Translation Inhibition of the *Salmonella* *fliC* Gene by the *fliC* 5' Untranslated Region, *fliC* Coding Sequences, and FlgM

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The 5'-untranslated region (5'UTR) of the *fliC* flagellin gene of *Salmonella* contains sequences critical for efficient *fliC* mRNA translation coupled to assembly. In a previous study we used targeted mutagenesis of the 5' end of the *fliC* gene to isolate single base changes defective in *fliC* gene translation. This identified a predicted stem-loop structure, SL2, as an effector of normal *fliC* mRNA translation. A single base change (–38C:U) in the *fliC* 5'UTR resulted in a mutant that is defective in *fliC* mRNA translation and was chosen for this study. Motile (Mot⁺) revertants of the –38C:T mutant were isolated and characterized, yielding several unexpected results. Second-site suppressors that restored *fliC* translation and motility included mutations that disrupt a RNA duplex stem formed between RNA sequences in the *fliC* 5'UTR SL2 region (including a precise deletion of SL2) and bases early within the *fliC*-coding region. A stop codon mutation at position 80 of *flgM* also suppressed the –38C:T motility defect, while *flgM* mutants defective in anti- σ^{28} activity had no effect on *fliC* translation. One remarkable mutation in the *fliC* 5'UTR (–15G:A) results in a translation defect by itself but, in combination with the –38C:U mutation, restores normal translation. These results suggests signals intrinsic to the *fliC* mRNA that have both positive and negative effects on *fliC* translation involving both RNA structure and interacting proteins.

Salmonella enterica serovar Typhimurium possess 5 to 10 peritrichous flagella that are utilized for motility (19). The bacterial flagellum is composed of three main substructures: (i) a basal body that transverse the cell membranes and acts a proton-driven rotary motor enabling the flagellum to rotate and propel the bacterium through a liquid environment and across surfaces; (ii) a hook that acts as flexible coupling between the external filament and the basal body; and (iii) a rigid, helical filament that typically extends up to 10 μ m from the cell surface and acts as a propeller. Flagellum assembly begins with the basal body, proceeds through the hook, and is completed by the filament (20). The assembly of extracellular structures such as the filament and the hook involves the transport of the component proteins through a central channel within the growing flagellum. The cost of the maintenance of a flagellar motility system is high due to the large number of genes and protein subunits and the energy required for flagellum synthesis and functioning, which is ca. 2% of biosynthetic energy expenditure of the cell (19). As a result, the flagellar system of *Salmonella* is highly regulated at multiple levels, including responses to environmental cues and the developmental stages of the organelle (4). The flagellar biosynthetic and chemotaxis systems require more than 60 genes that are arranged into a transcriptional hierarchy of three promoter classes (class 1, class 2, and class 3) whose expression corresponds to when their products are needed for assembly (16). The class 1 *flhDC* operon is the master control operon. Transcription from this promoter is influenced by a number of global regulatory signals

in a manner that is not fully understood (14). The FlhD and FlhC proteins form a heterotetrameric complex that is required for the transcription of class 2 promoters upstream of the Middle genes (18). Class 2 promoters mediate the transcription of genes necessary for the assembly and structure of the hook-basal body (HBB) complex in addition to the transcriptional regulators FlgM and FliA (σ^{28}). Class 3 promoters require σ^{28} -RNA polymerase for their transcription (23). FlgM is an inhibitor of σ^{28} and prevents class 3 transcription prior to completion of the HBB structure (13, 15). Upon HBB completion, FlgM is secreted outside of the cell resulting in a σ^{28} -dependent transcription from the class 3 promoters (13). Class 3 promoters direct transcription of the *flgKL* operon that encodes the hook-filament junction proteins, the *fliDST* operon that encodes the filament cap (FliD) and the secretion chaperones FliS (FliC chaperone) and FliT (FliD chaperone), the *flgMN* operon encoding the anti- σ^{28} factor FlgM and the secretion chaperone FlgN (FlgK and FlgL chaperone), the flagellin operons *fliC* and *fliBA*, the *fliAZY* operon that encodes σ^{28} (*fliA*), a regulator of class 2 transcription (*fliZ*), and a gene of unknown function (*fliY*) (11). In addition, class 3 promoters direct transcription of genes whose products are required for chemotaxis and flagellar rotation.

In *Salmonella*, little is known regarding the translational control mechanisms in flagellin biosynthesis. The best-studied system is in the *Caulobacter crescentus* flagellar system, where the FlbT protein has been discovered as a regulator of flagellin gene translation translational. FlbT acts by directly binding the 5' untranslated region (5'UTR) of flagellin mRNA to inhibit its translation (2). Recently, we found that transcription of the chromosomal *fliC* gene without the *fliC* 5'UTR region are nonmotile, suggesting an important role for the *fliC* 5'UTR in

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normal *fliC* mRNA expression associated with filament assembly (1). The 5' end of the *fliC* gene was analyzed, and regions essential for transcriptional and translational control were defined. A stem-loop region, SL2, of the *fliC* 5'UTR was discovered that when mutated was defective in *fliC* translation (1).

In the present study we isolated and characterized motile (Mot⁺) revertants of a translation-defective mutant within a putative stem-loop structure, SL2, of the *fliC* 5'UTR (−38C:U relative to the AUG start codon). This analysis led to the unexpected discovery that SL2 is an inhibitor of *fliC* mRNA translation. In addition, suppressors identified the C-terminal 17 amino acids of FlgM and a region in the *fliC* structural gene that codes for amino acids 13 and 14 that act to facilitate regulation of *fliC* mRNA translation. A model is presented that suggests an interaction between the SL2 of the *fliC* 5'UTR and the *fliC* coding region, including the 3' base of amino acid codon 11 through the initial base of amino acid codon 15.

MATERIALS AND METHODS

Bacterial strains and growth medium conditions. The bacterial strains used in the present study are listed in Table 1. Strains were grown in Luria-Bertani (LB) medium with aeration as described by Davis et al. (6). Motility assay was performed in motility agar plates that consisted of 10 g of tryptone, 5 g of NaCl, and 0.3% (wt/vol) agar per liter (pH 7.4). Plates were incubated at 37°C. Minimal E salts medium supplemented with 0.2% glucose was used as a minimal medium (28). Indicator media included X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), MacConkey-lactose (Difco), and 2,3,5-triphenyltetrazolium chloride (TTC)-lactose (17). Green plates were made up as described previously (27) and used to isolate phage-free bacteria after transductions. Selection of tetracycline-sensitive (Tc^s) clones was done on zinc-fusaric acid selection plates (21).

Strain construction. Markers were mobilized between *Salmonella* strains by generalized transduction using the mutant P22 bacteriophage HT105/int-201 (26). The following antibiotics were used for resistance marker selections (final concentrations): ampicillin, 30 µg/ml for chromosomal resistant gene expression or 100 µg/ml for plasmid-encoded resistant gene expression; chloramphenicol, 12.5 µg/ml; kanamycin, 50 µg/ml; and tetracycline, 15 µg/ml (21).

β-Galactosidase assays. β-Galactosidase assays were performed in triplicate as described previously (21). Cells were grown to a net Klett reading of 100 (optical density at 600 nm of ~0.6) prior to the assay.

FliC immunoblotting. Immunoblots were performed in triplicate as described previously (12). Samples were subjected to 10% Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Blots were probed using an anti-FliC polyclonal antibody (Difco) and an horseradish peroxidase-conjugated secondary antibody (Bio-Rad). Nonradioactive ECL Plus Kit (Amersham Biosciences) was used for blot detection. Quantification was performed with ImageQuant for the Macintosh (IQ v1.2; Molecular Dynamics). Protein levels were calculated as a percentage of the total protein at time *t* divided by the amount of protein at time zero and multiplied by 100.

Predicted RNA secondary structures. RNA secondary structures were predicted by using the M-FOLD program, which is available online (22). We analyzed two different partial RNA secondary structures. One structure included the *fliC* +1 translational start site and 12 nucleotides (nt) downstream the ATG start codon. The second structure included the *fliC* +1 translational start site, the full length of the 5'UTR (62 nt) and 42 nt downstream the ATG initiation codon. In the wild-type strain, TH6232, the examined RNA total length was 77 nt for the first structure and 108 nt for the second one. M-FOLD analyses were conducted at the fixed temperature of 37°C. In some cases more than one global folding pattern were displayed with an increase in the available free energy.

Construction of transcriptional and translational fusions. Transcriptional and translational fusions of the *fliC* gene to the *lac* operon and *lacZ* gene, respectively, were constructed in strains TH6232, TH7293, and TH7317 using the λ-Red system of Datsenko and Wanner (5). A segment of DNA including the entire *fliC* 5'UTR sequence from TH6232, TH7293, and TH7317 was transformed into strains TH6305 and TH6307, which contain the MudJ and MudK *lac* transcriptional and translational fusion vectors in the *fliC* coding region. The UTR sequence was amplified with primers carrying extensions (between 36 and 40 nt) homologous to the last portion of the targeted sequence (forward primer) and to a region downstream from it (reverse primer) to allow transfer of the

sequence by recombination into the chromosome. Primers were engineered to insert *fliC* promoter-down mutations to facilitate the measurement of β-galactosidase levels. Primers used to introduce promoter-down mutations (a ΔA residue between the −10 and 35 promoter regions that is 23 bp upstream of the transcriptional start site or a C:T in the −10 region that is 13 bp upstream of the transcriptional start-site) were as follows: 5' promoter 13-bp fliC2 (5'-GAA GTG AAA AAT TTT CTA AAG TTC GAA ATT CAG GTG CTG ATA CAA GG-3'), 5' promoter TH7044 (5'-GGA AGT GAA AAA TTT TCT AAA GTT CGA ATT CAG G-3'), and 3' fliC+39R (5'-CAG CGA CAG GCT GTT TG-3'). Both recipient strains TH6305 and TH6307 have a *tetRA* cassette insertion that removed a region of DNA, including the −10 sequence of *fliC* promoter through 10 bases before the ATG start codon, and are in the *fliC*^{ON} orientation. TH6305 has a *fliC-lac* transcriptional fusion where transcription of the *lac* operon is dependent on the *fliC* promoter, whereas *lacZ* is independently translated. TH6307 has the *fliC-lacZ* translational fusion, where both transcription and translation of *lacZ* are dependent on the *fliC* promoter and the mRNA translation initiation region. Competent cells were concentrated 250-fold and transformed with 100 ng of PCR product. Recombinant colonies were selected by plating transformed cells on Tc^s plates at 42°C. Loss of the plasmid pKD46 was confirmed by screening Tc^s colonies for growth on L-Ap plates at 37°C. Constructs were confirmed by PCR using the primers 5' fliC13 (5'-CTT TGT CAG GTC TGT C-3'), which contains homology to the upstream DNA immediately adjacent to *fliC* (75 bp from ATG of *fliD* reverse), and 3' fliC+39R. Sequencing of the PCR products obtained was performed at the Biochemistry DNA Sequencing Facility, University of Washington, Seattle.

Construction of *fliC*::Mud-lac transcriptional and translational fusion to the *fliC* gene in Mot⁺ revertant strains with promoter-down mutations. A *tetRA* cassette insertion was introduced in the *fliC* gene between codons 12 and 13 in strains TH8856, TH8862, TH8868, and TH8874. The primers were as follows: FliC cod 12-13 tetR (5'-G ATC ATG GCA CAA GTC ATT AAT ACA AAC AGC CTG TCG CTG TTAAGACCCACTTTCACATT-3') and FliC cod 12-13 tetA (5'-C CAG AGC GGA CTG GGA TTT GTT CAG GTT ATT CTG GGT CAA CTAAGCACTTGTCTCCTG-3'). The *tetRA* cassette was replaced with a PCR product (253 bp in length) from TH7312, TH7314, and TH7318 containing the respective Mot⁺ revertant *fliC* coding sequence mutation. The *fliC* sequence was amplified with the primers *fliC* reverse CELI (5'-ATG GAG ATA CCG TCG-3') and fliC-29 (5'-CAA GTT GTA ATT GAT AA-3'). Constructs were sequenced by using the primer fliC13 (5'-CTT TGT CAG GTC TGT C-3') to confirm the *fliC* 5'UTR −38C:T, the *fliC* promoter, and the *fliC* coding sequence mutations.

Construction of double mutants. Double mutants containing both the *fliC* 5'UTR motile revertant deletion of SL2 [*fliC* 5'UTR Δ(−47 through −36 relative to AUG start codon)] from TH7317 and, respectively, the *fliC* coding motile revertant mutations from strains TH7312 (+41C:G), TH7314 (+41 C:A), and TH7318 (+37 T:C) were constructed by using λ-Red (5). A *tetRA* cassette was introduced into strains TH7312, TH7314, and TH7318 in the *fliC* 5'UTR region, 40 bases upstream of the *fliC* AUG start codon. The primers were as follows: FLICUTR(−40BP)/tetR (5'-CTT TTC CTT ATC AAT TAC AAC TTG ATG TTA TTG GAC T TTAAGACCCACTTTCACATT-3') and FLICUTR(−40BP) tetA (5'-GCC GAT ACA AGG GTT ACG GTG AGA AAC CGT GGG CAAC CTAAGCACTTGTCTCCTG-3'). The *tetRA* cassette was replaced with a DNA fragment containing the TH7317 *fliC* 5'UTR deletion. The PCR product of 113 bp was obtained by using the primers fliC+39R (5'-CAG CGA CAG GCT GTT TG-3') and UTR (5'-TTC GAA ATT CAG GTG-3'). Strain constructs were confirmed by sequencing using the primer fliC+39R.

The strains with different *flgM* alleles in strains with the *fliC-lac* transcriptional reporter fusion (*fliC5050::MudJ*) were constructed as follows. Strain TH4107 (*pyrC691::Tn10 flgM5222::MudCm fliC5050::MudJ fljB^{mx} vh2*) was transduced to *pyrC⁺* with P22 transducing phage lysates prepared on strains TH4572 (*flgM5224* [R5C]), TH4576 (*flgM5228* [I82T]), TH4577 (*flgM5229* [E89-Stop]), TH5139 (*ΔflgM5628::FRT*), TH8891 (*flgM6318* [G80-Stop]), and LT2 selecting for growth on minimal glucose medium and screening for Tc^s and Cm^s. *PyrC⁺* transductants become Tc^s due to the loss of the *pyrC691::Tn10* allele, and transductants that also replace the *pyrC*-linked *flgM5222::MudCm* allele with the *flgM* allele of the donor strain are Cm^s. The resulting set of strains were then transduced to ampicillin resistance using a P22 transducing lysate prepared on strain TH6595 (*fliC5469::MudB*) and screened for Km^s. This resulted in replacement of the *fliC-lac* transcriptional reporter with a *fliC-lac* translational reporter, allowing assay of the effect of the *flgM* alleles on *fliC-lac* transcription and translation.

The set of strains with the different *flgM* alleles in strains with the *fliC5969*(−38C:T allele) and either the *fliC5050::MudJ* or *fliC5469::MudK* re-

TABLE 1. *S. enterica* serovar Typhimurium strains

Strain	Genotype	Source or reference ^a
LT2	Wild type	John Roth
TH3933	<i>motA5461::MudJ</i>	1
TH4107	<i>flhC5050::MudJ pyrC691::Tn10 flgM5222::MudCm fljB^{enx} vh2</i>	Strain collection
TH4572	<i>flgM5524</i> (R5C) <i>flhA</i> (ts) <i>flhC5050::MudJ</i>	Lakshmi Rajagopal
TH4576	<i>flgM5528</i> (I82T) <i>flhA</i> (ts) <i>flhC5050::MudJ</i>	Lakshmi Rajagopal
TH4577	<i>flgM5529</i> (E89-Stop) <i>flhA</i> (ts) <i>flhC5050::MudJ</i>	Lakshmi Rajagopal
TH5139	Δ <i>flgM5628::FRT</i>	Strain collection
TH6232	Δ <i>hin-5717::FRT (flhC^{ON})</i>	3
TH6234	Δ <i>hin-5717::FRT (flhC^{ON}) flhC5050::MudJ</i>	3
TH6235	Δ <i>hin-5717::FRT (flhC^{ON}) flhC5469::MudK</i>	3
TH6293	Δ <i>hin-5717::FRT (flhC^{ON}) flhC5050::MudJ flhC5532::tetRA</i>	3
TH6295	Δ <i>hin-5717::FRT (flhC^{ON}) flhC5469::MudK flhC5532::tetRA</i>	3
TH6305	pKD46/ Δ <i>hin-5717::FRT flhC5050::MudJ flhC5532::tetRA</i>	1
TH6307	pKD46/ Δ <i>hin-5717::FRT flhC5469::MudK flhC5532::tetRA</i>	1
TH6594	<i>flhC5469::MudK</i>	3
TH6595	<i>flhC5469::MudB</i>	3
TH7044	Δ <i>hin-5717::FRT flhC5894</i> (Δ A –23 bp from +1 UTR) <i>flhC5050::MudJ</i>	
TH7045	Δ <i>hin-5717::FRT flhC5894 flhC5469::MudK</i>	
TH7293	Δ <i>hin-5717::FRT flhC5969</i> (UTR –38C:T)	1
TH7312	Δ <i>hin-5717::FRT flhC5969</i> (UTR –38C:T) <i>flhC6350</i> (T14S, ACC:AGC)	
TH7314	Δ <i>hin-5717::FRT flhC5969</i> (UTR –38C:T) <i>flhC6352</i> (T14N, ACC:AAC)	
TH7317	Δ <i>hin-5717::FRT flhC5969</i> (UTR –38C:T) <i>flhC6355</i> (UTR Δ SL2)	
TH7318	Δ <i>hin-5717::FRT flhC5969</i> (UTR –38C:T) <i>flhC6356</i> (L13L, TTG:CTG)	
TH7321	Δ <i>hin-5717::FRT flhC5969</i> (UTR –38C:T) <i>flhC6359</i> (L13L, TTG:CTG)	
TH7328	Δ <i>hin-5717::FRT flhC5969</i> (UTR –38C:T) <i>flhC6366</i>	
TH7952	Δ <i>hin-5717::FRT flhC5532::tetRA</i>	
TH8670	pKD46/ Δ <i>hin-5717::FRT flhC5747::Tn10dTc</i>	
TH8860	Δ <i>hin-5717::FRT flhC5050::MudJ flhC6294</i> (Δ A –23 from +1 UTR; P [–])	
TH8862	Δ <i>hin-5717::FRT flhC5969 flhC5050::MudJ flhC6296</i> (Δ A –23 from +1; P [–])	
TH8864	Δ <i>hin-5717::FRT flhC5050::MudJ flhC6355</i> (Δ SL2) <i>flhC6298</i> (Δ A –23 from +1; P [–])	
TH8872	Δ <i>hin-5717::FRT flhC5469::MudK flhC6306</i> (Δ A –23 from +1 UTR; P [–])	
TH8874	Δ <i>hin-5717::FRT flhC5469::MudK flhC5969 flhC6308</i> (Δ A –23 from +1; P [–])	
TH8876	Δ <i>hin-5717::FRT flhC5469::MudK flhC6355</i> (Δ SL2) <i>flhC6310</i> (Δ A –23; P [–])	
TH8878	Δ <i>hin-5717::FRT flhC6350</i> (aa14 ACC:AGC)	
TH8879	Δ <i>hin-5717::FRT flhC6352</i> (aa14 ACC:AAC)	
TH8882	Δ <i>hin-5717::FRT flhC6356</i> (aa13 TTG:CTG)	
TH8891	Δ <i>hin-5717::FRT flhC5969 flgM6318</i> (G80Stop)	
TH8901	Δ <i>hin-5717::FRT flhC5969 flhC5050::MudJ</i>	
TH8902	Δ <i>hin-5717::FRT flhC6355</i> (Δ SL2) <i>flhC5050::MudJ</i>	
TH8904	Δ <i>hin-5717::FRT flhC5969 flhC5469::MudK</i>	
TH8905	Δ <i>hin-5717::FRT flhC6355</i> (Δ SL2) <i>flhC5469::MudK</i>	
TH9408	<i>flgM5524</i> (R5C) Δ <i>hin-5717::FRT flhC5969</i>	
TH9409	<i>flgM5528</i> (I82T) Δ <i>hin-5717::FRT flhC5969</i>	
TH9410	<i>flgM5529</i> (E89-Stop) Δ <i>hin-5717::FRT flhC5969</i>	
TH9411	Δ <i>flgM5628::FRT flhC5050::MudJ fljB^{enx} vh2</i>	
TH9412	<i>flgM6318</i> (G80-Stop) Δ <i>hin-5717::FRT flhC5969</i>	
TH9413	Δ <i>hin-5717::FRT flhC5969</i>	
TH10038	<i>flgM5524</i> (R5C) <i>flhC5050::MudJ fljB^{enx} vh2</i>	
TH10039	<i>flgM5528</i> (I82T) <i>flhC5050::MudJ fljB^{enx} vh2</i>	
TH10040	<i>flgM5529</i> (E89-Stop) <i>flhC5050::MudJ fljB^{enx} vh2</i>	
TH10041	<i>flgM5628::FRT flhC5050::MudJ fljB^{enx} vh2</i>	
TH10042	<i>flgM6318</i> (G80-Stop) <i>flhC5050::MudJ fljB^{enx} vh2</i>	
TH10043	<i>flhC5050::MudJ fljB^{enx} vh2</i>	
TH10055	<i>flgM5524</i> (R5C) <i>flhC5469::MudB fljB^{enx} vh2</i>	
TH10056	<i>flgM5528</i> (I82T) <i>flhC5469::MudB fljB^{enx} vh2</i>	
TH10057	<i>flgM5529</i> (E89-Stop) <i>flhC5469::MudB fljB^{enx} vh2</i>	
TH10058	Δ <i>flgM5628::FRT flhC5469::MudB fljB^{enx} vh2</i>	
TH10059	<i>flgM6318</i> (G80-Stop) <i>flhC5469::MudB fljB^{enx} vh2</i>	
TH10060	<i>flhC5469::MudB fljB^{enx} vh2</i>	
TH10067	Δ <i>pyrC::FKF flgM5222::MudCm flgBC::tetRA fljB^{enx} vh2</i>	
TH10152	<i>flgM5524</i> (R5C) <i>flhC5050::MudJ flhC5969</i>	
TH10153	<i>flgM5528</i> (I82T) <i>flhC5050::MudJ flhC5969</i>	
TH10154	<i>flgM5529</i> (E89-Stop) <i>flhC5050::MudJ flhC5969</i>	
TH10155	Δ <i>flgM5628::FRT flhC5050::MudJ flhC5969</i>	
TH10156	<i>flgM6318</i> (G80-Stop) <i>flhC5050::MudJ flhC5969</i>	
TH10157	<i>flhC5050::MudJ flhC5969</i>	
TH10158	<i>flgM5524</i> (R5C) <i>flhC5469::MudK flhC5969</i>	
TH10159	<i>flgM5528</i> (I82T) <i>flhC5469::MudK flhC5969</i>	

Continued on following page

TABLE 1—Continued

Strain	Genotype	Source or reference
TH10160	<i>flgM5529</i> (E89-Stop) <i>fliC5469::MudK</i> Δ <i>hin-5717::FRT fliC5969</i>	
TH10161	Δ <i>flgM5628::FRT fliC5469::MudK</i> Δ <i>hin-5717::FRT fliC5969</i>	
TH10162	<i>flgM6318</i> (G80-Stop) <i>fliC5469::MudK</i> Δ <i>hin-5717::FRT fliC5969</i>	
TH10163	<i>fliC5469::MudK</i> Δ <i>hin-5717::FRT fliC5969</i>	
TH10218	Δ <i>flgG-L2157</i> Δ <i>pyrC::FKF flgM5222::MudCm fljB^{enx} vh2</i>	
TH10231	<i>flgM5524</i> (R5C) <i>motA5461::MudJ</i> Δ <i>flgG-L2157 fljB^{enx} vh2</i>	
TH10232	<i>flgM5528</i> (I82T) <i>motA5461::MudJ</i> Δ <i>flgG-L2157 fljB^{enx} vh2</i>	
TH10233	<i>flgM5529</i> (E89-Stop) <i>motA5461::MudJ</i> Δ <i>flgG-L2157 fljB^{enx} vh2</i>	
TH10234	Δ <i>flgM5628::FRT motA5461::MudJ</i> Δ <i>flgG-L2157 fljB^{enx} vh2</i>	
TH10235	<i>flgM6318</i> (G80-Stop) <i>motA5461::MudJ</i> Δ <i>flgG-L2157 fljB^{enx} vh2</i>	
TH10236	<i>fliC5050::MudJ motA5461::MudJ</i> Δ <i>flgG-L2157 fljB^{enx} vh2</i>	
SJW1518	Δ <i>flgG-L2157 fljB^{enx} vh2</i>	S. Yamaguchi

^a Unless indicated otherwise, all strains were constructed during the course of this study.

porter fusions were constructed as follows. P22 transducing phage lysates prepared on strains TH6293 (Δ *hin-5717::FRT fliC5050::MudJ fliC5532::tetRA*) and TH6295 (Δ *hin-5717::FRT fliC5469::MudK fliC5532::tetRA*) were used to separately transduce strains TH9408, TH9409, TH9410, TH9411, TH9412, and TH9413 to Km^r and screened for a Tc^s phenotype. Because the *fliC5532::tetRA* Tc^r allele is a replacement of the region including the *fliC* 5'UTR through the -10 *fliC* promoter region with the *tetRA* genes from transposon Tn10, the Km^r, Tc^s transductants would have inherited the *fliC::MudJ* or *fliC::MudK* reporter but retained the *fliC* 5'UTR region of the recipient strains.

The set of strains with the different *flgM* alleles in the HBB mutant background (Δ *flgG-L*) used to measure FlgM anti- σ^{28} activity using a class 3 transcriptional reporter fusion (*motA5461::MudJ*) were constructed as follows. A P22 transducing lysate grown on strain TH10067 (Δ *pyrC::FKF flgM5222::MudCm* Δ *flgBC::tetRA fljB^{enx} vh2*) was used to transduce strain SJW1518 (Δ *flgG-L2157 fljB^{enx} vh2*) to Km^r, which selects for the linked Δ *pyrC::FKF* allele and screened for Cm^r and Tc^s, which includes Km^r transductants that also brought in the linked *flgM5222::MudCm* and retained the Δ *flgG-L2157* allele because in order to be Cm^r and Tc^s, a recombination even had to occur between the *flgM* and *flgB* genes. This resulted in the creation of strain TH10218. TH10067 was also used to transduce strains TH9408, TH9409, TH9410, TH9411, TH9412, and TH9413 to Tc^r, followed by a screen for Km^s and Cm^s transductants to place the Δ *flgBC::tetRA* adjacent to the six *flgM* alleles. P22 transducing lysates were grown on these six strains and used to transduce TH10218 to *pyrC*⁺, followed by a screen for Tc^s and Cm^s, which resulted in the placement of the Δ *flgG-L2157* allele adjacent to the six *flgM* alleles. These six strains were then transduced to Km^r with P22 lysate grown on strain TH3933 (*motA5461::MudJ*) to generate strains TH10231 through TH10236.

Isolation of the *fliC* coding sequence mutations from the *fliC* 5'UTR ($-38C:T$) mutant. Constructs were obtained by using λ -Red (5). As a recipient strain we used TH8670 that has a Tn10dTc insertion in the *fliC* gene, after coding base +71. Synthesis of the double-stranded DNA to be used for Tn10dTc replacement was performed by an in vitro polymerization reaction (fill-in) using two primers of different lengths. Long primers (91 bases) carried the *fliC* coding sequence second-site suppressor of the motility defect in the *fliC* 5'UTR $-38C:T$ mutant. The primers were as follows: Mot+4 *fliC*utr (5'-G TCG CTG TTG AGC CAG AAT AAC CTG AAC AAA TCC CAG TCC GCT CTG GGC ACC GCT ATC GAG CGT CTG TCT TCC GGT CTG CGT ATC AAC AGC-3') carrying the TH7312 mutation (+41 C:G), Mot+6 *fliC*utr (5'-G TCG CTG TTG AAC CAG AAT AAC CTG AAC AAA TCC CAG TCC GCT CTG GGC ACC GCT ATC GAG CGT CTG TCT TCC GGT CTG CGT ATC AAC AGC-3') carrying the TH7314 mutation (+41 C:A), Mot+10 *fliC*utr (5'-G TCG CTG CTG AAC CAG AAT AAC CTG AAC AAA TCC CAG TCC GCT CTG GGC ACC GCT ATC GAG CGT CTG TCT TCC GGT CTG CGT ATC AAC AGC-3') carrying the TH7318 (+37 T:C) mutation, and Mot + 3' Fillin (5'-GTT GAT ACG CAG ACC-3') complementary to the 3' ends of the latter primers and used to provide a free 3'-hydroxyl group for initiation of synthesis.

Construction of *flgM* alleles into strain TH7293. A Δ *flgM::tetRA* insertion-deletion was introduced in strain TH7293 by using λ -Red (5). The primers used were as follows: *flgM* TAA tetA (5'-C ATC TGG TCA AGT ATT TCT GAC AAA CGA GTC ATA CGC TTA CTAAGCACTTGCTCTCCTG-3') and *flgM* AUG tetR (5'-A GCT GGC CGC TAC AAC GTA ACC CTC GAT GAG GAT AAA TAA-3'). PCR verification was performed to confirm the correct structure with the primers T1Test (5'-AGG AGA GAT TTC ACC GC-3'), which is 750

bases upstream of the *tetA* stop codon reading out of *tetA*, and *flgM3'* (5'-CTG GTC AAG TAT TTC-3'). The *tetRA* cassette was then replaced with the *flgM* gene from the TH7293 motile revertant strain TH8891 (*flgM* G80Stop). The *flgM* gene was amplified with the primers *flgM3'* (5'-CTG GTC AAG TAT TTC-3') and FLGMAUG-40 (5'-TGG CCG CTA CAA CGT AA-3').

Flagellum staining. Cells were grown in LB to an optical density at 600 nm of ca. 0.6 to 0.8. A 0.5-ml volume of bacterial culture was gently mixed with 0.1 ml of fresh fixative solution (1 ml of 16% paraformaldehyde, 1.5 μ l of glutaraldehyde) and 20 μ l of 1 M NaPO₄ buffer (pH 7.4). A 10- μ l volume of sample was added into each well of a poly-L-lysine (Sigma) multitest slide by using a wide-bore Eppendorf tip. Samples were incubated for 20 min at room temperature. Fixed bacteria were washed three times with phosphate-buffered saline (PBS; pH 7.4) and allowed to dry completely. A 10- μ l volume of blocking solution (2% bovine serum albumin [BSA] in PBS) was added to each well, and the slides were incubated for 10 min at room temperature (9, 25). Cells were then treated with 10 μ l of the anti-FlaC antibody (Difco Salmonella H antiserum) purified by serum affinity chromatography as described previously (10) at a 1:200 dilution in BSA-PBS overnight at 4°C. Slides were placed on wetted 3MM paper in a petri dish and wrapped with Parafilm to prevent evaporation. Samples were washed 10 times with PBS and treated with 10 μ l of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (1:200 dilution in BSA-PBS) for 2 h at room temperature in the dark. Samples were washed eight times with PBS and equilibrated with 1 drop of SlowFade Light Antifade Equilibration Buffer (Molecular Probes). Cell membranes and DNA were stained with 1 μ l of FM4-64 (1 mg/ml; Molecular Probes) (24) and 2 μ l of DAPI (4',6'-diamidino-2-phenylindole; 2 ng/ml; Molecular Probes). Slides were mounted in SlowFade glycerol (Molecular Probes). Samples were observed with a Zeiss Axioscope 2 MOT microscope equipped with a $\times 100$ objective lens and photographed with an Hamamatsu ORCA-ER digital camera. Cells were illuminated with a 100 Mercury arc with the following cubes set (Chroma Technology): 41028 yellow green fluorescent protein (GFP) BP for FITC, 31058 FM4-64 for the FM4-64 probe, and 31041 blue GFP II for DAPI. Individual fluorescent channels and images were merged by using Open Lab modular software (Scientific Imaging) to create the final picture.

RNA isolation. Strains were grown in LB broth at 37°C to logarithmic phase (optical density at 600 nm of 0.5). Transcription was inhibited by rifampin (Sigma) treatment at a concentration of 500 μ g/ml. A 1.5-ml volume of culture was added to a guanidine (GuSCN)-phenol solution (7.5 M guanidine thiocyanate, 0.5% sodium dodecyl sulfate, 1 mM EDTA, 50 mM sodium acetate [pH 4.0]) at 0, 0.5, 1, 1.5, 2, 3, and 4 min after the addition of rifampin. Total RNA was prepared as described previously (8). RNA samples were analyzed by agarose gel electrophoresis to ensure RNA quality.

Probe construction. A [³²P]UTP radiolabeled RNA probe used to detect the *fliC* transcript was synthesized by using an in vitro transcription reaction (Ambion MAXIScript). Template DNA for the transcription reaction was amplified using the following primers: T7 *fliC* 3' (5'-TAC GAC TCA CTA TAG GGA GA CAC ATT CAG CGT ATC CAG AC-3') and 5' Vitrotran *fliC* (5'-TGC GCA GAC CAC TGA AG-3'). T7 *fliC* 3' has the T7 promoter sequence (19 bp) at the 5' end. Amplification of the target DNA yielded a PCR product that contained the T7 promoter upstream of the sequence of interest. The probe was complementary to the sequence of the *fliC* transcript located between 217 to 504 bases downstream of the *fliC* ATG start codon. The PCR product was examined on agarose gel to verify that the product was unique and of the expected size. A total

FIG. 1. (A) Motility phenotype of the Mot⁺ revertant TH7317, wild-type TH6232, and the mutant strain TH7293. Strains were tested on motility plates as described in Materials and Methods, and incubation was performed for 6 h at 37°C. (B) Potential RNA secondary structure of the *fliC* 5'UTR TH7317 including stem-loop (SL) regions and motile revertants of -38C:T mutant strain TH9293 (boxed). The structure included the *fliC*+1 translational start site and, 12 nt downstream, the AUG start codon. The RNA secondary structure was predicted by using the M-FOLD program (version 3.1). The *fliC* 5'UTR -47 to -37 (strain TH7317) deletion includes 12 nt of the second stem-loop SL2. A double mutant was also isolated that includes an insertion of an "A" base at the normal -41 (relative to the AUG start codon) location and a large duplication of -37 through -22, inclusive. A "+" symbol indicates that it is a double mutation.

of 0.1 µg of double-stranded DNA was used as a template in the in vitro transcription reaction.

RESULTS

Isolation and characterization of motile revertants of the *fliC* 5'UTR -38C:T allele. In an accompanying study, the 5' end of the *fliC* gene was mutagenized, and motility-defective mutants were isolated and characterized for defects in transcription, translation, and filament assembly (1). A stem-loop structure, SL2, within the *fliC* 5'UTR was identified that appeared to be critical for *fliC* translation because mutants defective in stem formation showed reduced *fliC* mRNA translation and motility. For example, the single mutants -45G:C and -38C:G in SL2 reduced translation and possessed a motility defect, while the -45G:C -38C:G double mutant had wild-type levels of *fliC* mRNA translation and was motile. We sought to characterize the role of SL2 in *fliC* mRNA translation through the isolation and characterization of motile revertants of one of the SL2 translation-specific mutants (Fig. 1). Strain TH7293 carries a *fliC*-5'UTR single base mutation in SL2 (-38C:U) that is defective in *fliC* mRNA translation,

resulting in a motility-defective phenotype (Mot⁻). Mot⁺ revertants of the -38C:T mutant were isolated and characterized. The Mot⁺ revertants were first screened for linkage to the *fliC* gene, and ca. 99% of the Mot⁺ revertants were linked to the *fliC* gene. Linkage was determined by growing a P22 transducing lysate on the Mot⁺ revertants strains, and the lysate was used to transduce strain TH7952 [Δ *hin-5717*::FRT (*fliC*^{ON}) Δ *fliC*5532::tetRA] to Tc^s. Strain TH7952 has a segment of DNA containing the -10 of the *fliC* promoter through base -10 from the *fliC* ATG start codon deleted and replaced by a *tetRA* cassette (1). Transduction to Tc^s will replace the *tetRA* cassette with the *fliC* 5' end from the donor strain. Tc^s transductants were tested directly for motility onto motility plates for linkage of the Mot⁺ revertants in the donor strain to the *fliC* region. We performed, as a control, the same genetic cross using as donor a P22 lysate grown on the parent strain TH7293. Selection was done for Tc^s colonies. As expected, all transductant colonies (100 of 100) from the control cross were Mot⁻.

DNA sequence analysis of *fliC*-linked motile revertants of the *fliC* -38C:T 5'UTR allele. Initially, 14 independent motile

(Mot⁺) revertants of strain TH7293 were isolated, and all were linked to *fliC*. DNA sequence analysis resulted in two categories of mutations (Fig. 1). Ten Mot⁺ revertants had mutations in the 5'UTR, including single base changes at positions -15, -39, -41, and -46 (relative to the ATG start codon); a single base deletion at -45; a duplication of bases -7 through -1; a deletion of the entire SL2 and a double mutant with a single base insertion of A at the original -40 position; and a large duplication from -37 through -22. In addition to the 5'UTR mutants, four mutants were obtained with single base changes in the *fliC*-coding region at amino acids 13 and 14. One of these, obtained twice, was a silent mutation at amino acid 13 (L13L), and the other two were at amino acid 14 (T14S and T14N).

We found the revertant resulting from a complete deletion of SL2 to be remarkable. Prior to the isolation of this mutant, we thought the fact that the -38C:T *fliC* 5'UTR mutant was defective in *fliC* mRNA translation meant that this region was required for translation. The fact that the entire SL2 could be deleted to restore translation is strong evidence that SL2 is a translational inhibitory domain of the 5'UTR, and the -38C:T allele resulted in an increase in the translational inhibitory activity of the domain.

A rare, unlinked motile revertant of the *fliC* -38C:T 5'UTR allele is in *flgM*. In an independent set of experiments, 160 independent Mot⁺ revertants of strain TH7293 were isolated and checked for linkage to *fliC*. All but one was linked. The unlinked Mot⁺ revertant was mapped through the isolation of a linked Tn10dTc transposon. One insertion that was 67% linked to Mot⁺ by P22-mediated transduction was determined by DNA sequence analysis to be in the *mviM* gene that encodes for a putative *Salmonella* virulence factor. The *mviM* gene is closely linked to the flagellar *flg* genes. Three-factor mapping analysis was performed using the *mviM*::Tn10dTc and a Δ pyrC::Cm allele to localize the *fliC*-unlinked Mot⁺ mutation to a small enough region of the chromosome that could be analyzed by DNA sequence analysis. The three-factor cross analysis placed the Mot⁺ allele clockwise of both *mviM* and *pyrC* on the standard *Salmonella* linkage map in the vicinity of the *flgAMN* genes. Further DNA sequence analysis revealed the Mot⁺ allele to be a single-base-change mutation in the *flgM* gene (+238 G:T) 53bases upstream the *flgM* gene stop codon that resulted in a TGA stop codon at the G80 amino acid codon position of *flgM* [GGA(Gly)80:TGA(Stop)].

Effect of *fliC*-linked -38C:T motile revertants on *fliC* expression. To investigate the contribution of the -38C:T mutation on *fliC* expression, *fliC-lac* transcriptional and translational fusions in *fliC* were introduced into strains that had either a wild-type *fliC* 5'UTR, the, -38C:T allele (*fliC*5969), or the deletion the *fliC* 5'UTR SL2 region (Δ SL2). Initial assays showed *fliC* expression of the translational reporter fusion to be elevated by the SL2 deletion (Table 2, compare TH6235 [row 1] to TH8905 [row 3]), although the standard deviation was too high in these assays to be conclusive. In order to get a more accurate measure on the effect of Δ SL2 on *fliC* translation, we introduced mutations in the *fliC* promoter region that had been isolated in our lab to downregulate *fliC* gene transcription (1). The purpose of using promoter-down mutations was twofold. First, the *fliC* gene has a strong promoter, and β -galactosidase activities are easier to measure when *fliC-lac*

TABLE 2. Effects of *fliC* 5'UTR mutations on *fliC-lac* transcription and translation

Row	Strain background [strain(s)]	Activity $\pm$ SD ^a	
		Transcriptional	Translational
1	Δ hin-5717::FRT (<i>fliC</i> ^{ON}) (TH6234, TH6235)	1,500 $\pm$ 90	2,600 $\pm$ 320
2	Δ hin-5717::FRT <i>fliC</i> 5969(-38C:T) (TH8901, TH8904)	1,300 $\pm$ 110	360 $\pm$ 75
3	Δ hin-5717::FRT <i>fliC</i> 6355(Δ SL2) (TH8902, TH8905)	1,300 $\pm$ 86	2,900 $\pm$ 210
4	Δ hin-5717::FRT P _{fliC} (TH8860, TH8872)	62 $\pm$ 1	110 $\pm$ 5.1
5	Δ hin-5717::FRT <i>fliC</i> 5969 P _{fliC} (TH8862, TH8874)	56 $\pm$ 4	22 $\pm$ 1
6	Δ hin-5717::FRT <i>fliC</i> 6355 P _{fliC} (TH8864, TH8876)	56 $\pm$ 4	160 $\pm$ 11
7	No Mud control strain (TH6232)	2.0 $\pm$ 0.1	2.0 $\pm$ 0.1

^a The numbers are β -galactosidase activity units in nmol/min/optical density at 650 nm ml. The levels of *fliC* transcription and translation were assayed using either a *fliC*::MudJ (*lac* transcriptional) or *fliC*::MudK (*lac* translational) reporter fusions (3). All strains are locked in the *fliC*^{ON} orientation (Δ hin-5717::FRT). These assays were performed in the presence or absence of a *fliC* promoter down mutation (Δ A between the -10 and -35 promoter regions [-23 relative to the transcription start site]). This was done to show that regulation was independent of *fliC* promoter strength and allows future Lac⁺ revertant selections to be done with the promoter-down mutant backgrounds. The following strains were used (listed in order of MudJ then MudK): wild type, wild-type promoter-down, TH8860 and TH8872; *fliC*5969(-38C:T) promoter-down, TH8862 and TH8874; Δ SL2 motile revertant promoter-down, TH8864 and TH8876. TH6232 was used as the background (no-Mud) control.

fusions are expressed at lower levels. Second, the -38C:T mutant is Lac⁺ on selective media with the normal *fliC* promoter, but Lac⁻ with the promoter-down mutation, which facilitates the isolation of revertants using Lac selections. The promoter-down mutation was a single base deletion in the spacer region between the -10 and -35 promoter sequences (-23 Δ A relative to +1 of the translation). We obtained statistically significant results with the promoter-down allele. The effect of the Δ SL2 mutation was to increase *fliC-lacZ* translation ca. 30% over the wild type (Table 2, compare strains TH8872 [row 4] and TH8876 [row 6]).

Transcriptional assays of strains with either a wild-type *fliC* 5'UTR region, the -38C:T, or the Δ SL2 allele all showed similar β -galactosidase levels, with or without the *fliC* promoter-down allele, indicating that the -38C:T and Δ SL2 mutations in the *fliC* 5'UTR had no apparent effect on *fliC* transcription or mRNA stability (Table 2). These results were confirmed by RNase protection assays (data not shown). In contrast a five- to sevenfold decrease in β -galactosidase activity was observed with the *fliC-lacZ* translational fusion with the -38C:T 5'UTR mutation (Table 2, compare TH6235 [row 1] and TH 8904 [row 2] and compare TH8872 [row 4] with TH8874 [row 5]). As mentioned above, deletion of SL2 restored *fliC* translation to a level greater than that observed with the wild-type *fliC* 5'UTR. To determine whether the SL2 deletion results in increased *fliC*⁺ mRNA translation compared to *fliC*⁺ mRNA with the wild-type 5'-UTR, FliC protein levels were measured in wild-type (TH6232), -38C:T (TH7293), and Δ SL2 (TH7317) strains. A fivefold decrease in FliC protein levels due to the -38C:T mutation and a 50% increase in FliC protein levels due to the Δ SL2 mutation over the wild-type levels were observed by Western blot analysis (Fig. 2).

The results presented above indicate that SL2 is a negative

FIG. 2. FliC levels in various *fliC* 5'UTR mutant strains with an intact *fliC* coding sequence. FliC was detected by using a polyclonal anti-FliC antibody (Difco). Immunoblots were performed in triplicate with log-phase cells. The following strains were tested: TH6232 (wild type [WT]), TH7293 (-38C:T), TH7317 (ΔSL2), TH7312 (-38C:T, +41C:G), TH7314 (-38C:T, +41G:A), TH7318 (-38C:T, +37T:C), TH8878 (+41C:G), TH8879 (+41G:A), and TH8882 (+37T:C).

regulator of *fliC* mRNA translation and suggest that the -38C:T substitution enhanced this inhibitory role. Four Mot⁺ revertants of the -38C:T allele had the second site mutation in the *fliC* coding sequence: TH7312 (+41C:G), TH7314 (+41C:A), TH7318, and TH7321 (+37T:C). The fact that one of these Mot⁺ revertant mutations (in both TH7318 and TH7321) resulted in a change of a nonrare leucine codon to another nonrare leucine codon suggested that the effect of these mutations was due to the changes in mRNA sequence independent of the amino acid codon change. The FliC protein was assayed for the *fliC*-coding Mot⁺ revertants in the -38C:T background (TH7312, TH7314, and TH7318) (Fig. 2). The +41C:A allele of strain TH7314 restored FliC levels to those seen in the wild-type strain TH6232. The other two coding revertant alleles, +37T:C (TH7318) and +41C:G (TH7312), had 50% higher FliC levels comparable to the SL2 deletion revertant strain (TH7317). The *fliC*-coding Mot⁺ revertant alleles were separated from the original -38C:T mutation. When separated from the -38C:T mutation, the FliC levels were at best 30% higher for +37 T:C (TH8882) and +41 C:A

FIG. 3. Diagram of potential stem structure formed between the SL2 region of the *fliC* 5'UTR and the *fliC* early coding region. Single base substitutions that suppress the -38C:T translation defect are also shown.

(TH8879) and the same for the +41 C:G allele (Fig. 2). Consistent with the FliC levels observed, the *fliC*-coding Mot⁺ revertant alleles exhibited a wild-type motility phenotype (data not shown).

Potential interaction of the *fliC* 5'UTR SL2 region with *fliC* coding sequences. The isolation of -38C:T motile suppressors in the *fliC* coding region at positions +37 and +41 suggested that these regions might interact. When these sequences were aligned, we found a remarkably strong potential stem structure that could form by base pairing between the SL2 region of the 5'UTR and a region of *fliC* coding sequence from positions +32 through +43 (Fig. 3). According to this model, a stretch of 11 bases in the SL2 region of the *fliC* 5'UTR can base pair with a stretch of 12 bases in the *fliC* coding sequence, leaving a single A residue unpaired. We decided to insert a base in the *fliC* 5'UTR between the -44C and -45G residues that could have the potential of base pairing with the unpaired A residue in the *fliC* coding sequence. An insertion of A, C, or G resulted in a motility phenotype similar to that of the wild type, whereas the insertion of a T residue resulted in a nonmotile phenotype (results not shown). This suggests that strengthening of the interaction of SL2 with the *fliC* coding region has an inhibitory effect on *fliC* translation. The insertion of A, C, or G was not expected to enhance the interaction between the SL2 region, and the resulting motile phenotypes are in agreement with this result.

Effect of *flgM* alleles on suppression of the -38C:T translation defect. Strain TH8891 [Δ *hin-5717::FRT*(*fliC*^{ON}) *fliC*5969

FIG. 4. Motility plate comparing the effects of various *flgM* mutants on the suppression of the -38C:T translation-defective mutation in the *fliC* 5'UTR.

(-38C:T) *flgM6318*(G80Stop)] harbors the *fliC*-unlinked Mot⁺ revertant of -38C:T that resulted in stop codon mutation at amino acid 80 of FlgM. To verify that this single base change at codon 80 was responsible for the Mot⁺ revertants phenotype, the *flgM* region from TH8891 was amplified by PCR, and the resulting DNA fragment was used to replace the Δ *flgM*::*tetRA* allele introduced into the parent strain TH7293 [Δ *hin*-5717::FRT(*fliC*^{ON}) *fliC5969*(-38C:T)] using selection for Tc^s in the presence of the λ -Red functions. The new strain, TH9213, acquired the Mot⁺ revertant phenotype of the donor strain, indicating that the *flgM6318*(G80Stop) allele could suppress the motility defect caused by decreased *fliC* mRNA translation of the -38C:T mutation. This was quite unexpected. FlgM inhibits *fliC* transcription as an anti- σ^{28} factor, but the -38C:T mutation does not affect *fliC* transcription (Table 2). However, increased *fliC* transcription could compensate for reduced *fliC* mRNA translation if an overall increase in *fliC* transcript led to more FliC gene product. However, why was the *flgM6318*(G80Stop) suppressor of the -38C:T motility-defective mutation such a rare occurrence (<1% of motile revertants)?

To determine whether the loss of FlgM resulted in suppression of the -38C:T-dependent motility-defective phenotype, a

set of *flgM* alleles isolated as having reduced or no anti- σ^{28} activity was transduced into the TH7293 Δ *flgM*::*tetRA* strain selecting for Tc^s. The set included *flgM*⁺ and a complete non-polar deletion (Δ *flgM5628*::FRT) of *flgM*, two point mutants—*flgM5524*(R5C) and *flgM5528*(I82T)—that are defective in interacting with σ^{28} , and *flgM5529*, which has a stop codon mutation at amino acid 89 of *flgM*. A gradient of motility suppression was observed (Fig. 4). A slight increase in motility was observed with the single amino acid substitution mutants, a little more with 89-Stop mutant, and even more with the complete *flgM* deletion. However, the *flgM6318*(G80-Stop) allele clearly suppressed the motility defect over all other *flgM* alleles. This suggests that truncated FlgM, missing its last 18 amino acids but not missing its last 9 amino acids, has an active role in suppressing the translation defect of the *fliC* -38C:T mutation independent of its anti- σ^{28} activity.

Effect of *flgM* alleles on σ^{28} -dependent transcription. The *flgM6318*(G80-Stop) suppressor was tested for its effect on *fliC* transcription and translation compared to the other *flgM* alleles by using *lac* transcriptional and translational reporter constructs (Table 3) (3). Both the *flgM6318*(G80-Stop) suppressor allele and the *flgM* allele with the entire *flgM* coding region deleted (Δ *flgM5628*::FRT) exhibited a comparable in-

TABLE 3. Effects of *flgM* alleles on *fliC-lac* transcription and translation

Row	Strain background	Mean activity $\pm$ SD ^a	
		Transcriptional	Translational
1	<i>flgM</i> ⁺ (TH10043, TH10060)	1,500 $\pm$ 80	1,700 $\pm$ 100
2	<i>flgM5524</i> (R5C) (TH10038, TH10055)	1,700 $\pm$ 50	2,100 $\pm$ 280
3	<i>flgM5528</i> (I82T) (TH10039, TH10056)	1,500 $\pm$ 90	2,000 $\pm$ 100
4	<i>flgM5529</i> (E89-Stop) (TH10040, TH10057)	1,600 $\pm$ 73	1,900 $\pm$ 110
5	Δ <i>flgM5628::FRT</i> (TH10041, TH10058)	2,200 $\pm$ 97	2,300 $\pm$ 250
6	<i>flgM6318</i> (G80-Stop, Mot ⁺) (TH10042, TH10059)	2,100 $\pm$ 84	2,400 $\pm$ 120
7	<i>fliC5969</i> (UTR-38C:T) <i>flgM</i> ⁺ (TH10157, TH10163)	1,100 $\pm$ 170	140 $\pm$ 19
8	<i>fliC5969 flgM5524</i> (R5C) (TH10152, TH10158)	1,200 $\pm$ 190	430 $\pm$ 23
9	<i>fliC5969 flgM5528</i> (I82T) (TH10153, TH10159)	1,100 $\pm$ 97	470 $\pm$ 51
10	<i>fliC5969 flgM5529</i> (E89-Stop) (TH10154, TH10160)	1,200 $\pm$ 200	430 $\pm$ 35
11	<i>fliC5969 flgM5628::FRT</i> (TH10155, TH10161)	1,500 $\pm$ 210	670 $\pm$ 82
12	<i>fliC5969 flgM6318</i> (G80-Stop) (TH10156, TH10162)	1,500 $\pm$ 180	650 $\pm$ 88
13	No Mud control strain (TH6232)	2.0 $\pm$ 0.1	2.0 $\pm$ 0.1

^a The numbers are β -galactosidase activity units in nmol/min/optical density at 650 nm ml. The levels of *fliC* transcription and translation were assayed using either a *fliC::MudJ* (*lac* transcriptional) or *fliC::MudK* (*lac* translational) reporter fusions (3). All strains are locked in the *fliC*^{ON} orientation using alleles defective in flagellar phase variation (either *fliB*^{enx} *vh2* or Δ *hin-5717::FRT*). The strain designations listed in the order transcriptional fusion, translational fusion.

creases in β -galactosidase activity in the *lac* transcriptional and translational fusion constructs compared to the *flgM*⁺ allele [Table 3, compare *flgM*⁺ levels of TH10043 and TH10060 (row 1) with Δ *flgM5628::FRT* levels of TH10041 and TH10058 (row 5) and with *flgM6318*(G80-Stop) levels of TH10042 and TH10059 (row 6)]. The other *flgM* alleles—*flgM5524*(R5C), *flgM5528*(I82T), and *flgM5529*(E89-Stop)—had only a small effect on *fliC-lac* expression (compare row 1 [*flgM*⁺] to rows 2, 3, and 4 in Table 3). The *fliC-lac* transcriptional and translational assays were repeated in the presence of the *fliC* –38C:T mutation (*fliC5969*). The *fliC5969* showed reduced *fliC-lac* transcription of ca. 25% and reduced *fliC-lac* transcription and translation of ca. 95% (Table 3, compare row 1 with row 7). Again, both the *flgM6318*(G80-Stop) and the Δ *flgM5628* alleles exhibited comparable increases in β -galactosidase activity in the *lac* transcriptional and translational fusion constructs compared to the *flgM*⁺ allele (Table 3, compare row 7 with rows 11 and 12), whereas the other *flgM* alleles had smaller effects (Table 3, compare row 7 with rows 8, 9, and 10). These results demonstrate that the *flgM* motile revertant of the *fliC* –38C:T 5'UTR mutant allele, *flgM6318*(G80-Stop), did not exhibit significant differences in its effect on *fliC* transcription and translation in the presence or absence of the –38C:T 5'UTR mutant allele compared to the complete deletion allele of *flgM* (Δ *flgM5628*).

Finally, we tested the effect of the *flgM* alleles on the expression of a *lac* transcriptional reporter to another σ^{28} -dependent promoter, the *motA* promoter, in a strain defective in HBB formation (Δ *flgG-L*). The purpose of these experiments was to determine whether FlgM(G80-Stop) was able to bind

TABLE 4. Anti- σ^{28} activity of *flgM* alleles

Row	Strain genotype	Mean transcriptional activity $\pm$ SD ^a
1	Δ <i>flgG-L2157 fljB</i> ^{enx} <i>vh2</i> (TH10236)	3.6 $\pm$ 0.5
2	Δ <i>flgG-L2157 fljB</i> ^{enx} <i>vh2 flgM5524</i> (R5C) (TH10231)	8.0 $\pm$ 2.3
3	Δ <i>flgG-L2157 fljB</i> ^{enx} <i>vh2 flgM5528</i> (I82T) (TH10232)	22 $\pm$ 4.5
4	Δ <i>flgG-L2157 fljB</i> ^{enx} <i>vh2 flgM5529</i> (E89-Stop) (TH10233)	17 $\pm$ 2.1
5	Δ <i>flgG-L2157 fljB</i> ^{enx} <i>vh2 flgM5628::FRT</i> (TH10234)	280 $\pm$ 20
6	Δ <i>flgG-L2157 fljB</i> ^{enx} <i>vh2 flgM6318</i> (G80-Stop) (TH10235)	230 $\pm$ 36
7	No Mud control strain (TH6232)	2.0 $\pm$ 0.1

^a The numbers are β -galactosidase activity units in nmol/min/optical density at 650 nm ml. Levels of σ^{28} -dependent transcriptional activities were assayed in strains carrying a *motA::MudJ* (*lac* transcription) reporter construct and a deletion of HBB genes (Δ *flgG-L*).

σ^{28} and show anti- σ^{28} activity. The Δ *flgG-L* mutation prevents FlgM secretion from the cell and σ^{28} -dependent transcription is completely inhibited (10). In this assay, the *flgM5524*(R5C), *flgM5528*(I82T), and *flgM5529*(E89-Stop) alleles show significant anti- σ^{28} activity (Table 4, compare *flgM*⁺ [row 1] with rows 2, 3, and 4), whereas the *flgM5529*(E89-Stop) allele showed only a small decrease in *motA-lac* expression compared to the Δ *flgM5628* allele [Table 4, compare *flgM*⁺ (row 1) with Δ *flgM5628* (row 5) and with *flgM5529*(E89-Stop) (row 6)]. These results demonstrate that the *flgM6318*(G80-Stop) allele did not exhibit significant differences in its effect on anti- σ^{28} activity compared to the complete deletion allele of *flgM* (Δ *flgM5628*).

Effect of the *fliC* –38C:T allele and motile revertant alleles on flagellum production. The effect of the *fliC* –38C:T 5'UTR allele on flagellum production was examined by microscopy. Immunofluorescence was performed with strains TH6232 (*fliC*⁺), TH7293 (–38C:T), and TH7317 (Δ SL2). The cells were treated with the live membrane stain FM4-64 (red) and DAPI (blue DNA stain). Flagellar filaments (FliC) were colored green by using a polyclonal anti-FliC antibody followed by the addition of anti-rabbit FITC-conjugated antibody as described in Materials and Methods (Fig. 5). TH6232 (*fliC*⁺) cells showed an average of five to six flagella per surface area, while TH7293 (*fliC*–5'UTR –38C:T) had an average of one or two short flagella per cell. TH7317 (Δ SL2) exhibited at least as many flagella as the TH6232 (*fliC*⁺) control. The results are consistent with a reduction of *fliC* mRNA translation by the *fliC* –38C:T 5'UTR allele and normal or slightly elevated (50%) *fliC* mRNA translation with the *fliC* Δ SL2 5'UTR deletion mutant.

DISCUSSION

We discovered a region of the *fliC* 5'UTR that is involved in the inhibition of *fliC* translation. A stem-loop region within the 5'UTR, called SL2, was identified as having a number of base substitutions that were defective in expression of a *fliC-lacZ* translational fusion reporter but did not affect the expression of a *fliC-lac* transcriptional fusion reporter (1). The mutants

FIG. 5. Characterization of flagella by immunofluorescence microscopy. Strains were grown in LB medium until log phase, fixed, and stained as follows: cell membranes were stained red with FM4-64, DNA was stained blue with DAPI, and flagellar filaments (FliC) were stained green with FITC-conjugated anti-FliC antibody. Three different filter sets were used for each fluorophore. Individual images were merged as described in Materials and Methods to create the final pictures.

with decreased translation all destabilized the stem of SL2. One pair of mutations that were in the different bases within a presumed SL2 base pair, $-38\text{C}:\text{G}$ and $-45\text{G}:\text{C}$, individually resulted in defective *fliC* mRNA translation, but the combination of the two resulted in wild-type translation levels. In the present study, motile revertants were analyzed for one translation-defective mutant of the SL2 stem, TH7293, that harbors the $-38\text{C}:\text{T}$ mutation. The analysis of motile revertants led to several surprising discoveries.

One surprising result from the DNA sequence analysis of motile revertants was that one motile revertant had SL2 completely and precisely deleted. This demonstrated that SL2 was not required for normal *fliC* mRNA translation. In fact, there was a slight increase in *fliC* mRNA translation in the SL2 deletion mutant. We can only conclude that SL2 does not function to facilitate *fliC* mRNA translation. The fact that translation-defective mutants arise from single base substitutions that disrupt the stem of SL2 suggests that SL2 has a role in the inhibition of *fliC* mRNA translation under some as-yet-unidentified condition. Mutations that disrupt the stem lead to the loss of controlled inhibition by SL2 so that SL2 inhibitory activity is constitutive. We suspect that *fliC* transcription is localized to the base of the flagellum to couple *fliC* mRNA translation to assembly. It is possible that SL2 has a role in preventing unwanted *fliC* mRNA translation in the cytoplasm where translation is not coupled to secretion and assembly. However, since cells deleted for the SL2 are perfectly motile, it cannot be essential for *fliC* translation and assembly. The SL2 segment probably contributes to the efficiency of the assembly process.

A second unexpected finding was that a number of suppressors of the translation-defective $-38\text{C}:\text{T}$ allele were in the *fliC* coding region. These suppressors were at positions +37 and +41 relative to the beginning of the ATG start codon. The finding that one suppressor did not change the amino acid codon is consistent with a translation-specific effect. This intriguing set of mutants is reminiscent of the signal recognition particle pathway in gram-negative bacteria involved in the co-translation assembly of inner membrane proteins. The N-terminal 50 amino acids of the FliC protein (1-MAQVINTNSL SLLTQNNLNK SQSALGTAIE RLSSGLRINS AKDDAAG QAI-50) include the secretion signal and are not visible in the final crystal structure (29) but could be visualized by using electron cryomicroscopy and image analysis (30). The N terminus of a peptide can affect the location of translation in the cell. The signal recognition particle pathway recognizes the nascent N-terminal peptide of proteins destined for the cytoplasmic membrane or for the Sec type II secretion pathway and facilitates the localized translation of these proteins (7). It may be that there are regions in the *fliC* mRNA sequence that cause ribosome stalling to prevent *fliC* mRNA translation when it is not coupled to secretion. Two such regions would be SL2 and the sequence around the amino acid 13,14 coding region. When these sequences are aligned, there is a remarkably strong stem structure that forms (Fig. 3). All of the motile revertants of the $-38\text{C}:\text{U}$ translation defect that were due to sequence changes in either SL2 or the +37/41 coding region disrupt this stem structure. Strengthening the interaction between the SL2 region of the *fliC* 5'UTR and the *fliC* coding region by insertion of a T residue at position -45 relative to

the ATG start codon resulted in a nonmotile phenotype, whereas insertion of A, G, or C, which are not predicted to strengthen this interaction, had no effect on motility. This supports the model that interaction between these regions plays an important role in the regulation of *fliC* mRNA translation. It is not clear how the duplication revertants affect this stem-loop structure since additional sequence could easily affect the secondary and tertiary conformations of the *fliC* 5' mRNA structure.

A third unexpected result was the isolation of the -15G:A substitution resulting in suppression of the motility defect of the strain with a -38C:T translation defective mutation. This is because that exact same mutation, the -15G:A transition in the *fliC* 5'UTR sequence, was among the single base substitution mutations in the original *fliC* translation-defective mutants (1). Somehow the -15 position can influence the SL2 region in both a positive and a negative manner, depending on what base is located at the -38 position (relative to the AUG start codon) of the *fliC* 5'UTR. The isolation and characterization of motile revertants of the -15G:A in a strain with the "C" base at the -38 position (the wild-type base at this position) should result in the isolation of -38C:T mutants along with other suppressors that might lead to a better understanding of how the -15 position functions in the regulation of *fliC* mRNA translation.

The final unexpected result was the isolation of a motile suppressor in the *flgM* gene. This rare suppressor (<1% of motile suppressors of TH7293) was unusual because the degree of suppression was dependent on the loss of the last 18 amino acids of FlgM and not on an FlgM-null phenotype. Mutants isolated as defective in anti- σ^{28} activity did not suppress the *fliC* mRNA translation defect caused by the -38C:T mutation. This result leads us to conclude that the very C terminus of FlgM is involved in regulation of *fliC* mRNA translation. It fits with the position of single amino acid substitution mutations in FlgM that are defective in anti- σ^{28} activity. These all reside in the region from amino acid 57 through amino acid 82. We have isolated a stop codon mutant at amino acid codon 89 of *flgM* with reduced anti- σ^{28} activity, but it retains the translation defect caused by the -38C:T mutation in the *fliC* 5'UTR. The fact that no single amino acid substitutions in FlgM relieved the *fliC* mRNA translation inhibitory activity suggests that there are multiple amino acids in the C terminus of FlgM that contribute to this activity. A good future experiment would be a targeted mutagenesis of the C-terminal 18-amino-acid coding region of *flgM*, followed by a screen for increased or decreased translation of *fliC* mRNA.

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