA was PCR amplified from pKK1012 with primers ICS3E and ICE4B. After digestion with EcoRI and BamHI, the amplified product was inserted into the corresponding site of pSPT18 to obtain pIC18UTR. This plasmid DNA was linearized by digestion with EcoRI and used as a template for in vitro RNA synthesis from SP6 RNA polymerase with a digoxigenin (DIG) RNA labeling kit (Roche, Basel, Switzerland). The DIG-labeled RNA was precipitated by ethanol and dissolved in diethylpyrocarbonate-treated H_2O.

The plasmid for preparation of the RNA probe complementary to the region from positions +590 to +1109 of *fliC* mRNA (probe 2) was constructed as follows. The DNA fragment corresponding to this region was PCR amplified from pKK1012 with primers ICiPr1Bm and ICiPr2Ec. After digestion with BamHI and EcoRI, the amplified product was inserted into the corresponding site of pSPT19 to obtain pSPT19*fliC*. After this plasmid DNA was linearized by digestion with BamHI, DIG-labeled probe 2 was prepared as described above.

The plasmid for preparation of the RNA probe complementary to the region

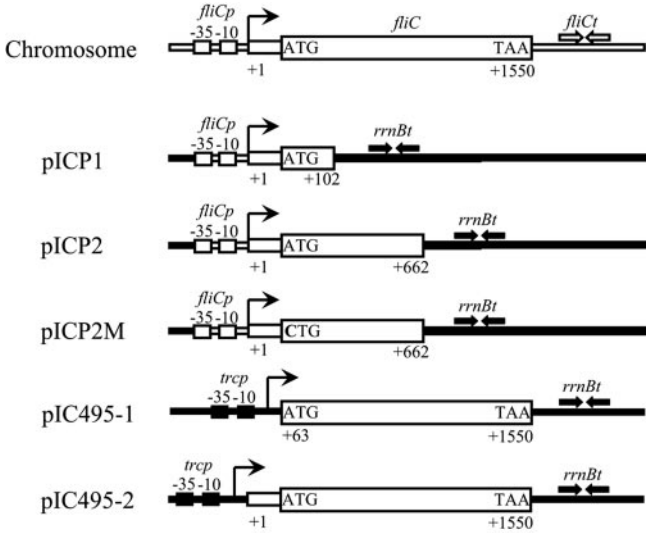

FIG. 1. Schematic representation of the structure of the *fliC* genes on the chromosome and plasmids used in this study. DNAs derived from the native *fliC* gene and pTrc vectors are shown by open and filled rectangles, respectively. *fliCp* and *trcp* indicate the *fliC* and *trc* promoters, respectively. Convergent arrows labeled *fliCt* and *rrnBt* indicate the *fliC* and *rrnB* terminators, respectively. Hooked arrows indicate transcriptional start sites and directions, and the transcriptional start site of the *fliC* gene is numbered +1.

from positions +586 to +1138 of *fljB* mRNA (probe 3) was constructed as follows. The DNA fragment corresponding to this region was PCR amplified from pKK1001 with primers JBPr1Bm and JBPr2Ec. After digestion with BamHI and EcoRI, the amplified product was inserted into the corresponding site of pSPT19 to obtain pSPT19*fljB*. Using this plasmid, DIG-labeled probe 3 was prepared by the same procedure as that for probe 2.

Northern blotting analysis. Cells were grown at 37°C to an OD_{600} of 0.4 in 1.5 ml of LB. If necessary, ampicillin was added to the medium at a final concentration of 100 μg/ml. RNAs were extracted from the cells with a TRI reagent (Sigma Chemical, MO) according to the manufacturer's instructions. A mixture containing 0.5 μg of the RNA sample, 5.25% formaldehyde, and 50% formamide in MOPS buffer [20 mM 3-(*N*-morpholino)propanesulfonic acid, 1 mM EDTA, 5 mM sodium acetate, pH 7.0] was heated at 85°C for 5 min and cooled quickly. The RNAs were then separated electrophoretically on a 1.5% agarose gel containing 2% formamide in MOPS buffer. After electrophoresis, RNAs were transferred to a Hybond-N^+ nylon membrane (Amersham Biosciences, NJ) by capillary blotting in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer (3 M NaCl, 0.3 M trisodium citrate, pH 7.0). After being baked at 120°C for 30 min, the membrane was treated for 2 h at 65°C with a hybridization buffer (0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 0.1 mM $Na_2H_2PO_4$ [pH 6.5], 0.5% SDS, 50% formamide, 0.1 mg of salmon sperm DNA/ml in 5× SSC). The membrane was then incubated for 12 h at 65°C in the hybridization buffer containing the DIG-labeled RNA probe. After hybridization, the membrane was first washed under low-stringency conditions in 2× SSC containing 0.1% SDS for 10 min at room temperature and was then washed under high-stringency conditions in 0.2× SSC containing 0.1% SDS for 30 min at 65°C. DIG-labeled bands were visualized using the DIG luminescence detection kit (Roche) according to the manufacturer's instructions.

In vitro transcription and translation. A 611-bp DNA fragment encompassing the *trc* promoter, the *fliC* 5′-UTR, and the first 79 codons of the *fliC* gene was PCR amplified from pIC495-2 with primers PTRCP7 and ICE4B and used as a template for the in vitro transcription experiment. The transcription reaction mixture (50 μl) contained 16 nM template DNA, 1 unit of *E. coli* RNA polymerase holoenzyme (Epicenter, WI), 40 mM Tris-HCl (pH 7.5), 150 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 units of RNase inhibitor (Takara), and various concentrations (0 to 1,600 nM) of His-FljA. After preincubation for 30 min at 37°C, the reaction was initiated by the addition of four nucleotides containing [α-^{32}P]UTP (Amersham Biosciences) at a final concentration of 0.2 mM. After incubation for 20 min at 37°C, the reaction was terminated by the addition of 83 μl of a stop solution (0.6 M sodium acetate [pH 5.5], 20 mM EDTA, 200 μg tRNA/ml). RNAs were precipitated by ethanol and separated on a 6% polyacrylamide gel containing 6 M urea. The labeled transcripts were detected by autoradiography.

The DNA-directed coupled transcription-translation and RNA-directed translation experiments were performed using an *E. coli* S30 extract (Promega, WI) according to the manufacturer's instruction as described previously (31). For the coupled transcription-translation system, the reaction mixture (70 μl) contained 11 nM DNA template linearized by HindIII digestion and 0 or 1,100 nM His-FljA. After incubation for 4 h at 37°C, proteins were precipitated by acetone and vacuum dried. Synthesized FliC proteins were detected by Western blotting using an anti-FliC antibody.

The structure of the RNA template used in the RNA-directed translation experiment is shown in Fig. 2. It was prepared as follows. DNA of pSPT18*fliC*UTR-ORF was linearized by digestion with HindIII and used as a template for in vitro transcription by T7 RNA polymerase (Roche). The RNA transcript was purified as described above. The translation mixture (70 μl), containing 21 nM RNA template, various concentrations (0, 210, and 2,100 nM) of His-FljA, 21 MBq [^{35}S]methionine/ml (Amersham Biosciences), and 50 units of RNase inhibitor (Takara), was incubated for 4 h at 37°C and then mixed with 220 μl of 10 mM Tris-HCl (pH 7.5) containing 5 μg of trypsin inhibitor and 5 μl of anti-FliC antiserum. After incubation for 12 h at 4°C with gentle shaking, 5 mg of protein A-Sepharose CL-4B (Amersham Biosciences) was added to the mixture, and gentle shaking was continued for another 1 h at 4°C. The ternary complex consisting of protein A-Sepharose CL-4B, FliC, and the antibody was collected by centrifugation; washed twice with 300 μl of a wash buffer containing 50 mM Tris-HCl (pH 8.0), 0.15 M NaCl, 0.1 mM EDTA, and 0.1% lubrol; and then subjected to electrophoresis on an SDS–12% polyacrylamide gel. ^{35}S-labeled proteins were detected by fluorography using an Amplify fluorographic reagent (Amersham Biosciences).

RNA stability analysis. Growing cells in LB were treated with rifampin at a final concentration of 200 μg/ml, and aliquots were sampled at 0, 1, 5, 10, and 20 min after rifampin addition. RNAs extracted from the cells were analyzed by Northern blotting as described above.

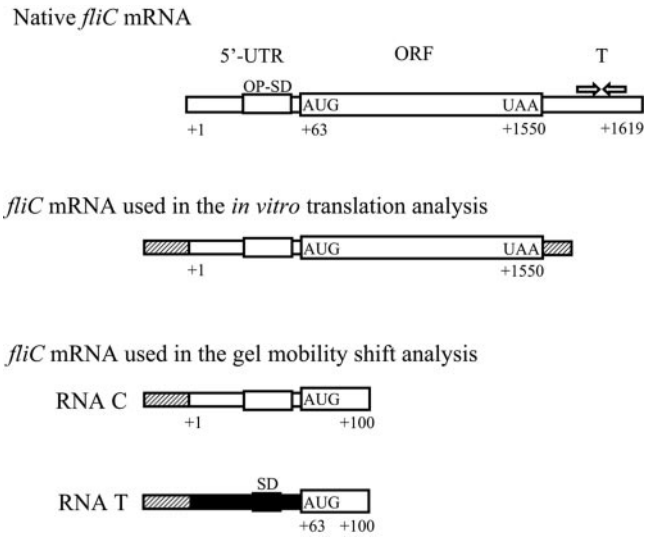

FIG. 2. Schematic representation of the structure of the native and engineered *fliC* mRNAs. RNAs derived from the native *fliC* gene, pTrc vectors, and pSPT18 are shown by open, filled, and hatched rectangles, respectively. OP and SD indicate the operator and Shine-Dalgarno sequences of the *fliC* gene on RNAs. T indicates the terminator sequence of *fliC* mRNA. The 5′-terminal nucleotide of the native *fliC* mRNA is numbered +1.

RNA binding assay. The structure of the RNA fragments used for the gel mobility shift assay is shown in Fig. 2. They were prepared as follows. DNAs of pSPT18*fliC*UTR-ORF and pSPT18*trc*UTR-*fliC*ORF were linearized by digestion with HincII and used as templates for runoff transcription by T7 RNA polymerase to obtain 121- and 98-nucleotide transcripts, respectively. The former (named RNA C) contains a 5′ portion (positions +1 to +100) of native *fliC* mRNA, and the latter (named RNA T) contains the *trc* 5′-UTR (positions +1 to +39) and the 5′ portion of the *fliC* ORF (positions +63 to +100). These RNAs were gel purified, dephosphorylated with calf intestinal alkaline phosphatase (Toyobo), and then labeled at the 5′ end with [γ-^{32}P]ATP (Amersham Biosciences) by T4 polynucleotide kinase (Toyobo). The binding reaction mixture (20 μl) contained 0.5 nM labeled RNA, 10 mM Tris-acetate (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl, 50 mM KCl, 10 mM dithiothreitol, 5% glycerol, and various concentrations of His-FljA. In the competition assay, unlabeled RNA competitors were added to the reaction mixture at a final concentration of 50 nM. The reaction mixture was incubated for 30 min at 37°C and subjected to electrophoresis at 4°C on native 6% polyacrylamide gels containing 5% glycerol in 0.5× TBE buffer (46 mM Tris base, 46 mM boric acid, 1 mM EDTA). Labeled RNAs were detected by autoradiography.

RESULTS

Absence of *fliC* mRNA in phase 2 cells. In order to resolve the confusion regarding two previously reported conflicting results on the *fliC* mRNA level in phase 2 cells (2, 50), we performed Northern blotting analysis of flagellin mRNAs using *fliC*- and *fljB*-specific RNA probes. The probes used were designed to be complementary to the central regions of the respective flagellin mRNAs. Since the central region corresponds to the sequence-variable domain of flagellin (26), the respective probe was expected to hybridize specifically with either *fliC* or *fljB* transcript. In hybridization with the *fliC*-specific probe (probe 2), an approximately 1.6-kb band was detected in the mRNA sample prepared from phase 1 cells (KK211), whereas no band was observed in the mRNA sample prepared from phase 2 cells (KK212) (Fig. 3A). Since the size of this band is equivalent to the expected size of *fliC* mRNA,

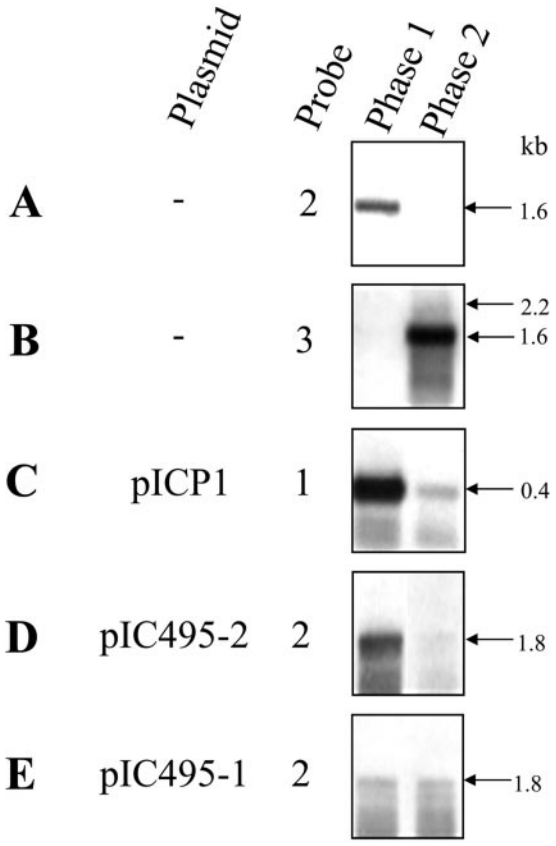

FIG. 3. Steady-state level of *fliC* and *fljB* mRNAs. Total RNAs were isolated from cells, separated on agarose gels, and transferred onto nylon membranes. *fliC* and *fljB* mRNAs were detected by Northern blotting. Strains used: A and B, KK211 (phase 1) and KK212 (phase 2); C, KK211CT (phase 1) and KK212CT (phase 2) harboring pICP1; D, KK211CT (phase 1) and KK212CT (phase 2) harboring pIC495-2; E, KK211CT (phase 1) and KK212CT (phase 2) harboring pIC495-1. Probes used: A, D, and E, probe 2; B, probe 3; C, probe 1. The approximate length of each RNA band is indicated on the right.

we conclude that this band corresponds to *fliC* mRNA. Conversely, in the hybridization with the *fljB*-specific probe (probe 3), an approximately 1.6-kb band was detected in the mRNA sample prepared from phase 2 cells, whereas no band was detected in the mRNA sample prepared from phase 1 cells (Fig. 3B). The full-length size of mRNA for the *fljBA* operon is estimated to be approximately 2.2 kb, which is larger than the size of the band detected in Fig. 3B. It was known that a ρ-independent terminator exists between the *fljB* and *fljA* genes and that 99% of mRNAs initiated from the *fljB* promoter stop transcription at this terminator, yielding a 1.6-kb RNA transcript (16). Therefore, we conclude that the 1.6-kb band hybridized with the *fljB* probe corresponds to the *fljB* transcript. These results clearly indicate that *fliC* mRNA is absent from phase 2 cells, which is consistent with results previously reported by Suzuki and Iino (50).

Bonifield and Hughes (2) used a T2 RNase protection assay to show that *fliC* mRNA was produced even in phase 2 cells. The RNA probe that they used is complementary to the first 200 nucleotides of *fliC* mRNA. This region is highly homologous (92.1% identity) to the corresponding region of *fljB* mRNA, suggesting that this probe could hybridize with not only *fliC* mRNA but also *fljB* mRNA. Therefore, we suppose that the amount of *fliC* mRNA in phase 2 cells might have been overestimated in their T2 protection assay. This supposition is supported by our Northern blotting analysis with probe 1, which covers the first 300 nucleotides of *fliC* mRNA. As expected, this probe hybridized with the 1.6-kb RNA extracted from phase 2 cells as well as that from phase 1 cells (data not shown).

Gene fusion analysis of *fliC* expression in the presence of FljA. The results described above led us to reexamine the effect of FljA on the expression of *fliC-lacZ* transcriptional and translational fusion genes. Two types of transcriptional fusions were used in this study. One was a chromosomal *fliC-lacZ* fusion (30), in which the Mu*d1*(*amp lac*) phage DNA had been inserted into the *fliC* ORF after nucleotide position +941 from the transcriptional start site, and the other was a *fliC-lacZ* fusion gene on a plasmid, pRLIC0, in which a DNA segment (positions −35 through +1550) containing the promoter-operator region and the entire *fliC* ORF with its stop codon was fused transcriptionally to the *lacZ* gene on a promoter-probe vector, pRL124. In the chromosomal fusion, β-galactosidase activity was reduced 2.5-fold in phase 2 cells compared with that in phase 1 cells (Table 4). With the fusion gene on the plasmid, a small reduction in the enzyme activity was also observed in phase 2 cells.

The *fliC-lacZ* translational fusion gene used was on a plasmid, pMCIC0, in which the DNA segment (positions −35 through +1547) containing the promoter-operator region and the entire ORF of *fliC* without its stop codon was fused translationally to the *lacZ* gene on a vector, pMC1871. In phase 2 cells, β-galactosidase activity was reduced 10-fold compared with that in phase 1 cells (Table 4). Therefore, in the analysis with *fliC-lacZ* fusion genes, FljA is able to repress *fliC* expression at both transcriptional and translational levels, and the translational repression is more profound. These results coincide with those from the chromosomal gene fusion study reported previously by Bonifield and Hughes (2).

Effect of FljA on stability of *fliC* mRNA. The above-mentioned results mean that *fliC* mRNA is absent from phase 2 cells, although a low but considerable level of transcription of the *fliC* gene occurs in phase 2 cells. This raised the possibility

TABLE 4. Effect of FljA on the expression of *fliC-lacZ* fusion genes

fliC-lacZ fusion	β-Galactosidase activity (Miller units ± SD) in:	
	Phase 1 cells[a]	Phase 2 cells[a]
Transcriptional fusion		
Chromosome	1,270 ± 50	510 ± 20
pRLIC0	3,590 ± 200	2,810 ± 140
pRL124	90 ± 10	110 ± 20
Translational fusion		
pMCIC0	4,640 ± 110	470 ± 30
pMC1871	60 ± 10	70 ± 30

[a] Strains used for the β-galactosidase assay are as follows: chromosomal *fliC-lacZ* fusion gene, KK211CL (phase 1) and KK212CL (phase 2); plasmid-borne *fliC-lacZ* fusion gene, KK211 (phase 1) and KK212 (phase 2).

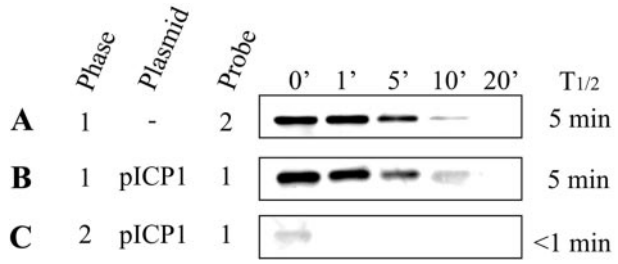

FIG. 4. Decay of *fliC* mRNA. Cells were grown in LB at 37°C. At time intervals after the inhibition of transcription with rifampin, total RNAs were isolated from the cells. Equal amounts of each RNA sample were then analyzed by Northern blotting. Strains used: A, KK211; B, KK211CT harboring pICP1; C, KK212CT harboring pICP1. Probes used: A, probe 2; B and C, probe 1. The length of the half-life ($T_{1/2}$) for each RNA is shown on the right.

that the *fliC* gene might be transcribed efficiently in phase 2 cells but that the synthesized *fliC* mRNA might be degraded rapidly in the presence of FljA. In order to test this possibility, we examined the effect of FljA on the stability of *fliC* mRNA.

Total RNA was isolated from phase 1 cells at various time points after rifampin addition and analyzed by Northern blotting with probe 2 (Fig. 4A). The half-life of the *fliC* mRNA calculated from the band density was approximately 5 min. In order to make it possible to detect *fliC* mRNA in phase 2 cells, we used cells harboring pICP1, a multicopy plasmid carrying a DNA segment between positions −35 and +102 of the *fliC* gene followed by the *rrnB* terminator, producing a 415-nucleotide mRNA transcribed from the *fliC* promoter. As shown in Fig. 3C, the steady-state level of the *fliC* mRNA encoded by pICP1 was much reduced in phase 2 cells compared with that in phase 1 cells. This indicates that the *fliC* DNA sequence downstream of position +103 is dispensable for regulation by FljA. However, a low but detectable amount of *fliC* mRNA was produced from pICP1 even in the phase 2 cells (Fig. 3C), probably because of a high level of expression of *fliC* mRNA from a multicopy plasmid. This enabled us to measure the half-life of *fliC* mRNA in the presence of FljA. In phase 1 cells, the half-life of *fliC* mRNA from pICP1 was approximately 5 min (Fig. 4B), which is equivalent to that of the full-length *fliC* mRNA transcribed from a chromosomal *fliC* gene. In contrast, the half-life of *fliC* mRNA in phase 2 cells was less than 1 min (Fig. 4C), which is much shorter than that in phase 1 cells. This result suggested that the absence of *fliC* mRNA in phase 2 cells might result from its loss of stability in the presence of FljA.

Effect of FljA on *fliC* mRNA expression from a foreign promoter. The operator mutations that rendered *fliC* expression insensitive to FljA were all mapped within the 5′-UTR of the *fliC* gene, but their positions are far downstream (positions +46 to +59) of the *fliC* promoter (21). This suggested that the DNA sequence around the *fliC* promoter is not important for regulation by FljA. To address this issue, we constructed a hybrid plasmid, pIC495-2, in which a full-length *fliC* mRNA including its native 5′-UTR is transcribed from the *trc* promoter and transcription terminates at the *rrnB* terminator, yielding a 1,868-nucleotide transcript (Fig. 1). Total RNA isolated from cells harboring this plasmid was analyzed by Northern blotting with probe 2. The amount of *fliC* mRNA was much reduced in phase 2 cells compared with that in phase 1 cells (Fig. 3D), indicating that FljA can repress the *fliC* gene even under the control of the *trc* promoter.

Next, we analyzed *fliC* expression using cells harboring pIC495-1 in which not only the promoter but also the entire 5′-UTR of the *fliC* gene had been replaced with those of *trc* (Fig. 1). From this plasmid, approximately equal amounts of the hybrid *fliC* mRNA were produced in phase 1 and phase 2 cells (Fig. 3E). Therefore, we conclude that the 5′-UTR of the *fliC* gene or transcript that includes the putative operator sequence plays an important role in FljA-dependent regulation.

Translational repression of the *fliC* gene by FljA. His_6-tagged FljA (His-FljA) was purified as described in Materials and Methods and used for in vitro analysis of *fliC* expression. In the in vitro transcription-translation-coupled system with an *E. coli* S30 extract, two plasmids, pIC495-1 and pIC495-2, were used as templates. The FliC proteins synthesized were analyzed by Western blotting with anti-FliC antibody (Fig. 5A). In the absence of His-FljA, FliC was synthesized from both templates. However, when His-FljA was added at a 100-fold molar excess over the template DNA, FliC was not synthesized from pIC495-2. In contrast, FliC synthesis from pIC495-1 was not affected significantly in this condition.

In order to know whether transcription or translation of the *fliC* gene was inhibited by FljA, we performed an in vitro transcription experiment using *E. coli* RNA polymerase holoenzyme. It was found that the addition of His-FljA at up to a 100-fold molar excess over the DNA template did not affect the production of *fliC* transcript (Fig. 5B). Next, we performed an in vitro translation experiment with an *E. coli* S30 extract using in vitro-synthesized *fliC* mRNA as a template. FliC was synthesized in the absence of His-FljA, whereas FliC synthesis was completely inhibited in the presence of His-FljA at a 10-fold molar excess over the template RNA (Fig. 5C). In this experiment, stability of *fliC* mRNA was also monitored by Northern blotting. It was shown that the presence of His-FljA did not affect the stability of *fliC* mRNA (data not shown). These results indicate that FljA inhibits translation, and not transcription, of the *fliC* gene in vitro.

Stability of *fliC* mRNA in the absence of its translation. According to the results described above, FljA facilitates the degradation of *fliC* mRNA in vivo, whereas FljA inhibits the translation of *fliC* mRNA in vitro. This raised the possibility that FljA-enhanced degradation of *fliC* mRNA in vivo may result from the inhibition of its translation by FljA. In order to test this possibility, two plasmids, pICP2 and pICP2M, were constructed (Fig. 1). pICP2 encodes a C-terminally truncated FliC. In pICP2M, the initiation codon, ATG, of the *fliC* gene on pICP2 was replaced with CTG, which was expected to inhibit the translation of the *fliC* gene. Cells harboring either one of these plasmids were examined for production of *fliC* mRNA and FliC protein by Northern blotting with probe 1 and Western blotting with anti-FliC antibody, respectively. As expected, FliC protein was not produced from pICP2M even in phase 1 cells (Fig. 6B). It was shown that the steady-state level of *fliC* mRNA from pICP2M was much reduced even in phase 1 cells (Fig. 6B). This suggests that *fliC* mRNA is destabilized in the absence of its translation. In order to confirm this, we compared the half-lives of *fliC* mRNAs from pICP2 and pICP2M in phase 1 cells (Fig. 6C and D). As expected, the

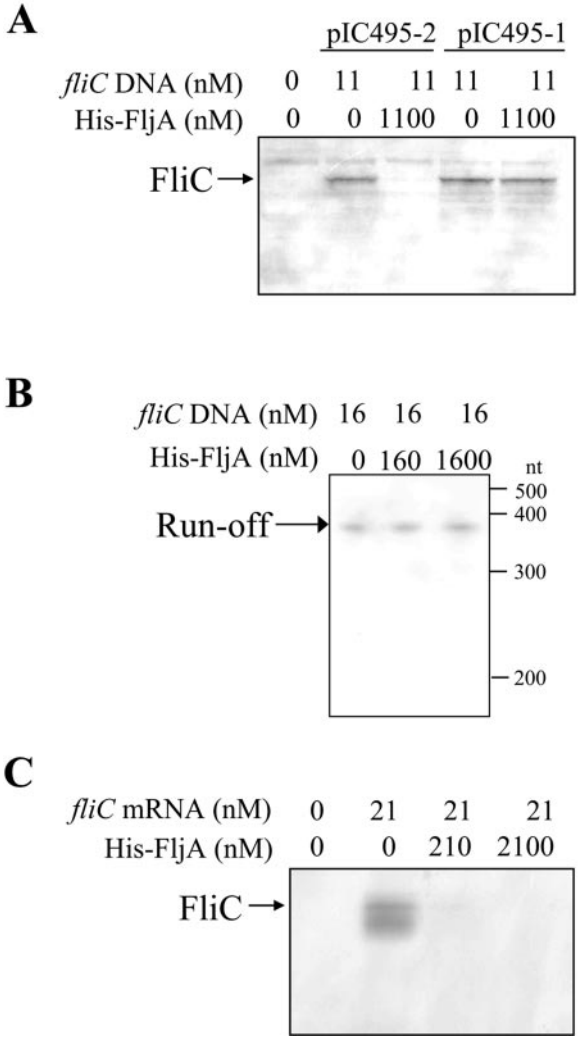

FIG. 5. Effect of FljA on the *fliC* expression in vitro. (A) DNA-directed transcription-translation-coupled system. The *fliC* gene was expressed from the *trc* promoter. The *fliC* transcript encoded by pIC495-2 contains the *fliC* UTR, whereas that encoded by pIC495-1 contains the *trc* UTR. Proteins were synthesized in an *E. coli* S30 extract with or without His-FljA. Proteins were separated by SDS-PAGE, and FliC was detected by Western blotting with anti-FliC polyclonal antibody. (B) In vitro transcription. The DNA fragment containing the *trc* promoter and the *fliC* UTR was transcribed in vitro with *E. coli* RNA polymerase holoenzyme with or without His-FljA in the presence of [α-^{32}P]UTP. Synthesized RNAs were separated on a polyacrylamide gel containing 6 M urea and detected by autoradiography. nt, nucleotide. (C) RNA-directed protein synthesis. An RNA template used was synthesized in vitro and contained the *fliC* UTR and ORF. Protein synthesis was carried out in an *E. coli* S30 extract with or without His-FljA in the presence of [^{35}S]methionine. Synthesized FliC protein was immunoprecipitated, separated by SDS-PAGE, and detected by fluorography. An extra band with a higher mobility might have been derived from *fliC* mRNA of incomplete length.

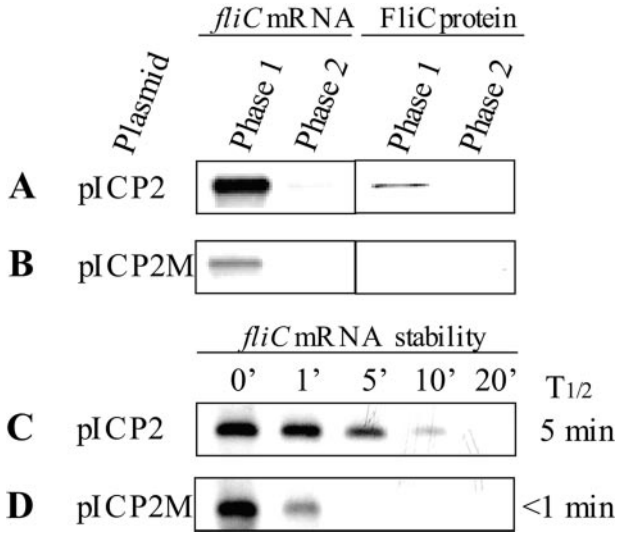

FIG. 6. Stability of *fliC* mRNA in the absence of its translation. (A and B) Steady-state levels of *fliC* mRNA and FliC protein encoded by the *fliC* gene with and without the start codon. *fliC* mRNA was detected by Northern blotting with probe 1. FliC protein was detected with Western blotting with anti-FliC antibody. (C and D) Decay of *fliC* mRNA with and without the start codon. Decay of *fliC* mRNA was measured by Northern blotting with probe 1 as described in the legend of Fig. 4. Strains used: A, KK211CT (phase 1) and KK212CT (phase 2) harboring pICP2; B, KK211CT (phase 1) and KK212CT (phase 2) harboring pICP2M; C, KK211CT harboring pICP2; D, KK211CT harboring pICP2M. $T_{1/2}$, half-life.

half-life of *fliC* mRNAs from pICP2M (less than 1 min) was much shorter than that from pICP2 (approximately 5 min).

Binding of FljA to *fliC* mRNA. DNA binding activity was not observed in the purified His-FljA protein in a gel mobility shift assay using ^{32}P-labeled *fliC* DNA as a probe (data not shown). The fact that FljA inhibits the translation of *fliC* mRNA suggested that FljA binds to *fliC* mRNA. In order to test this, a gel mobility shift assay was performed using ^{32}P-labeled *fliC* mRNA (RNA C) synthesized in vitro. This RNA was composed of the first 100 nucleotides of *fliC* mRNA containing the putative operator sequence. A shifted band was detected in the presence of 5 nM His-FljA. When ^{32}P-labeled RNA lacking the putative operator sequence (RNA T) was used, no shifted band was observed even in the presence of 500 nM His-FljA (Fig. 7). Binding specificity was examined by a competition experiment using unlabeled RNAs as competitors. As a result, the unlabeled RNA C behaved as an effective competitor, whereas the unlabeled RNA T did not, indicating a high specificity in binding of FljA to *fliC* mRNA containing the operator sequence.

DISCUSSION

The dual controlling system governs flagellar phase variation of *Salmonella*; one part of the system is Hin-mediated inversion of the H segment containing the promoter for the *fljBA* operon, and the other is FljA-mediated inhibition of the *fliC* expression. Although the mechanism of the former system has already been well documented at a molecular level (18, 24, 25), there have been very few studies on the mechanism of the latter system. Suzuki and Iino (50) showed that phase 2 cells did not contain mRNA with FliC-synthesizing activity. Since then, FljA-mediated repression of the *fliC* gene has long been believed to be achieved at a transcriptional level. However, Bonifield and Hughes (2) showed evidence suggesting that this repression might occur mainly at a posttranscriptional level.

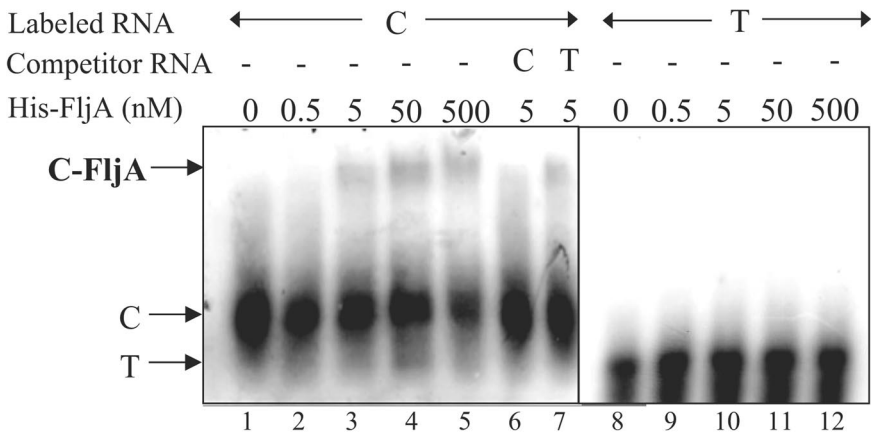

FIG. 7. Gel mobility shift of *fliC* mRNA with His-FljA. RNAs were 5′-end labeled with ^{32}P. After incubation with His-FljA, the RNA-protein mixture was separated on a native polyacrylamide gel, and labeled RNA was detected by autoradiography. RNAs used: C, RNA C (native *fliC* mRNA); T, RNA T (*trc-fliC* hybrid mRNA). In the competition experiments (lanes 6 and 7), 50 nM unlabeled RNA C (C) or RNA T (T) was used. Positions of free and bound RNAs are shown by arrows on the left.

This study was carried out to clarify this discrepancy. We showed that *fliC* mRNA was not detected in the presence of FljA, which coincides with the observation previously reported by Suzuki and Iino (50). However, in the *fliC-lacZ* gene fusion analysis, FljA-dependent repression was not so strong at the transcriptional level but was severe at the translational level, which conforms to the observation reported previously by Bonifield and Hughes (2). In order to explain these conflicting results, we examined the effect of FljA on the stability of *fliC* mRNA and found that it was much shorter in the presence of FljA than in the absence of FljA. Therefore, we can explain the above-mentioned conflicting results as follows: in phase 2 cells, transcription of the *fliC* gene occurs, but synthesized *fliC* mRNA is degraded rapidly in the presence of FljA.

Using purified His-tagged FljA protein, we showed that FljA binds specifically to *fliC* mRNA and inhibits the translation in vitro from *fliC* mRNA. Consistent with this, FljA belongs to a superfamily of Lsm ribonucleoproteins, which includes important modulators of RNA biosynthesis and function (17, 27). The binding affinity of FljA to *fliC* mRNA seems to be very low, because a complete shift could not be attained in a gel mobility shift assay in the presence of FljA, even at a 1,000-fold molar excess over *fliC* mRNA (Fig. 7). This suggests a possibility that another protein(s) may be required for maximal interaction between FljA and *fliC* mRNA.

Previously isolated operator mutations that rendered *fliC* expression insensitive to FljA defined the putative operator site around the SD sequence of the *fliC* gene (21). It is reasonable to postulate that this putative operator sequence acts as the FljA-binding site. This site locates within the 5′-UTR of the *fliC* gene and thus is transcribed into mRNA. Therefore, we propose that FljA binds to *fliC* mRNA at this site. Consistent with this, the *fliC* mRNA lacking this sequence showed no activity to bind FljA in the gel mobility shift assay (Fig. 6). Because the SD sequence should act as a ribosome-binding site (46), binding of FljA to this site may prevent ribosomes from binding to this site, which leads to translation inhibition of the *fliC* gene. Therefore, like some ribosomal proteins such as S4 and S15 of *E. coli* (10, 11, 41) and Reg protein of bacteriophage T4 (52), FljA must act as a translational repressor.

Next, we consider a possible mechanism whereby *fliC* mRNA is destabilized in phase 2 cells. In several genes, translating ribosomes have been known to interfere with the degradation of mRNAs (3, 6, 22, 23, 42, 54). For example, ribosomes inhibit an RNase E cleavage of *rpsO* mRNA in *E. coli* (3). By analogy with this, we propose that in phase 2 cells, FljA binds to *fliC* mRNA and inhibits its translation, which in turn facilitates its degradation. This is supported by our observation that an engineered untranslatable *fliC* mRNA was degraded rapidly even in the absence of FljA (Fig. 6). It is unlikely that FljA itself may act as an RNase responsible for the degradation of *fliC* mRNA, because FljA did not facilitate the degradation of *fliC* mRNA in vitro (data not shown). In our preliminary experiment, *fliC* mRNA was shown to be stabilized in the mutant defective in the RNase E gene even in the presence of FljA (our unpublished results), suggesting that the degradosome including RNase E (4) may be implicated in this regulation.

In the *fliC-lacZ* transcriptional fusion gene, a weak decrease in β-galactosidase activity was observed in the presence of FljA (Table 4). This suggests that transcriptional repression may also be involved in FljA-mediated inhibition of *fliC* expression. The *fljA* gene is known to be transcribed inefficiently, owing to the ρ-independent terminator between the *fljB* and *fljA* genes (16). This is also supported by our observation that a full-length *fljBA* mRNA was hardly observed in phase 2 cells (Fig. 3). Therefore, the cellular level of FljA should be significantly low. However, in this study, the chromosomal *fljA* gene was found to be able to inhibit the expression of the *fliC* gene on multicopy plasmids (Fig. 3). This indicates that multiple DNA copies of the operator sequence cannot efficiently titrate the intracellular FljA proteins. Consistent with this, the DNA fragment containing the operator sequence showed no activity to bind FljA in a gel mobility shift assay (data not shown). Therefore, it is unlikely that FljA may repress transcription of the *fliC* gene by binding to the operator site on DNA. At present,

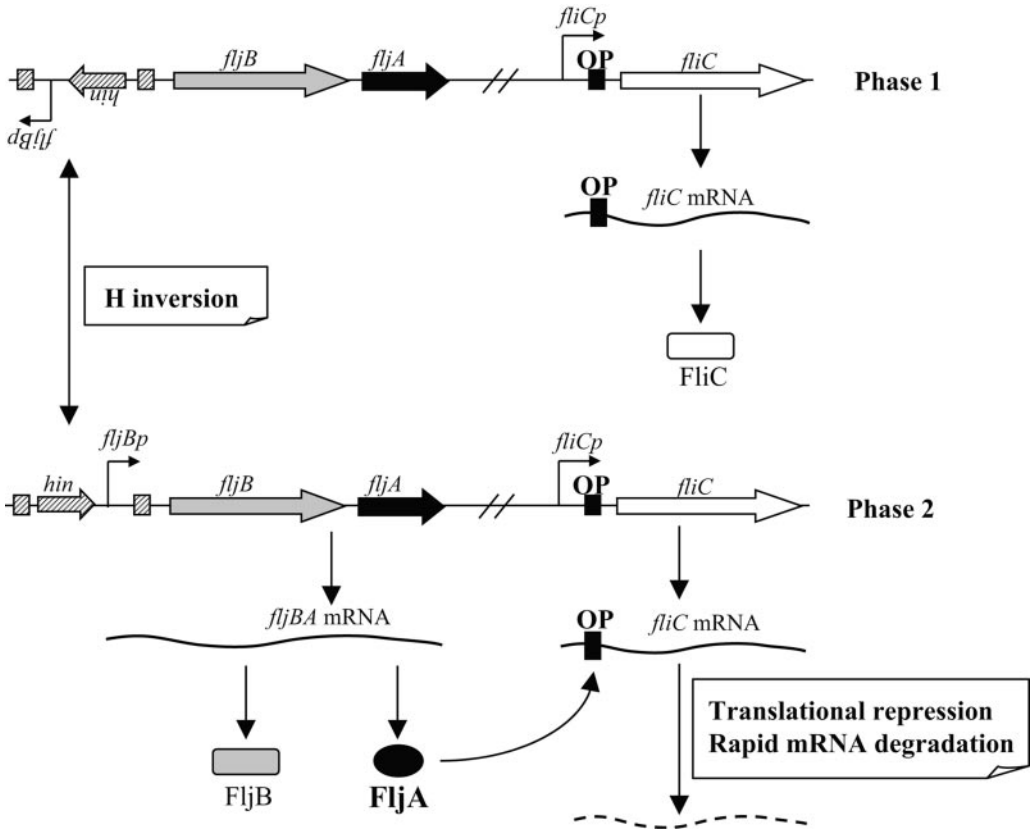

FIG. 8. Model for the mechanism of genetic control of flagellar phase variation in *Salmonella*. The dual controlling system governs flagellar phase variation; one part of the system is Hin-mediated inversion of the H segment containing the promoter for the *fljBA* operon, and the other is FljA-mediated inhibition of *fliC* expression. FljA binds to the 5′-UTR of *fliC* mRNA, which inhibits its translation and facilitates its degradation. OP, operator sequence.

we cannot think of a special mechanism for transcriptional repression by FljA. However, there is a possibility that the *fliC-lacZ* hybrid mRNA transcribed from the *fliC-lacZ* transcriptional fusion gene may be susceptible to RNase attack due to the presence of an FljA-binding site, which may reduce β-galactosidase activity to some extent in the presence of FljA.

We also showed that the chromosomal *fljA* gene is sufficient to inhibit *fliC* expression from the *trc* promoter, which is known to be one of the strongest promoters in bacteria (38). This suggests that overproduced *fliC* mRNAs cannot titrate intracellular FljA proteins either. This is not a surprising result, because *fliC* mRNA is degraded rapidly in the presence of FljA (Fig. 4). However, the possibility that the binding affinity of FljA to *fliC* mRNA is too low to titrate out the cellular FljA proteins cannot be ruled out.

In Fig. 8, we summarize the present understanding of the mechanism controlling flagellar phase variation of *Salmonella*. H inversion is an on-off switch of transcription of the *fljBA* operon, whereas FljA is a posttranscriptional regulator of the *fliC* operon. It is believed that posttranscriptional control enables bacteria to quickly adjust their gene expression to environmental changes (8, 14, 43, 44, 49). Flagellar filaments or flagellin subunits act as potent antigens in host organisms, and there have been several reports suggesting that flagellar phase variation might influence the virulence phenotype of *Salmonella* (7, 20, 45, 51). Therefore, it is possible that the mode of its genetic control may have some effect on the growth or survival of *Salmonella* cells in hosts. However, at present, the biological significance of *Salmonella* employing posttranscriptional control for FljA-mediated inhibition of *fliC* expression remains unknown.

Two FljA paralogues are known to exist: one is HCM2.0081, encoded by a plasmid, pHCM2, found in *S. enterica* serovar Typhi CT18 (39), and the other is Y1019, encoded by a plasmid, pMT1, found in *Yersinia pestis* KIM (35). More than 30% of their amino acids are identical to those of FljA (data not shown). Therefore, although their function has not yet been characterized, it is reasonable to postulate that they may regulate gene expression through a mechanism similar to FljA.

ACKNOWLEDGMENTS

We thank Naoya Tanaka for his assistance in construction of some plasmids used in this study. Radioisotope facilities were provided by the Advanced Science Research Center Tsushima Division, Okayama University.

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