Posttranscriptional Control of the *Salmonella enterica* Flagellar Hook Protein FlgE

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Previous work suggested that the FlgE (flagellar hook subunit) protein in *Salmonella enterica* serovar Typhimurium was posttranscriptionally regulated in response to the stage of flagellar assembly. Specifically, the FlgE protein could be detected in flagellar mutants defective at the stages of assembly before or after rod assembly but not in rod assembly mutants, yet *flgE* mRNA levels were unaffected. To elucidate posttranscriptional mechanisms involved in the coupling of *flgE* gene expression to hook assembly, the RNA sequences at the 5' and 3' ends of the *flgE*-containing mRNA processed from the large *flgBCDEFGHIJKL* operon were determined by rapid amplification of cDNA ends, and secretion of the FlgE protein in different flagellar assembly mutant strains was analyzed. The sequences 5' and 3' of the *flgE* gene where RNA processing occurred was within 15 bases upstream of the *flgD* stop codon and at bases 145 to 147 downstream of the *flgF* start codon, respectively. The ribosome binding site of the *flgD* gene was found to be inhibitory to *flgE* translation in strains deleted for the upstream *flgD* gene, unless the region 15 bases upstream of the *flgD* stop codon was present. Secretion of FlgE into the periplasm was monitored using β -lactamase (Bla) fusions as a periplasm-specific reporter, which conferred resistance to ampicillin when FlgE-Bla was secreted into the periplasm. Using this assay, we found that the effect of rod assembly mutants on FlgE levels was due to FlgE turnover in the periplasm and that the FliE rod component protein was required for efficient FlgE-Bla secretion.

Many bacteria can swim through liquid environments or crawl across surfaces by the controlled rotation of extracellular organelles called flagella. Bacteria can access favorable environments or escape from unfavorable environments using flagellum-dependent motility. *Salmonella enterica* serovar Typhimurium possesses 6 to 10 peritrichous flagella per cell (23). Each flagellum is composed of three distinct structures: basal body, hook, and filament (1). More than 60 genes are required for the assembly and function of the flagellum in *S. enterica* serovar Typhimurium (8). Flagellar gene expression is regulated temporally, and flagellar biosynthesis follows an ordered assembly process (16). The flagellar genes are expressed in a transcriptional hierarchy of three temporal promoter classes (8). At the top of the flagellar transcriptional hierarchy in *S. enterica* serovar Typhimurium is the *flhDC* master operon, which is expressed from a class 1 promoter and is sensitive to environmental and cellular signals (20, 33). Early gene products, FlhD and FlhC, form a transcriptional activator complex that directs the σ^{70} -dependent transcription of class 2 promoters (22). The class 2 promoters mediate the transcription of genes whose products are required for the structure and assembly of the hook-basal body (HBB). In addition, two regulatory proteins, flagellum-specific sigma factor σ^{28} (FliA) and its cognate anti-sigma factor, FlgM, are expressed along with hook-basal body genes. The class 3 promoters transcribe genes whose products are needed late in the assembly process, including the structural components of the filament, the motor force generators, and the chemosensory genes. The σ^{28} protein is required for the transcription of class 3 promoters (31) but

prior to HBB completion is tightly associated with anti- σ^{28} factor FlgM (7, 30). Thus, the transcription of class 3 promoters is inhibited until completion of the intermediate hook-basal body structure (16). Upon HBB completion, FlgM is secreted from the cell to free σ^{28} and to allow transcription of class 3 promoters (13, 21).

In addition to sophisticated transcriptional regulation mechanisms that control the expression of flagellar genes, a layer of posttranscriptional regulation mechanisms has recently been uncovered. A posttranscriptional control mechanism is involved in flagellar phase variation in *S. enterica* serovar Typhimurium (5). The FljA protein, previously thought to be a transcriptional repressor of the *fliC* flagellin gene, has been shown to be an RNA-binding translational regulator of *fliC* mRNA translation (P. Aldridge et al., unpublished results). The hook structural gene (*flgE*) expression also appeared to be coupled to flagellum assembly by a posttranscriptional regulatory mechanism (6). The FlgE protein was absent in strains defective in rod assembly, yet the *flgE* mRNA level was unaffected. Recently, similar data were reported for *fimA* regulation of *Porphyromonas gingivalis* (32) and *flaA* regulation of *Borrelia burgdorferi* (29). The mRNA levels of *fimA* and *flaA* in PG2131 and *flaB* mutants, respectively, did not change, but their protein levels were decreased compared with those of their wild types, consistent with a posttranscriptional control mechanism.

Little is known about *S. enterica* serovar Typhimurium *flgE* mRNA translation in coordination with hook assembly. The *flgB-L* operon has 11 genes, and the stoichiometry of individual proteins per flagellum is highly variable (24). The stoichiometry of the FlgE protein is 5 to 20 times higher than that of other *flgB-L* operon proteins. Northern blot analysis of *Rhodobacter sphaeroides flgE* suggested a role for mRNA processing in the

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TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Genotype	Source or reference ^a
Strains		
<i>S. enterica</i> serovar Typhimurium		
LT2	Wild type	J. Roth
TH4702	pKD46/LT2	5
TH6733	<i>flgE5315::MudA</i>	
TH8547	$\Delta flgD6219(\Delta aa2-232) fljB^{e,n,x} vh2$	
TH9924	$\Delta flgD6534(\Delta aa57-232)$	
TH9925	$\Delta flgD6558(\Delta aa57-232)::tetRA \Delta pyrC::FRT-Km-FRT$	
TH9926	$\Delta flgD6535(\Delta aa34-232)$	
TH9927	$\Delta flgD6536(\Delta aa14-232)$	
TH9928	$pflgD/\Delta flgD6558(\Delta aa57-232)::tetRA \Delta pyrC::FRT-Km-FRT$	
TH9929	$\Delta flgD6537(\Delta aa2-56)$	
TH9930	$\Delta flgD6538(\Delta aa2-177)$	
TH9931	$\Delta flgD6539(\Delta aa2-200)$	
TH9932	$\Delta flgD6540(\Delta aa2-220)$	
TH9933	$\Delta flgD6541(\Delta aa2-224)$	
TH9934	$\Delta flgD6542(\Delta aa2-228)$	
TH9935	$\Delta flgD6543(\Delta aa2-231)$	
TH9936	$pflgD/\Delta flgD6219(\Delta aa2-232) flgC6544(G828T Mot^+) fljB^{e,n,x} vh2$	
TH9937	$pflgD/\Delta flgD6219(\Delta aa2-232) flg-6545(Mot^+) fljB^{e,n,x} vh2$	
TH9938	$pflgD/\Delta flgD6219(\Delta aa2-232) flgC6546(G827A Mot^+) (Mot^+) fljB^{e,n,x} vh2$	
TH9939	$pflgD/\Delta flgD6219(\Delta aa2-232) flgC6547(A829T Mot^+) (Mot^+) fljB^{e,n,x} vh2$	
TH9940	$pflgD/\Delta flgD6219(\Delta aa2-232) flg-6548(Mot^+) fljB^{e,n,x} vh2$	
TH9941	$pflgD/\Delta flgD6219(\Delta aa2-232) flg-6549(Mot^+) fljB^{e,n,x} vh2$	
TH9942	$pKD46/\Delta flgD6219(\Delta aa2-232) fljB^{e,n,x} vh2$	
TH9943	$\Delta flgD6550(\Delta aa2-227)$	
TH9944	$\Delta flgD6551(\Delta aa2-232)::165 \text{ bp of } tetA \text{ 3' region}$	
TH9945	$\Delta flgD6219(\Delta aa2-232) flgC6552(G828T)$	
TH9946	$\Delta flgD6219(\Delta aa2-232) flgC6553(G827A)$	
TH9947	$pflgD/\Delta flgD6219(\Delta aa2-232) fljB^{e,n,x} vh2$	
TH9948	<i>flgE6554::bla</i>	
TH9949	<i>flgE6554::bla \Delta flgBC6557</i>	
TH9950	<i>flgE6554::bla \Delta flgBC6557 flhF5036::Tn10dCm</i>	
TH9951	<i>flgE6554::bla \Delta flgBC6557 flhD5704::Tn10dTc</i>	
TH9952	<i>flgE6554::bla \Delta flgBC6557 \Delta fliE6555::tetRA</i>	
Plasmids		
pTrc99A	P _{trc} expression vector	Pharmacia
pKD46	pBAD γ β <i>exo</i>	9
<i>pflgD</i>	<i>flgD</i> ⁺ in pTrc99A	
<i>pflgDE</i>	<i>flgD</i> ⁺ <i>E</i> ⁺ in pTrc99A	

^a Unless otherwise indicated, all strains and plasmids were constructed for this work.

regulation of *flgB-J* operon expression and identified two different *flgE*-containing transcripts (4).

In the present study, we investigated the posttranscriptional mechanism of *flgE* in *S. enterica* serovar Typhimurium. We used rapid amplification of cDNA ends (RACE) (3) to map the mRNA processing sites of *flgB-L* mRNA in *S. enterica* serovar Typhimurium. In addition, an FlgE- β -lactamase (FlgE-Bla) fusion protein was constructed in order to assay whether the FlgE protein was secreted into the periplasm in rod mutant strains. We found that the FlgE-Bla protein was secreted into the periplasm and that the activity of FlgE-Bla was fully dependent on the flagellar basal structure. We also present evidence that the proximal rod FliE protein is required for efficient FlgE-Bla secretion but is not absolutely required, as was previously suggested (27).

MATERIALS AND METHODS

Strains and plasmids. All bacterial strains and plasmids used in this work are listed in Table 1.

Growth conditions and bacterial genetic and molecular methods. Growth conditions, media, P22 transductional methods, λ -Red swaps, and motility assay were performed as described previously (9, 10).

Construction of the *flgE-bla* fusions. The *flgE-bla* fusions were constructed by the replacement of chromosomally targeted *tetRA* elements with specific DNA fragments as described previously (15). The *tetRA* element from transposon Tn10 includes the tetracycline resistance gene, *tetA*, and the adjacent, divergently transcribed *tetA* repressor gene, *tetR* (17). The *tetRA* element was inserted just before the stop codon of *flgE* in strain LT2 using λ -Red-mediated recombination (9). The *tetRA* element was amplified by PCR with primers flgE403tetR and flgE403tetA (Table 2). The PCR-amplified *tetRA* fragment is flanked at its 5' and 3' ends by 40 bases of DNA sequence identical to the 40 bases upstream of the *flgE* stop codon and the 40 bases downstream of the last amino acid codon of *flgE*. Selection for tetracycline resistance (Tc^r) allows the isolation of the desired recombinants that have the *tetRA* element between the last amino acid codon of *flgE* and the stop codon. The *tetRA* element was replaced by λ -Red-mediated recombination with a PCR-amplified β -lactamase gene (*bla*) that was missing its N-terminal 23-amino-acid Sec-dependent secretion signal and flanked by the same 40-bp sequences identical to the upstream and downstream regions of the stop codon of *flgE*. Primers flgE5amp and flgE3amp (Table 2) were used to amplify the *bla* gene. The replacement of *flgE::tetRA* was achieved by electroporation of the PCR-amplified *bla* gene into the *flgE::tetRA*-containing strain on tetracycline-sensitive (Tc^s) selection medium (26) to create strain TH9948 (*flgE*-

TABLE 2. DNA primers used in this study

Primer	Sequence
5SF	5'-GCGAGAGTAGGGAAGTCCCA-3'
flgE403tetR	5'-AACCCAGGACCAGATCCTCAATACGCTGGTTAACCTGCGCTTAAGACCCACTTTTCACATT-3'
flgE403tetA	5'-TATAAATTGCGTGATCCATTAAGCTATCCCGTCAGGCGCTTACTAAGCACTTGTCTCCTG-3'
flgE5amp	5'-AACCCAGGACCAGATCCTCAATACGCTGGTTAACCTGCGCCACCCAGAAAACGCTGGTGAA-3'
flgE3amp	5'-TATAAATTGCGTGATCCATTAAGCTATCCCGTCAGGCGCTTACCAATGCTTAATCAGTGA-3'
flgBCtetR	5'-AAGCTGTCGGCTGAATTTTGCCATTTGCGGAGGAGATATGTTAAGACCCACTTTTCACATT-3'
flgBCtetA	5'-TCATTTCATATTTACGGCAATAGACATACGCGCCTCCTTTACTAAGCACTTGTCTCCTG-3'
flgBCdelet	5'-TTTAAGCTGTCGGCTGAATTTTGCCATTTGCGGAGGAGATTAAAGGAGGCGCGTATGTCT-3'
flgDrtR	5'-ATGGGTTAGTCGGGTCCTGG-3'
flgCtetR	5'-AAAAAGCATGATGCTTAAAACGCTGACATTAGGCCAGTAATTAAGACCCACTTTTCACATT-3'
flgD-p-5tetRA	5'-TTGAAGAACCAGGACCCGACTAACCCATTACAAAATAATGTTAAGACCCACTTTTCACATT-3'
flgD-p-3tetRA	5'-GAGAAAAAGCATGACCGGACTACTCTGATAAGTGTAAGGGCTTACTAAGCACTTGTCTCCTG-3'
flgBseq5	5'-CATTTTGCGTTTATTCCGGCG-3'
flgER	5'-CCACTTTGGAACCGGCGAAC-3'
flgDF	5'-CTCGCCATGGCAGCTATCAGGCAA-3'
flgDR	5'-TGC GGCTGCAGTAGAACACGGAA-3'
flgERHindIII	5'-TGGTTAAGCTTGCCCGGTCTGGA-3'
flgDdelet	5'-TTAAAACGCTGACATTAGGCCAGTAAAGGAGGCGCGTATGTAAGCCCTTACACTTATCAG-3'
flgDFRTdel	5'-ATTGAAGAACCAGGACCCGACTAACCCATTACAAAATAATTAAGCCCTTACACTTATCAG-3'
flgD5'deletI	5'-CAGCGGATCGATGACCGGAAGCAACGCTGCCATCTGCAATAAGCCCTTACACTTATCAG-3'
flgD5'deletII	5'-TATGTCTATTGCGGTAAATATGAATGACCCGACCAACACGTAAGCCCTTACACTTATCAG-3'
flgD3'delet1	5'-TTAAAACGCTGACATTAGGCCAGTAAAGGAGGCGCGTATGGAGTTAACGACACAGTTGGC-3'
flgD3'delet2	5'-TTAAAACGCTGACATTAGGCCAGTAAAGGAGGCGCGTATGGGTTCTTACAACATTGCGAT-3'
flgD3'delet3	5'-TTAAAACGCTGACATTAGGCCAGTAAAGGAGGCGCGTATGGCTCTGGTACAGGCGGTGAC-3'
flgD3'delet4	5'-TTAAAACGCTGACATTAGGCCAGTAAAGGAGGCGCGTATGGGACACACCACTCGACGA-3'
flgD3'delet5	5'-TTAAAACGCTGACATTAGGCCAGTAAAGGAGGCGCGTATGCTCGACGAAGTTTCGGCAAAT-3'
flgD3'delet6	5'-TTAAAACGCTGACATTAGGCCAGTAAAGGAGGCGCGTATGCGGCAAATAATCTAAGCCCT-3'
flgD3'delet8	5'-TTAAAACGCTGACATTAGGCCAGTAAAGGAGGCGCGTATGATCTAAGCCCTTACACTTAT-3'
flgD3'delet11	5'-TTAAAACGCTGACATTAGGCCAGTAAAGGAGGCGCGTATGGTTTCGGCAAATAATCTAAGC-3'
flgDtetdelet	5'-TTAAAACGCTGACATTAGGCCAGTAAAGGAGGCGCGTATGATTGGCCATTACTGTTTAC-3'
flgE3'RT-PCR	5'-CCACTTTGGAACCGGCGAAC-3'
flgFrtR	5'-TGGTTAACTGCCCGGTCTGGA-3'

bla). Deletion of the flagellar rod structural genes *flgBC* was accomplished by targeted deletion using λ -Red-mediated recombination with a *tetRA* fragment that was PCR amplified with primers flgBCtetR and flgBCtetA. The resulting DNA fragment contains the *tetRA* element flanked by 40 bases of sequence identical to the 40 bases upstream of the start codon of *flgB* and the 40 bases downstream of the *flgC* stop codon. Selection for Tc^r by λ -Red-mediated recombination resulted in the construction of the $\Delta flgBC::tetRA$ allele. The $\Delta flgBC::tetRA$ element was replaced using λ -Red-mediated recombination with a PCR-amplified DNA fragment designed with the 40 bases upstream of the *flgB* start codon fused to the 168 bases downstream of *flgC* stop codon. Primers flgBCdelet and flgDrtR were used to amplify the PCR products.

Site-directed mutagenesis. The $\Delta flgD(\Delta aa2-232)$ (missing amino acids 2 through 232) G828T (TH9945) and $\Delta flgD(\Delta aa2-232)$ G827A (TH9946) alleles were obtained by first constructing a deletion/insertion of the region between the two stop codons of *flgC* and *flgD* in LT2 with a *tetRA* element as described above using λ -Red-mediated recombination. The *tetRA* element was amplified by PCR with primers flgCtetR and flgD-p-3tetRA. The PCR-amplified *tetRA* fragment is flanked at its 5' and 3' ends by 40 bases of DNA sequence identical to the 40 bases upstream of the *flgC* stop codon and the 40 bases downstream of the *flgD* stop codon. The *tetRA* element was replaced using λ -Red-mediated recombination and Tc^r medium with PCR products that contain the *flgBC* sequence and 140 bp of 5' *flgE* sequence from TH9936 and TH9938, respectively, as described above. The PCR products were amplified using primers flgBseq5 and flgER.

Construction of plasmids pflgD and pflgDE. The *flgD* region of LT2 was amplified by PCR using primers flgDF and flgDR (Table 2). The PCR products were digested with NcoI and PstI and ligated into pTrec99A, digested with NcoI and PstI, to make pflgD. The *flgDE* region of LT2 was also amplified by PCR using primers flgDF and flgERHindIII (Table 2). The PCR products were digested with NcoI and HindIII and ligated into pTrec99A, digested with NcoI and HindIII, to make pflgDE.

Construction of *flgD* deletion strains. The *flgD* gene was replaced with a *tetRA* element in LT2 using λ -Red-mediated recombination as described above. The *tetRA* element was amplified by PCR with primers flgD-p-5tetRA and flgD-p-3tetRA (Table 2). The *tetRA* fragment is flanked at its 5' and 3' ends by 40 bases of DNA sequence identical to the 40 bases upstream of the 56th amino acid

codon of *flgD* and the 40 bases downstream of the *flgD* stop codon. To construct *flgD* deletion strains, the $\Delta flgD::tetRA$ element was replaced using λ -Red-mediated recombination and Tc^r medium with PCR products that contain the relevant *flgD* regions. Primers flgDdelet, flgDFRTdel, flgD5'deletI, flgD5'deletII, flgD3'delet1, flgD3'delet2, flgD3'delet3, flgD3'delet4, flgD3'delet5, flgD3'delet11, flgD3'delet6, and flgD3'delet8 (Table 2) were used to make TH8547, TH9924, TH9926, TH9927, TH9929, TH9930, TH9931, TH9932, TH9933, TH9943, TH9934, and TH9935, respectively. Each of the 5' 40 bases of these primers are identical to each of the 40 bases upstream of the deleted regions, and each of the 3' 20 bases of the primers are identical to the 20 bases downstream of the deleted regions. Primer flgE3'RT-PCR was used as a reverse primer that is complementary to the region 20 bases upstream of base 146 of the *flgE* coding sequence. The genomic DNA of LT2 was used as a PCR template. Primer flgDtetdelet, whose 5' 40 bases are identical to the 40 bases upstream of second amino acid codon of *flgD* and whose 3' 20 bases are identical to the 20 bases downstream of the 1,041st base of *tetA*, and primer flgE3'RT-PCR were used to make TH9944. The genomic DNA of the $\Delta flgD::tetRA$ strain was used as a PCR template.

Assay for FlgE- β -lactamase activity. One-milliliter cultures in LB medium were grown overnight at 37°C with aeration. The cells were diluted 100-fold into 3 ml LB medium and grown at 37°C with aeration to mid-log phase (optical density at 600 nm [OD₆₀₀] of 0.5 to 0.7). The cells were diluted 50-fold in LB medium containing variable concentrations of ampicillin (Ap) (twofold serially diluted from 800 μ g/ml to 3.125 μ g/ml). After 6 h of growth at 37°C, the OD₆₀₀ of each sample was measured. If the OD₆₀₀ was lower than 0.05, the Ap concentration in LB was considered as a MIC.

Preparation of RNA. Three-milliliter cultures grown to about 5×10^8 cells/ml were pelleted and resuspended in 50 μ l protoplasting buffer (15 mM Tris-HCl [pH 8], 0.45 M sucrose, 8 mM EDTA). A 5- μ l portion of Lysozyme (10 mg/ml) was added, and the mixture was incubated for 5 min at 25°C. A 500- μ l portion of RNAwiz (Ambion Inc., Austin, TX) was added, and the sample was mixed thoroughly by vortexing vigorously for 10 s and incubated for 5 min at 25°C. After the addition of 100 μ l of chloroform (Sigma), the sample was mixed by vortexing vigorously for 20 s and incubated for 10 min at 25°C. The sample was centrifuged at $10,000 \times g$ for 15 min at 4°C. The 250- μ l aqueous-phase sample was extracted and added to 250 μ l of water. After the addition of 500 μ l of isopropyl alcohol

FIG. 1. Flow diagram of 5' RACE. Total RNAs were randomly ligated and then reverse transcribed using a random primer. PCR amplification was performed with 5SF and gene-specific primers. The PCR products were cloned and sequenced.

(Sigma), the sample was mixed and incubated for 10 min at 25°C. The precipitate was collected by centrifugation at $10,000 \times g$ for 15 min at 4°C and washed with 500 μ l of 75% cold ethanol. The precipitate was then dissolved in 50 μ l of RNA storage buffer (Ambion Inc., Austin, TX). The concentration of RNA was determined by measuring the absorbance at 260 nm.

RACE. Cells were grown at 37°C in LB medium. The RNA was extracted as described above, and when required, 5' phosphate groups were removed by treatment with tobacco acid pyrophosphate (Epicenter). RNA ligation was performed at 37°C for 3 h in a 25- μ l volume containing 2.5 μ g of total RNA, 10 units of T4 RNA ligase (Ambion Inc., Austin, TX), and 20 units of RNasin (Promega). The ligated RNA was cleaned up using a G-50 column (Amersham Biosciences), and 1 μ g of RNA was reverse transcribed at 37°C for 1 h in a 20- μ l reaction volume containing 4 units of Ominiscript reverse transcriptase (QIAGEN), 0.5 mM each deoxynucleotide triphosphate, 0.5 mM random hexamer (Invitrogen), and 10 units of RNasin. PCR amplification was performed with a gene-specific primer and primer 5SF (5'-GCGAGAGTAGGGAAGTCCCA-3'), complementary to the 5S rRNA gene, using Hot Start *Taq* polymerase (QIAGEN). The PCR products were purified by using a PCR purification kit (QIAGEN) and separated by agarose gel electrophoresis. The desired band was eluted, cloned into the pGEM-T-Easy vector (Promega), and sequenced (Fig. 1).

Complementation tests. Complementation tests were performed using a motility plate assay. The *pflgD* or *pflgDE* plasmid was introduced into *flgD* mutants, and the motility activity of those strains was measured on motility agar plates (3 g Difco agar, 10 g Bacto tryptone, and 5 g NaCl per liter) without IPTG (isopropyl- β -D-thiogalactopyranoside). Motility plates were illuminated with a dark-field colony counter (Reichert) and photographed using a digital camera (Nikon E990).

Isolation and characterization of motile revertants from strain TH9947 [*pflgD*/ Δ *flgD*(aa2-232)]. Motile revertants of strain TH9947 (*pflgD*/ Δ *flgD*) were isolated by transferring cells from isolated colonies with a round toothpick into motility plates by poking the cells through the agar followed by incubation at 30°C until revertant motility flares appeared (up to 3 days). Motile (Mot⁺) revertants were single-colony isolated two times on an LB agar plate for purification. The Mot⁺ phenotypes were mapped to the *flg* operon by cotransduction

with the *pyrC* locus. A P22 transducing phage grown on the motile revertants was used to infect *pflgD*/TH9925 [Δ *pyrC*::FRT-Kan-FRT (FKF) Δ *flgD*::*tetRA*], and the mixture was incubated on minimal glucose plates to select *pyrC*⁺ transductants. One-hundred *pyrC*⁺ Tc^s colonies were screened for motility. The screening of only the *pyrC*⁺ Tc^s transductants ensured that the region of the *flg* operon between *pyrC* and *flgD* was replaced (although a low level of double recombination events that separately replaced the *pyrC* and *flgD* regions of the recipient DNA [a few percent] was also possible). If the Mot⁺ revertant allele was upstream of *flgD*, we expected nearly 100% of the *pyrC*⁺ Tc^s transductants to be Mot⁺. If the Mot⁺ revertant allele was downstream of the Δ *flgD*::*tetRA* allele, we expected significantly less than 100% of the *pyrC*⁺ Tc^s transductants to be Mot⁺. The percentage of *pyrC*⁺ Tc^s transductants was determined for each cross, and revertants predicted to be upstream of the Δ *flgD*::*tetRA* allele were subjected to DNA sequence analysis of the region upstream of *flgD*.

RESULTS

Analysis of mRNA processing in the *flgB-L* operon. The finding that FlgE was posttranscriptionally regulated in response to flagellar assembly led us to examine a possible role of mRNA processing in the expression of the 9.9-kbp class 2 *flgBCDEFGHIJKL* operon. Also, cleavage of the *flgBCDEF GHIJ* operon has been implicated in posttranscriptional regulation of the *flgE* gene in *Rhodobacter sphaeroides* (4, 18).

The mRNA processing sites in the upstream and downstream regions of the *flgE* gene of *S. enterica* serovar Typhimurium were determined by the technique of RACE (Fig. 1) (3). Following the ligation of genomic RNA to the 5S rRNA gene, two primers, *flgE*3'RT-PCR and *flgF*rtR, which are complementary to *flgE* and *flgF*, respectively, were used as gene-specific primers for performing PCR of the region. The PCR products showed discrete DNA bands by agarose gel electrophoresis (date not shown). The DNA bands were eluted, cloned, and analyzed by DNA sequencing. We analyzed 20 samples of each PCR product to determine the mRNA processing sites that were 5' and 3' of the *flgE* coding sequence (Fig. 2). The mRNA processing sites in the *flgD* and *flgF* genes were located just upstream of the *flgD* stop codon and 250 bases into the *flgF* coding region. Major (more than 5 out of 20 samples) and minor (less than 5 out of 20 samples) mRNA processing sites were mapped in each of the mRNA processing sites (Fig. 2B).

Deletion of the *flgD* coding sequence inhibits *flgE* gene expression. As described above, we detected mRNA processing sites at the end of the *flgD* coding sequence. To test whether the upstream *flgD* sequence might affect the production of FlgE, the *flgD* gene was deleted, leaving just the start and stop codons (strain TH8547). Our rationale was that if mRNA processing of the *flgD* region is necessary for the expression of *flgE*, then the Δ *flgD* allele would not be complemented with a plasmid expressing *flgD* alone but would be complemented with a plasmid expressing both *flgD* and *flgE*. This was found to be the case. The *flgD* and *flgDE* genes were cloned separately into the pTrc99A expression plasmid, resulting in the *pflgD* and *pflgDE* expression plasmids, respectively. Plasmid *pflgD* or *pflgDE* was introduced into the Δ *flgD* strain and screened for motility on tryptone soft-agar plates. Plasmid *pflgD* did not restore motility, while the *pflgDE* plasmid did (Fig. 3). This result suggested that the loss of the *flgD* coding region has a negative effect on expression of the *flgE* gene, perhaps due to the loss of the mRNA processing site.

FIG. 2. RNA processing sites in the *flgB-L* operon. (A) Organization of genes in the *flgB-L* operon. The mRNA processing sites are indicated. (B) Major (large arrows) and minor (small arrows) mRNA processing sites were mapped. The stop codon of *flgD* is indicated in RP1. RP, RNA processing.

The effects of *flgD* deletions on downstream *flgE* gene expression. The deletion of the *flgD* coding region from amino acid 2 to the stop codon failed to express the downstream *flgE* gene. Deletions of *flgD* that left different amounts of the 5' or 3' coding sequences were constructed to determine which sequences of *flgD* are required for *flgE* gene expression (Fig. 4). Deletions of the 5' coding sequence of *flgD*, leaving 15 to 528

bases of the 3' coding sequence, exhibited normal complementation by *pflgD* alone, indicating normal expression of *flgE*. The deletions leaving 12 or 3 bases of the 3' coding sequence did not express *flgE*. There was a very specific cutoff in *flgE* expression going from 15 bases at the 3' end to 12 bases. This corresponds exactly to the very 3' mRNA processing site for the *flgE* gene located by RACE in the 5' end of the *flgD* coding

FIG. 3. Motility assay of *flgD*-deficient strain TH8547 with *pflgD* (A) or *pflgDE* (B). pTrc99A (vector control), *pflgD*, or *pflgDE* was transformed into the wild type (LT2) or TH8547. pTrc99A and *pflgD* did not affect the motility of the wild type. TH8547 was not complemented with *pflgD* (A) but was complemented with *pflgDE* (B).

FIG. 4. Construction of *flgD* strains and motility assay of *flgD*-deficient strains with *pflgD* or *pflgDE*. The motility activities (percentage of wild-type motility with *pflgD* or *pflgDE*) of *flgD* strains are indicated in the left two columns.

sequence. Deletions of the 3' coding sequence of *flgD* appeared to have a gradient effect on *flgE* expression. Deletions leaving 36, 96, or 165 bases of the *flgD* 5' coding sequence showed weak, intermediate, and normal levels of *flgE* expression, respectively. Also, a deletion of 5' and 3' *flgD* coding sequences that left 165 bases from the middle of the *flgD* gene showed normal *flgE* expression. These results suggest that the 3' 15 bases of the *flgD* coding sequence are critical (depending on the context) for normal *flgE* expression, but this can be suppressed if there is a sufficient coding sequence of any kind (between 96 and 165 bases) upstream of the *flgD* stop codon.

The *flgD* RBS prevents *flgE* gene expression. The complete deletion of the *flgD* coding sequence left 26 bp of DNA sequence between the putative *flgD* and *flgE* ribosome-binding sites (RBSs) (Fig. 5A). It was possible that the *flgD* RBS was interfering with the ability of ribosomes to initiate at the *flgE* RBS unless a sufficient amount of *flgD* coding sequence (between 96 and 165 bases) was translated. In an attempt to identify bases that interfered with *flgE* gene translation due to Δ *flgD* mutation, motile revertants of the *pflgD*/ Δ *flgD*6219 strain were isolated and characterized. Motile revertants were isolated from 16 independent cultures and divided into four groups according to the degree of motility observed (Fig. 5B). The motile revertants were screened for linkage to the *flgD* region (see Materials and Methods). Some revertants showed 100% cotransduction with the *pyrC*-*flgD* region. Other revertants showed variable linkages, ranging from 10% to 60% cotransduction with the *pyrC*-*flgD* region. We expected that the revertants showing 100% cotransduction with the *pyrC*-*flgD* region were mutated in the *flgBC* region. Thus, we sequenced *flgBC* and 140 bp of the 5' *flgE* region from three independent

mutants (TH9936, TH9938, and TH9939) (Fig. 5A and B). Interestingly, all three mutants had different base substitution mutations in the RBS of *flgD* (G828T, G827A, and A829T, numbered relative to the *flgB* start codon in the wild type) (Fig. 5A and B). We constructed mutants with either the G828T (TH9945) or G827A (TH9946) substitution mutations by using site-directed mutagenesis to confirm that the phenotype observed was specific for these mutations. These strains were motile, suggesting that the *flgD* RBS was interfering with the ability of ribosomes to initiate at the *flgE* RBS in the Δ *flgD* mutant (Fig. 5C). We also sequenced the *flgBC* genes and 140 bp of the 5' *flgE* region of three other motile revertants (TH9937, TH9940, and TH9941), which were less than 100% linked to the *pyrC*-*flgD* region. As expected, there were no mutations present in *flgBC* and 140 bp of the 5' *flgE* region of these revertants. The cotransduction frequencies suggest that these mutations reside in the *flgG-L* operon downstream of the *flgE* gene and may affect the mRNA secondary structure in a manner that inhibits the ability of ribosomes to recognize the *flgD* RBS.

An FlgE- β -lactamase fusion is secreted into the periplasm in rod mutant strains. Previous work from our laboratory showed that the FlgE (flagellar hook subunit) protein was regulated in response to the stage of flagellum assembly independent of transcriptional regulation (6). Mutants early in flagellar assembly and late in flagellar assembly had cell-associated FlgE levels comparable to those observed in wild-type cells. Mutants defective in rod assembly (*flgB*, *flgC*, *flgF*, *flgG*, and *flgI* mutants), with the exception of a *fliE* mutant, had little or no detectable FlgE. Mutants defective in the stages between rod assembly and hook polymerization, including the hook-

FIG. 5. Isolation and characterization of motile revertants of *pflgD*/TH8547. (A) The sequences of two RBSs are shown. The sites of the mutations that restored the motility activity in TH9936, TH9938, and TH9939, respectively, are indicated by asterisks. The numbers are relative to the *flgB* start codon, ATG. (B) Motility assay of motile revertants. Three point mutants are indicated. (C) TH9945 and TH9946 were made by site-directed mutagenesis. Their motility activities are similar to those of TH9936 and TH9938, respectively.

scaffold gene, *flgD*, or P- and L-ring assembly genes *flgA*, *flgH*, and *flgI*, had between 15 and 30% of wild-type levels of FlgE protein. In all these flagellar mutant backgrounds, *flgE* mRNA levels were similar to those seen in the wild-type strain. It was previously shown that the hook-capping protein, FlgD, could be detected in the periplasm in rod and ring mutant strains (except in a *fliE* mutant) but not in strains defective in the flagellar type III secretion apparatus (27). It is possible that both FlgD and FlgE are secreted into the periplasm of rod mutants, but FlgE is much less stable. This would be consistent with the results reported previously by Bonifield and Hughes, which showed that FlgE was barely detectable or absent in the rod mutants but present at low levels in the ring mutants (6). In ring mutants, hook polymerization initiates in the absence of the ring structures (19), so some FlgE protein is stabilized by polymerization into nascent hook structures. Our hypothesis is that both FlgD and FlgE proteins are secreted into the periplasm of rod mutants but that FlgE is more sensitive to proteolysis and is rapidly degraded.

To test for FlgE secretion into the periplasm, we took advantage of the recent finding that the fusion of β -lactamase lacking its type II secretion signal (Bla) to the C terminus of FlgM had the dual benefit of preventing FlgM proteolysis in the periplasm and providing a direct assay for FlgM-Bla secretion by conferring an ampicillin-resistant (Ap^r) phenotype to

the cells but only if the fusion was secreted into the periplasm (2). Retention of FlgM-Bla in the cytoplasm or complete secretion from the cell resulted in an Ap^s phenotype. C-terminal fusions of Bla to FlgE were constructed and assayed for secretion of FlgE-Bla into the periplasm in different flagellar mutant strains. The results are presented in Table 3. Wild-type strain LT2 is the Ap^s control with a MIC of <3 μ g/ml for Ap. Strain TH6733, the positive Ap^r control that has wild-type β -lactamase with its intact secretion signal expressed from a *MudA* transposon insertion (11), has a MIC of 400 μ g/ml for Ap. A chromosomally expressed *flgE-bla* fusion in which the N-ter-

TABLE 3. MICs of ampicillin for the *flgE-bla* mutants in *S. enterica* serovar Typhimurium

Strain	Relevant genotype	MIC (μ g/ml)
LT2	Wild type	<3
TH6733 ^a	<i>flgE5315::MudA</i>	400
TH9948	<i>flgE-bla</i>	100
TH9949	<i>flgE-bla</i> $\Delta flgBC$	400
TH9950	<i>flgE-bla</i> $\Delta flgBC$ <i>fliF5036::Tn10dCm</i>	3
TH9951	<i>flgE-bla</i> $\Delta flgBC$ <i>fliD5704::Tn10dTc</i>	<3
TH9952	<i>flgE-bla</i> $\Delta flgBC$ $\Delta fliE::tetRA$	50

^a We used TH6733, which has a promoter and an intact *bla* gene (complete with type II secretion signal sequence) in *MudA*, as a positive control.

minal 23 amino acids of the β -lactamase protein, the type II secretion signal (14), was removed and the rest of the *bla* coding region was fused to the very C-terminal codon of *flgE* was constructed. The resulting FlgE-Bla fusion confers resistance to high levels of Ap in a strain deleted for the proximal rod genes *flgB* and *flgC* (TH9949). The level of Ap^r (MIC of 400 μ g/ml) was the same level observed in cells expressing the wild-type *bla*⁺ gene, whose protein product has its intact N-terminal type II secretion signal (TH6733). An intermediate Ap^r level (MIC of 100 μ g/ml) was observed in a strain with intact flagellar rods, and the only flagellar mutation present was the fusion of Bla to the C terminus of FlgE (TH9948). The Ap^r observed in the Rod⁺ and rod mutant strains was dependent on both flagellar gene expression (TH9951) and the presence of a flagellar secretion apparatus (TH9950). The assembly of the flagellar type III secretion system is completely blocked in strain TH9950, which contains a transposon insertion in the *fliF* gene. The FliF protein constitutes the MS ring on which the flagellar type III secretion apparatus is constructed (24, 25).

We also tested the effect of the rod protein FliE on the secretion of FlgE-Bla in a strain deleted for *flgB* and *flgC* and the rod gene, *fliE* (strain TH9952) (Table 3). FlgE was present in cell extracts from the *fliE* mutant background, but no FlgE was detectable in extracts from the other rod-specific mutant strains (*flgB*, *flgC*, *flgF*, *flgG*, and *flgI* mutants) (6). Also, in separate studies, the secretion of FlgD was dependent on FliE (12, 27), and FliE was shown to interact with the proximal rod protein FlgB (28). Taken together, these data suggest that FliE is both a part of the flagellar type III secretion apparatus and a rod component.

The introduction of the *fliE* deletion into the Δ *flgBC flgE-bla* strain lowered the Ap MIC from 400 μ g/ml to 50 μ g/ml, suggesting a strong defect in secretion, but secretion could still occur, compared to the complete absence of the flagellar secretion system (Ap MIC of 3 μ g/ml for TH9950 [*fliF*]). Thus, FliE is not essential for flagellar type III secretion but stimulates secretion eightfold by our assay.

DISCUSSION

In a previous study, we determined that the level of cellular FlgE protein was affected by a mutation in the flagellar assembly pathway, while *flgE* mRNA levels were unaffected (6). This led us to examine the possibility that the *flgE* gene, encoding the flagellar hook structural subunit protein, might be regulated by a posttranscriptional mechanism in a manner that couples *flgE* gene expression to FlgE assembly into the flagellum.

In this study, we determined the location of mRNA processing sites that flank the *flgE* gene of *S. enterica* serovar Typhimurium. Processing occurred within the last 15 bases of the *flgD* coding region and 250 bases into the coding region for the *flgF* gene. By examining the effect of *flgD* deletions on downstream *flgE* expression, we also determined that translation of *flgD* could have a negative effect on downstream *flgE* translation. The negative effect of *flgD* translation on *flgE* gene expression did not occur if enough *flgD* sequence was present (at least 100 bases) or if the last 15 bases upstream of the *flgD* stop codon were present. Somehow, the presence of the last 15 bases of the *flgD* coding sequence had the same effect as the

presence of 165 bases of the N terminus on the internal *flgD* coding sequence. There are 27 bases between the *flgD* stop and *flgE* start codons. The 15 bases upstream of the *flgD* stop codon might play a role in the secondary structure of the *flg* mRNA that allows the binding of ribosomes to the *flgE* RBS. The identification of mRNA processing sites in this 15-bp region is consistent with the possibility that this sequence is needed for *flgE* processing and that the processing of the *flgE* gene from the *flgB-L* mRNA transcript might be critical for normal *flgE* expression. This might also be part of a mechanism that prevents FlgE secretion until the rod structure is completed. Thus, the processing of the *flgE* gene from the *flgB-L* mRNA transcript may play a role in the coupling of *flgE* translation to assembly as a mechanism to increase the efficiency of the assembly process.

In this study, we also present evidence to support the hypothesis that the absence of FlgE in rod mutant strains reported previously (6) was due to the export of FlgE into the periplasm, where it was degraded. The fusion of β -lactamase to FlgE (FlgE-Bla) conferred a strong Ap^r phenotype that is consistent with the export of FlgE-Bla into the periplasm, and the fusion of Bla to the C terminus of FlgE appears to stabilize periplasmic FlgE. It is not known if rod and hook proteins are exported in the order they are assembled. Our results suggest that rod and hook subunits can be exported simultaneously and that specific protein subunits that are exported before they are needed for assembly, such as a hook subunit secreted before the rod is completed, are simply degraded in the periplasm.

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