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Flagellar Phase Variation in *Salmonella enterica* Is Mediated by a Posttranscriptional Control Mechanism

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***Salmonella enterica* has two antigenically distinct flagellin genes, *fliC* and *fljB*, that are alternatively expressed. The *fljA* gene is cotranscribed with *fljB* and encodes a protein that has been characterized as a transcriptional repressor of the unlinked *fliC* gene when FljB is expressed. In this study we report genetic evidence that FljA prevents the production of FliC protein through an interaction with the 5′-untranslated region of the *fliC* mRNA transcript. Studies with operon and gene fusions, Western analyses, and T_2 RNase protection assays were performed for strains with the *fljBA* promoter locked in either the on or the off orientation. β-Galactosidase assays of *fliC* transcriptional and translational fusions to the *lac* operon demonstrated that while FljA inhibits *fliC* transcription fivefold in the *fljBA*^{ON} orientation, it has a 200-fold effect on both *fliC* transcription and translation, indicating that the FljA inhibitor might act at both the transcriptional and translational level. T_2 RNase protection assays also demonstrated a fivefold decrease in *fliC* transcript levels for cells locked in the *fljBA*^{ON} orientation compared to those in the *fljBA*^{OFF} orientation, and an eightfold decrease in FliC protein levels was observed by Western analysis. This reduction in FliC protein levels is greater than the decrease observed for the transcript. These results are consistent with a new model whereby FljA inhibits FliC expression by an attenuation or translational control mechanism.**

Bacterial flagella facilitate the mobility of the organism within its environment, allowing it to move towards attractants and away from repellants (reviewed in reference 4). *Salmonella enterica* serovar Typhimurium has approximately 6 to 10 flagella that are peritrichously arranged around the cell. The individual flagella are composed of three distinct substructures: the basal body, a transmembrane motor; a hook that links the motor and the filament; and the filament that acts as a propeller (reviewed in references 1 and 36). The filament is approximately 10 μm in length and composed of approximately 20,000 subunits of flagellin protein, either FliC or FljB. During assembly, structural subunit proteins are secreted by a flagellum-specific type III secretion system (36a), assembled at the base of the flagellum, through the elongating structure, and added onto the distal tip (9, 24).

Flagellum biogenesis is a highly ordered process whereby gene expression closely parallels expression and assembly of the subunit proteins (5, 27). The expression of more than 50 genes is required for the assembly, function, and maintenance of these structures. The promoters of the flagellar regulon can be organized into three classes that determine their temporal expression. The class 1 promoter directs transcription of the *flhDC* master operon and includes six known transcriptional start sites that respond to different environmental signals (52). FlhD and FlhC form a heterotetrameric complex that is a transcriptional activator for σ^{70}-dependent transcription of the class 2 promoters (3, 34, 35). Class 2 promoters mediate the transcription of genes required for the structure and assembly of the hook-basal body (HBB) in addition to flagellum-specific sigma factor σ^{28}, FliA (41), and its cognate anti-sigma factor FlgM (42). Class 3 promoters require σ^{28}-RNA polymerase for their transcription (21). FlgM has been found to associate with σ^{28} and prevent class 3 transcription until completion of the intermediate HBB structure (13, 29). Upon HBB completion, FlgM is secreted outside of the cell, resulting in σ^{28}-dependent transcription from the class 3 promoters (22, 30) which direct transcription of the hook-associated genes, flagellin genes, and genes whose products are required for chemotaxis and flagellar rotation.

S. enterica alternately expresses two different flagellar filament proteins, FljB and FliC, in a process known as flagellar phase variation (2) (Fig. 1). The molecular mechanism mediating flagellar phase variation occurs by a site-specific DNA inversion event in the chromosome (reviewed in reference 20). The promoter for the FljB flagellin protein is flanked by the recombination sites *hixL* and *hixR* (Fig. 1). The *Hin* recombinase, in conjunction with the recombination enhancer proteins Fis (factor for inversion stimulation) and HU, mediates a reversible recombination reaction between the *hix* sites, resulting in the inversion of the DNA segment containing the *fljBA* promoter. In one orientation the *fljBA* promoter directs transcription of the *fljBA* operon and FljB flagellin is produced. The *fljA* gene is cotranscribed with *fljB* and encodes a transcriptional inhibitor of the unlinked *fliC* gene (11, 31, 33, 43, 46, 49). In the alternate orientation, the *fljBA* operon is not expressed and transcription of the *fliC* gene ensues.

The FliC and FljB flagellin proteins themselves are identical for the first 71 amino acids and last 46 amino acids, but surface-exposed amino acids in the middle are divergent, resulting in distinct antigenicities (40). *S. enterica* alternates between expressions of these proteins at a rate of 10^{-3} to 10^{-5} per cell generation (14, 48). In fact, most *Salmonella* phase vary, at similar rates, between expression of two different flagellin genes (10). As with FliC and FljB, there is a great deal of

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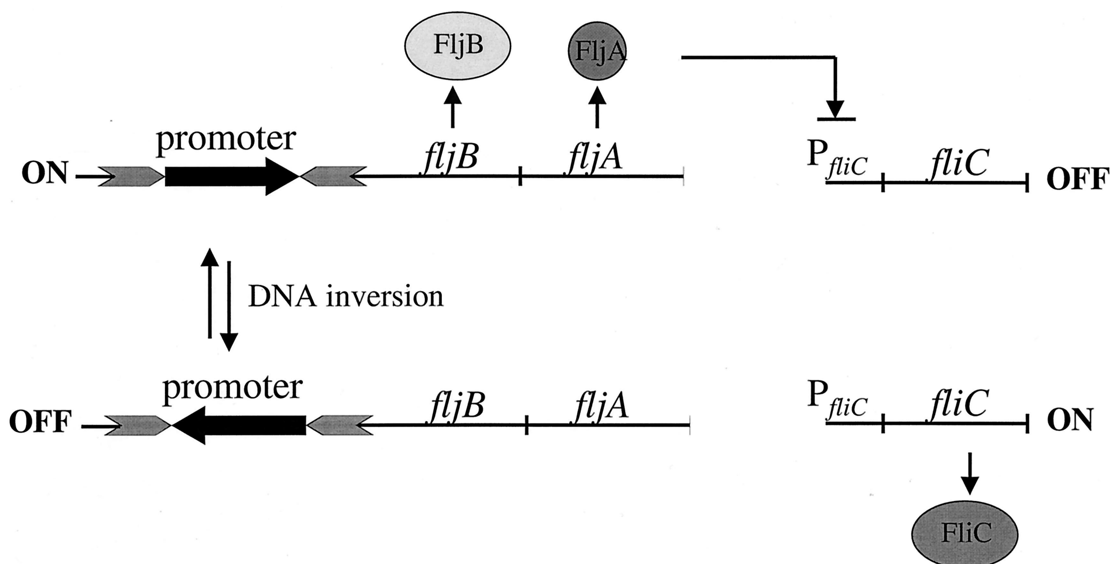

FIG. 1. A schematic representation of flagellar phase variation in *S. enterica*. The promoter for the *fljBA* operon is located within an invertible DNA segment whereby inversion of the promoter is mediated by the *Hin* recombinase. In one orientation, the *fljBA* operon is expressed and FljB flagellin is produced along with FljA, repressor of the unlinked *fliC* gene that encodes FliC flagellin. In the opposite orientation, the *fljB* gene is not expressed, nor is the repressor FljA, thus allowing transcription of the *fliC* gene.

variation in the central portion of these flagellins, while the amino- and carboxy-terminal domains are highly conserved (51). Flagellin protein itself is a potent antigen that stimulates the innate immune response in many plants and animals. Recently, Hayashi et al. (19) showed that stimulation of the TLR5 toll-like receptor by bacterial flagellin protein, including the *Salmonella* FliC protein, resulted in the mobilization of the nuclear factor NF-κB and stimulation of tumor necrosis factor alpha production. In addition, most *Salmonella*-specific $CD4^+$ T lymphocytes generated in response to a *Salmonella* infection have been shown to be directed at flagellin epitopes (6). Although flagellar phase variation has been postulated to play a role in the pathogenesis of the organism by providing a mechanism for the bacteria to temporarily avoid cellular immunity (10, 25), its role in *S. enterica* pathogenesis is not understood.

In contrast to our limited understanding of the biological significance of phase variation, the molecular mechanisms mediating this phenomenon have been well elucidated. For example, current dogma suggests that the FljA protein, encoded by the *fljA* gene downstream of *fljB*, is a transcriptional repressor of the unlinked *fliC* gene when FljB is expressed (16, 36, 47). In this study we provide evidence to support a mechanism by which FljA prevents production of FliC protein at the posttranscriptional level. Our results indicate that in addition to inhibiting *fliC* transcription, FljA regulates *fliC* translation. Because previous studies have identified mutations in the 5′-untranslated region (UTR) of the *fliC* transcript that bypass FljA regulation, we propose a model in which FljA regulates both *fliC* transcription and translation via interactions with the 5′-UTR of the transcript.

MATERIALS AND METHODS

Strains. The bacterial strains used in this study are presented in Table 1. Unless noted otherwise, all strains were constructed for this work.

Culture and growth medium conditions. Strains were cultured in Luria-Bertani medium with aeration as described by Davis et al. (8). Strains containing the pKD46, pKD3, or pKD4 plasmids (7) were grown at either 30°C (pKD46) or 37°C (pKD3 and pKD4) in Luria-Bertani medium with aeration in the presence of 100 μg of ampicillin (Sigma, St. Louis, Mo.)/ml.

Construction of *S. enterica* strains. Markers were mobilized between *Salmonella* strains by generalized transduction using the mutant P22 HT/*int* bacteriophage (37). Resistance markers were selected for by using the following antibiotic concentrations: ampicillin, 30 or 100 μg/ml; chloramphenicol, 12.5 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 12.5 μg/ml.

Quantitative immunoblot assays for FliC. Cells were grown overnight with aeration and then subcultured and grown to an optical density at 600 nm (OD_{600}) of ~0.6. One milliliter of culture was centrifuged, and the pellet was resuspended in 50 μl of sample buffer (32). Samples were run on 10% Tricine–sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (44), and proteins were transferred to polyvinylidene difluoride membranes (Schleicher & Schuell, Inc., Keene, N.H.) in 3-(cyclohexylamine)-1 propanesulfonic buffer (38) and probed with rabbit anti-FliC antibody (Becton Dickinson, Sparks, Md.) purified according to the methods of Hughes et al. (22). Primary antibody was detected, and protein levels were determined as previously described (28). Protein levels for each sample were recorded as phosphorimager units per OD_{600}.

Isolation of a translational fusion to the *fliC* gene. Translational fusions of the *fliC* gene to the *lacZ* gene were made using the Mu*d*K-*lac*(Mu*d*II1734) fusion vector (17a). Strain TH1059 contains a Tn*10d*Cm insertion in the IS*200* element adjacent to the *fliC* gene. Random Mu*d*K insertion mutants were introduced into TH1059 by the transitory *cis*-complementation method (23). The Mu*d*K insertion mutants were pooled, P22 transducing lysates were prepared on these pools, and Mu*d*K insertions linked to the Tn*10d*Cm insertion were identified. Isolates with linked insertions were tested for motility and phase variation. Potential Mu*d*K insertions within the *fliC* gene were confirmed by PCR amplification using a primer homologous to the adjacent *fliD* gene reading towards *fliC* (*fliD* 5′ out, 5′-ACAGAAGCTTCATAGGCGGTTAGCTTTGC; Life Sciences, Boston, Mass.) and a primer homologous to end of the Mu*d*K (mur4, 5′-ATGTAATG AATAAAAAGC; Life Sciences). Products were sequenced using an ABI 377 apparatus (Life Sciences). One Mu*d*K insertion (*fliC5469*::Mu*d*K) was found to be inserted 567 nucleotides into the *fliC* gene, resulting in a translational fusion of the first 189 amino acids of FliC to LacZ, and was used for further studies.

Construction of the *fljA* null mutation. A deletion of the *fljA* gene was constructed using the λ-red system as described by Murphy et al. (39) and modified by Datsenko and Wanner (7). The kanamycin Flp recombinase target site (FRT) cassette was amplified from pKD4 (7) using the following oligonucleotides: 5′

TABLE 1. List of strains

Strain	Genotype	Reference or source[a]
TH1059	IS*200-5548*::Tn*10d*Cm	
TH1077	*fliC5050*::Mu*d*J	12
TH1208	*fliC5050*::Mu*d*A	12
TH6594	*fliC5469*::Mu*d*K	
TH6596	*fliC5469*::Mu*d*Cm	
TH6595	*fliC5469*::Mu*d*B	
TH331	*proAB47 pyrB64*/F′ 128 *zzf-1066*::Mu*d*A	J. Roth
TH2748	*thrA49 leuBCD39 ara-7*/F⁺ *zzf-6820*::Mu*d*Cm	T. Elliot
TH1123	*nadA56*/F′ 152 *zzf-1093*::Mu*d*B	J. Roth
TH4702	LT2 pKD46	
TH5855	Δ*fljA5576*::FRT-Km-FRT	
TH5361	Δ*fliA5648*::FRT-Km-FRT	
TH5947	*fliC5050*::Mu*d*J Δ*hin-5717*::FRT-Cm-FRT	
TH5951	*fliC5469*::Mu*d*K Δ*hin-5717*::FRT-Cm-FRT	
TH5955	*fliC5050*::Mu*d*J Δ*hin-5718*::FRT-Cm-FRT	
TH5959	*fliC5469*::Mu*d*K Δ*hin-5718*::FRT-Cm-FRT	
TH5979	*fliC5050*::Mu*d*A Δ*hin-5718*::FRT-Cm-FRT *fljA5576*::FRT-Km-FRT	
TH5983	*fliC5469*::Mu*d*B Δ*hin-5718*::FRT-Cm-FRT *fljA5576*::FRT-Km-FRT	
TH6592	Δ*hin-5717*::FRT-Cm-FRT *fliA5648*::FRT-Km-FRT	
TH6593	Δ*hin-5718*::FRT-Cm-FRT *fliA5648*::FRT-Km-FRT	
TH5949	*fliC5050*::Mu*d*J Δ*hin-5717*::FRT-Cm-FRT Δ*flgM5301*	
TH5953	*fliC5469*::Mu*d*K Δ*hin-5717*::FRT-Cm-FRT Δ*flgM5301*	
TH5957	*fliC5050*::Mu*d*J Δ*hin-5718*::FRT-Cm-FRT Δ*flgM5301*	
TH5961	*fliC5469*::Mu*d*K Δ*hin-5718*::FRT-Cm-FRT Δ*flgM5301*	
TH5980	*fliC5050*::Mu*d*A Δ*hin-5718*::FRT-Cm-FRT Δ*flgM5301 fljA5576*::FRT-Km-FRT	
TH5984	*fliC5469*::Mu*d*B Δ*hin-5718*::FRT-Cm-FRT Δ*flgM5301 fljA5576*::FRT-Km-FRT	
TH5971	Δ*hin-5717*::FRT-Cm-FRT	
TH5975	Δ*hin-5717*::FRT-Cm-FRT *fljA5576*::FRT-Km-FRT	
TH5862	Δ*hin-5718*::FRT-Cm-FRT	
TH5990	Δ*hin-5718*::FRT-Cm-FRT *fljA5576*::FRT-Km-FRT	

[a] Unless otherwise noted, all strains were constructed for this work.

fljA-FRT (5′-CGGGGCTTTTTCATTTAGCATAGATGAATATATATTTTG TAGGCTGGAGCTGCTTCG) and 3′ *fljA*-FRT (5′-CTTTTCTCACGGAATT TTTTATTACCGTAGGCGCATATGAATATCCTCCTTAG; MWG Biotech, Inc., High Point, N.C.) that contain homology to the upstream and downstream DNA immediately adjacent to the *fljA* gene. TH4702 (LT2/pKD46) (7) was prepared for electroporation such that the cells were concentrated 250-fold and transformed with 50 to 100 ng of PCR product. Recombination into the chromosome of the FRT cassette and loss of the pKD46 plasmid were simultaneously selected for by growing the cells in the presence of 50 μg of kanamycin/ml at 37°C. Constructs were confirmed using the K1-test primer (7) and the hinSspI primer (homologous to the *hin* gene upstream of *fljA*; 5′-CGGCAGCAATTAG CTATTATTTTTAATATTG; MWG Biotech, Inc.).

Construction of phase-variation mutants. The chloramphenicol FRT cassette was amplified from pKD3 using the following oligonucleotides: *hin*-A-FRT, 5′-CCGCTCTGCGATTTTTATAGCGCATCAGCCACACGATTTTGTAGG CTGGAGCTGCTTCG, and *hin*-C-FRT, 5′-TCCTGTTCGTGTCTATTGATC GCCCGAGGGTGCCCTCCCAGCATATGAATATCCTCCTTAG (Life Technologies, Boston, Mass.). The *hin*-A-FRT oligonucleotide contains DNA homologous to the region upstream of the *hixL* site, and the *hin*-C-FRT primer contains DNA homologous to the middle of the *hin* gene reading toward the 5′ end of the gene. The PCR product was transformed into TH4702 with the *fljBA* promoter in the on orientation. Recombination into the chromosome resulted in a deletion of the *hixL* site and 448 nucleotides of the *hin* gene. The *hixR* site and the *fljBA* promoter were still present. The FRT chloramphenicol cassette was also amplified using the *hin*-A-FRT primer in conjunction with the *hin*-B-FRT primer (5′-CTGGGAGGGCACCCTCGGGCGATCAATAGACACGAACAG GACATATGAATATCCTCCTTAG). The *hin*-B-FRT primer has homology to the middle of the *hin* gene reading towards the 3′ end of the gene. Transformation and recombination of this PCR product into TH4702 with the *fljBA* promoter in the off orientation for *fljBA* expression resulted in the deletion of the *hixL* site, the 3′ end of the *hin* gene, and the *fljBA* promoter. Both of these strains are unable to undergo phase variation, with the former constructs being locked in the on orientation for *fljBA* expression and the latter constructs locked in the off orientation.

β-Galactosidase assays. β-Galactosidase assays were performed as described by Maloy (37). Cells were grown to an OD_{600} of ~0.8. Each sample was assayed in triplicate, and the values were recorded as β-galactosidase units (nanomoles per minute per OD_{650} unit per milliliter).

T_2 RNase protection assays. Cells were grown to an OD_{600} of ~0.6, and RNA was isolated as described previously (17). RNase T_2 protection assays of transcripts from the bacterial chromosome were performed as described elsewhere (50). A radiolabeled RNA probe complementary to the first 200 nucleotides of the *fliC* transcript was synthesized with T7 polymerase and the Riboprobe in vitro transcription system (Promega, Madison, Wis.). Template DNA for the in vitro transcription reaction was amplified using the following primers: *fliC*200R-T7P, 5′-TAATACGACTCACTATAGGGCCTGCCGCATCGTCTTTCG, and *fliC*60F, 5′-CGGTGAGAAACCGTGGGC (Integrated DNA Technologies, Inc., Coralville, Iowa). A 15-μg aliquot of total RNA from each strain was added to the hybridization mixture. Transcript levels were quantified with a Storm 840 Imager (Molecular Dynamics), and band intensity was determined using ImageQuant software (Molecular Dynamics).

RESULTS

FliC expression during phase variation is posttranscriptionally regulated. FliC production is known to be repressed when the *fljBA* promoter is in the on orientation (46), but to evaluate the contribution of transcriptional and posttranscriptional mechanisms in the regulation of *fliC* gene expression during phase variation we examined transcription and translation of *fliC* in strains that were locked in either the *fljBA*^{ON} orientation or the *fljBA*^{OFF} orientation (Fig. 2). Transcription of the *fliC* promoter was measured by determining β-galactosidase activities of a *lac* operon fusion to the *fliC* gene. Translation was determined using a *fliC-lacZ* gene fusion, where both transcription and translation of the *lacZ* gene are dependent upon *fliC* gene transcription and translation.

β-Galactosidase levels were down fivefold for the *fliC-lac* transcriptional fusion when the cells were locked in the *fljBA*^{ON} orientation compared to cells locked in the *fljBA*^{OFF} orientation (Fig. 3, columns 1 and 2), indicating that *fliC* transcription is somewhat inhibited when FljB is expressed. In contrast, a 200-fold decrease in β-galactosidase activity was observed with the FliC-LacZ translational fusion for cells locked in the *fljBA*^{ON} orientation compared to the *fljBA*^{OFF} orientation (Fig. 3, columns 4 and 5). The fact that there is only a fivefold effect of FljA on *fliC* transcription, but a 200-fold effect of FljA on both *fliC* transcription and translation, suggested that posttranscriptional regulation is a key factor mediating FliC expression during phase variation and that the FljA inhibitor might act at both the level of *fliC* transcription and translation.

To verify that the high basal level of *fliC-lac* transcription in the *fljBA*^{ON} strains was not an artifact of the *fliC-lac* reporter systems, we performed T_2 RNase protection assays to measure actual *fliC* mRNA transcript levels in the presence and absence

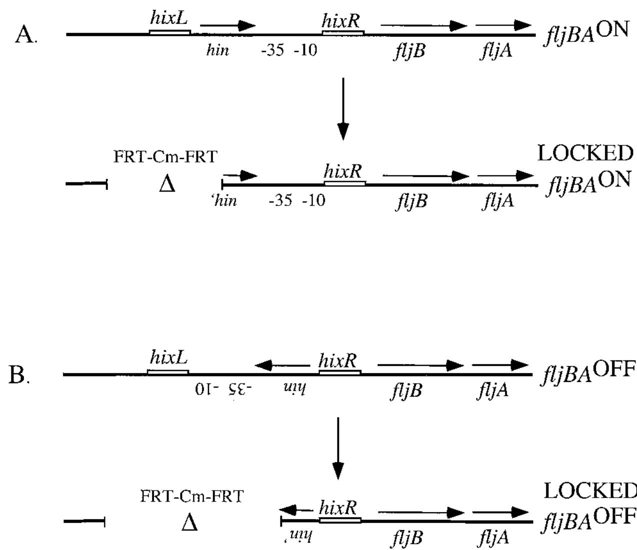

FIG. 2. Construction of strains locked in either the *fljBA*^{ON} or *fljBA*^{OFF} orientation. An FRT-chloramphenicol-FRT cassette (see Materials and Methods) was amplified using oligonucleotides containing DNA homologous to the region upstream of the *hixL* site and to the middle of the *hin* gene reading either towards the 5′ end of the gene (A) or towards the 3′ end of the gene (B). Recombination of the FRT-Cm-FRT cassette into the chromosome of an isolate with the *fljBA* promoter in the on orientation resulted in a deletion of the *hixL* site and a portion of the *hin* gene. The *hixR* site and the *fljBA* promoter are still present. In contrast, recombination of the second FRT-Cm-FRT cassette into an isolate with the *fljBA* promoter in the off orientation resulted in a deletion of the *hixL* site, the *fljBA* promoter, and the 3′ end of the *hin* gene. Both of these strains are unable to undergo phase variation with the constructs locked in either the on orientation (A) or the off orientation (B) for *fljBA* expression.

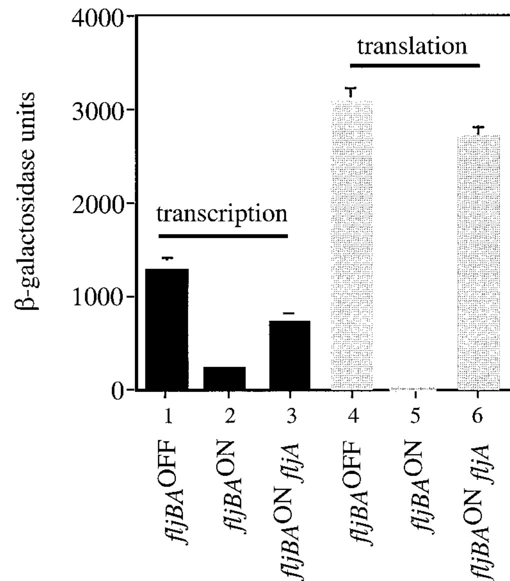

FIG. 3. β-Galactosidase activities for *fliC-lac* transcriptional and translational fusions in the *fljBA*^{OFF} and *fljBA*^{ON} orientations and for the *fljBA*^{ON} orientation in the absence of *fljA*. The levels of transcription and translation are recorded as β-galactosidase units. The average of three independent experiments and the standard deviations are shown. β-Galactosidase units for the *fliC-lac* transcriptional fusion were as follows: *fljBA*^{OFF}, 1,300 ± 75; *fljBA*^{ON}, 250 ± 32; *fljBA*^{ON} *fljA*, 730 ± 114. β-Galactosidase units for the *fliC-lac* translational fusion were as follows: *fljBA*^{OFF}, 3,100 ± 170; *fljBA*^{ON}, 15 ± 2; *fljBA*^{ON} *fljA*, 2,700 ± 175.

of FljA. A fivefold decrease in *fliC* transcript levels was observed in cells locked in the *fljBA*^{ON} orientation compared to *fliC* transcript levels in the *fljBA*^{OFF} orientation (Fig. 4, columns 1 and 2), thus corroborating our studies with the transcriptional fusions. As a negative control, T_2 RNase protection assays were performed in *fljBA*^{ON} and *fljBA*^{OFF} phase-locked strains containing a deletion of the flagellum-specific sigma factor FliA that is required for *fliC* transcription. The *fliC* transcript detected in these backgrounds was negligible (Fig. 4, columns 4 and 5).

FliC protein levels were directly determined by Western analysis with anti-FliC antibody in the *fljBA*^{ON} and *fljBA*^{OFF} orientations (Fig. 5, columns 1 and 2). An eightfold decrease in FliC protein levels in the *fljBA*^{OFF} orientation was observed compared to that in the *fljBA*^{ON} orientation. This reduction is greater than that observed for the transcripts, suggesting that *fliC* translation in addition to its transcription is regulated during phase variation and, therefore, FljA might not only inhibit *fliC* transcription but also inhibit its translation. However, the decrease of protein levels indicated by Western analysis was not as large as the decrease observed with the translational fusions (Fig. 3, columns 4 and 5). This could be due to posttranscriptional effects on FliC levels.

FljA is a translational regulator of *fliC* expression. The above results with the operon and gene fusions suggested that FljA might inhibit *fliC* transcription by 5-fold and *fliC* translation by an additional 40-fold. To test if inhibition of *fliC* translation in the *fljBA*^{ON} phase is mediated by FljA, the activities from *fliC-lac* transcriptional and translational fusions were assayed in the presence and absence of FljA protein in strains that have the *fljBA* promoter locked in the on orientation. A 3-fold increase in *fliC-lac* transcription was observed in the absence of FljA protein (Fig. 3, columns 2 and 3). In contrast, a 180-fold increase was observed for the FliC-LacZ translational fusion in the absence of FljA (Fig. 3, columns 5 and 6). These results implicate FljA as a negative regulator of both *fliC* transcription and translation, with a greater effect on *fliC* translation.

We performed T_2 RNase protection assays to measure transcript levels in the presence and absence of FljA in the *fljBA*^{ON} orientation. As with the *fliC-lac* transcriptional fusion, a threefold increase in *fliC* transcript levels was observed in the absence versus the presence of FljA (Fig. 4, columns 2 and 3). Because the *fljA* gene is cotranscribed with *fljB* and thus not expressed in the *fljBA*^{OFF} orientation, we did not expect to observe a FljA affect on *fliC* transcription in strains with the *fljBA* promoter in the off orientation. As predicted, *fliC* transcript levels were not significantly different in the presence or absence of FljA in these strains (data not shown).

To further characterize the role of FljA in regulating FliC expression, FliC protein levels were also determined by Western analysis in the presence and absence of FljA in the *fljBA*^{ON} orientation. In the absence of FljA, we observed a fivefold increase in FliC protein levels (Fig. 5, columns 2 and 3). This increase is greater than the threefold increase in transcription

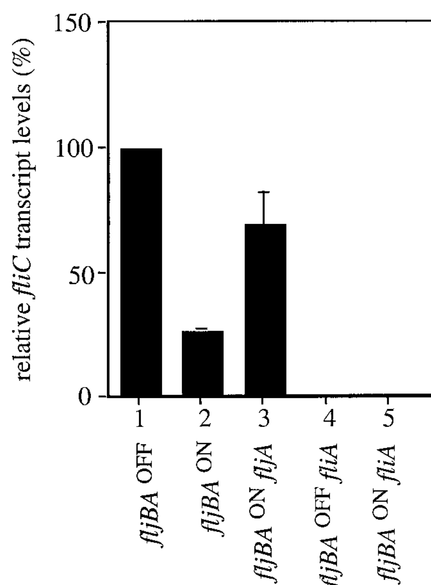

FIG. 4. Quantification of *fliC* transcript levels in the *fljBA*^{OFF} and *fljBA*^{ON} orientations in the presence and absence of FljA using T_2 RNase protection assays. Radiolabeled RNA probes covering the first 200 nucleotides of the *fliC* transcript were hybridized to 15 μg of total RNA for each strain tested. Band intensities were quantified using a Storm 840 PhosphorImager, and relative transcript levels were recorded as a percentage of the amount observed in the *fljBA*^{OFF} orientation. The averages of three independent experiments and the standard deviations are shown.

observed with both the fusion studies and the RNase protection assays (Fig. 3 and 4), consistent with the model that FljA inhibits both *fliC* transcription and translation when FljB flagellin is expressed, i.e., when the *fljBA* promoter is in the ON orientation. However, the increase in FliC protein levels is not as large as the 180-fold increase observed with the translational fusions (Fig. 3, columns 5 and 6).

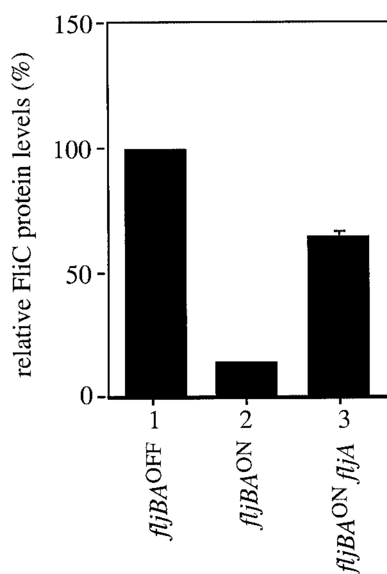

FIG. 5. Western blot analysis of FliC protein levels in strains locked in the *fljBA*^{ON} and *fljBA*^{OFF} orientations and the *fljBA*^{ON} in the absence of FljA protein. Relative FliC protein levels are shown as the percentage of those observed for cells locked in the *fljBA*^{OFF} orientation. The values represent the averages of three independent experiments, and standard deviations are shown.

TABLE 2. Effects of *fljA5576* or *flgM5301* disruptions on β-galactosidase activities of transcriptional and translational fusions of the *fliC* gene to the *lac* operon

Presence of:		Orientation of *fljBA* promoter	β-Galactosidase activity (β-galactosidase units)	
fljA	*flgM*		Transcription	Translation
+	+	OFF	830 (± 140)	2,000 (± 150)
+	−	OFF	1,100 (± 170)	2,300 (± 480)
+	+	ON	270 (± 60)	24 (± 1)
+	−	ON	260 (± 30)	17 (± 2)
−	+	ON	710 (± 140)	4,010 (± 1,100)
−	−	ON	1,800 (± 210)	3,900 (± 1,300)

Regulation of FliC expression by FljA functions independently of the anti-sigma factor FlgM. The FlgM protein is known to inhibit σ^{28}-dependent transcription of the *fliC* gene. We examined the effect of uncoupling *fliC* transcription from the regulatory control of FlgM (*flgM* deletion mutants) on the regulation of *fliC-lac* transcription and translation by FljA protein. In the absence of the anti-sigma factor FlgM, levels of flagellin transcription have been shown to increase above those observed in a wild-type background (15) (Table 2). We wanted to determine if the absence of FlgM would allow for *fliC* expression in the *fljBA*^{ON} orientation in the presence of FljA.

β-Galactosidase assays with the *fliC-lac* transcriptional and translational fusions were used to measure FliC expression. Introduction of a *flgM* null allele had no significant effect on *fliC* transcription or translation in the presence of FljA (Table 2). These data suggest that the modulation of FliC expression by FljA is maintained in the absence of the regulatory control of FlgM. We did not observe an increase in β-galactosidase activity for the *fliC*::Mu*d*K fusion in the absence of FlgM in the *fljA fljBA*^{ON} background (Table 2). This is because, in the absence of FlgM, the translational fusion is unstable and throws off Lac^{-} revertants at a high frequency, which resulted in a reduction in β-galactosidase levels.

fliC transcription and translation were still tightly regulated by FljA in the *fljBA*^{ON} orientation in *flgM* null strains, suggesting that FljA-dependent inhibition of FliC expression is not easily titratable. That is, in the presence of increased σ^{28} and, thus, increased initiation of *fliC* transcription, FljA is able to maintain both transcriptional and translational *fliC* inhibition. However, the *fljB* promoter belongs to the late promoter class and is regulated by the interaction between σ^{28} and FlgM and, therefore, the levels of FljA protein are likely elevated in *flgM* null strains, and this alone may account for the maintenance of FliC inhibition. However, if σ^{28}-independent factors were required for FliC inhibition during FljB expression, we would have expected to observe an increase in *fliC-lac* transcription or translation in the absence of FlgM. Alternatively, excess *fliC* transcripts or FliC protein in the *flgM* mutant backgrounds might be hypersensitive to degradation.

DISCUSSION

Since the early 1920s (2), it has been known that *S. enterica* undergoes a phenomenon known as phase variation, a molecular mechanism that allows for the alternate expression of the two different unlinked flagellar filament proteins, FliC and FljB (16). Transcription of the *fljB* gene is initiated from an invertible promoter, and a reversible site-specific DNA recombination event turns *fljB* transcription on and off (Fig. 1) (45). It has been postulated that the FljA protein, which is cotranscribed with the *fljB* gene (11, 43, 49), represses *fliC* transcription when the *fljB* promoter is in the on orientation (31, 43).

We found that *fliC* transcription is indeed regulated by FljA during phase variation. However, FljA-dependent inhibition of FliC expression is at the level of transcription and translation (Fig. 3, 4, and 5). Specifically, β-galactosidase assays with *fliC-lac* operon and gene fusions were performed for strains with the *fljBA* promoter locked in either the on or the off orientation. These experiments demonstrated that while FljA inhibits *fliC* transcription fivefold in the *fljBA*^{ON} orientation compared to the *fljBA*^{OFF} orientation, it has a 200-fold effect on both *fliC* transcription and translation, indicating that the FljA inhibitor might act at both the transcriptional and translational levels. T_2 RNase protection assays also demonstrated a fivefold decrease in *fliC* transcript levels, while FliC protein levels as determined by Western analysis showed an eightfold decrease in FliC protein levels in the *fljBA*^{OFF} orientation compared to *fljBA*^{ON}, further suggesting that FljA not only regulates *fliC* transcription in the *fljBA*^{ON} orientation but also inhibits its translation.

An eightfold decrease in FliC protein levels was observed by Western analysis (Fig. 5), while a 200-fold decrease in FliC-LacZ levels was observed in strains locked in the *fljBA*^{ON} orientation compared to that in the *fljBA*^{OFF} orientation (Fig. 3). The differences in FliC expression between the fusion studies and Western analysis likely represent differential stability of *fliC-lacZ* transcript or FliC-LacZ protein fusions compared to that of wild-type *fliC* transcript or FliC protein. It is important that wild-type flagellin is secreted and polymerized into flagellar filaments where it is highly stable (data not shown) while, in contrast, the fusion protein cannot be secreted and assembled (data not shown) but is retained within the cell, where it would be subject to proteolysis. Thus, the FliC-LacZ fusion protein may be turned over more rapidly than wild-type FliC protein, resulting in lower levels of the reporter protein in the presence of translational regulation by the FljA inhibitor.

In the absence of FljA protein in the *fljBA*^{ON} orientation, both *fliC* transcription and translation were reduced compared to the *fljBA*^{OFF} orientation (Fig. 3, 4, and 5). Because the *fljBA* promoter is maintained in strains locked in the *fljBA*^{ON} orientation, competition for σ^{28}-RNA polymerase is likely occurring between the *fliC* and *fljBA* promoters. In fact, both FliC and FljB flagellin can be detected by Western blotting in this genetic background (data not shown). It is possible that factors other than FljA are regulating FliC expression during phase variation and are responsible for the remaining inhibition of FliC expression in the absence of FljA.

Previous experiments demonstrated that strains locked in the *fljBA*^{ON} orientation but containing a defective *fljB* gene were nonmotile due to FljA-dependent inhibition of *fliC* gene expression (12). We were initially surprised that only a 5-fold reduction in *fliC* transcription by FljA activity could prevent motility, but that the further 40-fold reduction in *fliC* translation in the presence of FljA could account for the complete loss of motility. But yet, by Western blotting we were able to detect FliC protein within strains locked in the *fljBA*^{ON} orientation, indicating that FliC expression is not completely inhibited during phase variation. In fact, it has been shown that in the absence of *fljB* expression, these strains do not produce enough flagellin to allow motility (12). Thus, other mechanisms may be regulating the secretion and assembly of FliC protein during phase variation, or the amount of protein that is expressed is insufficient for full filament assembly.

Model of posttranscriptional regulation of FliC expression by FljA. Because the regulation of FliC expression by FljA during phase variation was thought to occur at the transcriptional level, genetic experiments were performed to identify the operator site for the FljA protein (12, 26). This work involved the isolation of motile revertants from strains which were transcribing *fljBA* but contained a nonfunctional *fljB* allele. Because these strains were also blocked for phase variation, mutations alleviating the negative regulation of FliC expression would result in a motile phenotype. The authors of this study identified nine *cis*-acting mutations that mapped downstream of the *fliC* promoter within the 5′-UTR of the *fliC* mRNA transcript. These mutations did not conform to the classical operator regulatory sequences observed in bacteria, which are often located close to or within transcriptional promoter regions, but instead clustered to a 15-bp sequence within the 5′-UTR of the *fliC* transcript immediately adjacent to and overlapping the ribosome binding sequence. In fact, one operator-constitutive *fliC* mutant (SJW57) was found to have a 28-bp tandem duplication that included 13 bases upstream of the *fliC* translational initiation site through base 15 of the coding sequence. This *fliC*-O^c mutant was dependent upon the presence of FljA for motility, consistent with an interaction of FljA with the mRNA to allow *fliC* gene expression. The 5′-UTR of bacterial transcripts has often been implicated in the translational regulation of protein expression. The work presented here suggests that the FljA protein functions as both a transcriptional and translational regulator of FliC expression (Fig. 3, 4, and 5) and, therefore, these "operator mutants" may be defective in FljA binding to mRNA as a regulator of transcription (attenuation) and translation.

Therefore, we put forward the following model for the transcriptional and posttranscriptional regulation of FliC expression during phase variation. We propose that FljA regulates *fliC* gene expression by binding to the 5′-UTR of the transcript, within or near the Shine-Dalgarno sequence, to inhibit transcription by an attenuation mechanism and to inhibit ribosome binding and thus translation. The presence of ribosomes at or near the Shine-Dalgarno site has previously been demonstrated to be particularly important for mRNA stability by protecting the 5′-terminal extremity from initiation of mRNA degradation (18). In addition, decreased stability of the *fliC* transcript would further reduce *fliC* translation. Finally, if FljA does indeed bind to *fliC* transcripts and prevent ribosome binding, it may also affect FliC expression by masking positive regulatory sequences contained within the 5′-UTR of the *fliC* transcript.

Importance of posttranscriptional regulation of FliC expression during phase variation. Historically, translational regulation was not thought to be an important factor mediating protein expression because mRNA half-lives in bacteria are typically on the order of a few minutes, but recent studies have demonstrated that mRNA turnover alone is often insufficient to provide necessary regulation of protein levels (18), as may be the case for regulation of FliC expression during phase variation. For example, translational regulation would allow for the inhibition of FliC expression from existing transcripts after inversion of the *fljBA* promoter to the on orientation and a more rapid transition to filaments composed of FljB flagellin. Although the central domain of a particular flagellin sequence is the peripheral molecular region exposed on a flagellar filament and helps define the diameter of the filament and the character of its surface, including topography, physiochemistry, and antigenicity, the importance of filament diameter and surface properties and the reasons for variability are not well understood (51). Further investigations into the biological significance of *S. enterica* phase variation may elucidate the importance of posttranscriptional regulatory mechanisms to mediate flagellar phase variation.

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