JOURNAL OF BACTERIOLOGY, Sept. 2010, p. 4752–4762 Vol. 192, No. 18
0021-9193/10/$12.00 doi:10.1128/JB.00093-10

Multiple Promoters Contribute to Swarming and the Coordination of Transcription with Flagellar Assembly in *Salmonella*▽†

Christopher E. Wozniak,[1]‡ Fabienne F. V. Chevance,[2,3] and Kelly T. Hughes[2,3]*

Department of Microbiology, University of Washington, Seattle, Washington 98195[1]; Department of Biology, University of Utah, Salt Lake City, Utah 84112[2]; and Department of Medicine, University of Fribourg, Fribourg CH1700, Switzerland[3]

Received 27 January 2010/Accepted 7 July 2010

In *Salmonella*, there are three classes of promoters in the flagellar transcriptional hierarchy. This organization allows genes needed earlier in the construction of flagella to be transcribed before genes needed later. Four operons (*fliAZY*, *flgMN*, *fliDST*, and *flgKL*) are expressed from both class 2 and class 3 promoters. To investigate the purpose for expressing genes from multiple flagellar promoters, mutants were constructed for each operon that were defective in either class 2 transcription or class 3 transcription. The mutants were checked for defects in swimming through liquids, swarming over surfaces, and transcriptional regulation. The expression of the hook-associated proteins (FlgK, FlgL, and FliD) from class 3 promoters was found to be important for swarming motility. Both *flgMN* promoters were involved in coordinating class 3 transcription with the stage of assembly of the hook-basal body. Finally, the *fliAZY* class 3 promoter lowered class 3 transcription in stationary phase. These results indicate that the multiple flagellar promoters respond to specific environmental conditions and help coordinate transcription with flagellar assembly.

Flagella provide a competitive advantage to many bacteria by enabling them to swim toward nutrients and away from harmful substances. Flagella are complex machines that are constructed from about two dozen structural proteins in *Salmonella* and *Escherichia coli* (26). At least two dozen additional proteins are involved in regulation, assembly, or function (5). Flagellar genes in many bacterial species are organized into transcriptional hierarchies. These hierarchies express groups of genes at different stages during flagellar biogenesis (32). In *Salmonella*, there are three classes of flagellar promoters in the transcriptional hierarchy (24). The single class 1 promoter responds to information related to the metabolic and environmental state of the cell to decide when to transcribe the genes for the FlhDC activator complex. Together with σ^{70}, FlhDC directs RNA polymerase to initiate transcription from class 2 promoters (37). Class 2 expressed gene products are used to build the hook-basal body (HBB). The basal body is a structure that spans the membranes and peptidoglycan layer and consists of the rotor, the rod, ringed structures, and the flagellar secretion system. The hook is a flexible, extracellular linker between the basal body and the rigid filament that acts as a propeller (31). A class 2 promoter also transcribes the gene for the flagellar sigma factor σ^{28}, which directs transcription to class 3 promoters (29). Class 3 promoters transcribe the genes for the filament, motor force generators, and chemosensory system. This organization allows genes needed earlier in the construction of flagella to be transcribed before genes needed later (5).

The regulatory protein FlgM helps coordinate the transcriptional hierarchy with the assembly status of HBBs. Before completion of the HBBs, FlgM binds to σ^{28} to prevent σ^{28} from initiating transcription at class 3 promoters. When the HBBs are complete, the flagellar secretion apparatus switches to the secretion of filament-type substrates, such as FlgM (12, 20). Lower intracellular levels of FlgM allow σ^{28} to turn on the class 3 promoters. As a result, tens of thousands of filament subunits are then produced and added onto the completed HBBs. This coordination by FlgM may prevent the HBB proteins and filament subunits from simultaneously competing for secretion (12), which could slow flagellar assembly. Moreover, not synthesizing and secreting flagellar proteins before they can be assembled into the flagellar structure could be an important resource conservation mechanism for the cell (12, 19, 20).

The purpose for other regulatory arrangements within the transcriptional hierarchy is less clear. In particular, 10 genes are expressed from both class 2 and class 3 promoters in *Salmonella* (Fig. 1). These include the genes for activators (σ^{28} and FliZ) and inhibitors (FlgM and FliT) of flagellar transcription (10, 14, 21). The filament cap protein FliD and the FlgK and FlgL proteins that form the junction between the hook and the filament are also expressed from both the class 2 and class 3 promoters (21). FlgK, FlgL, and FliD are collectively known as the hook-associated proteins (HAPs) because they assemble at the end of the hook before the filament is added. The HAPs enable the efficient polymerization of the filament subunits. In addition, secretion chaperones for the HAPs (FlgN and FliT) and the filament subunits (FliS) are produced from both class 2 and class 3 promoters (21). While the reason for this organization has not yet been determined, most of these proteins act at the point in assembly after completion of the HBB and before polymerization of the filament.

To investigate why flagellar genes are expressed from both class 2 and class 3 promoters, mutants were constructed that

* Corresponding author. Mailing address: Department of Biology, 257 S 1400 E, Salt Lake City, UT 84112-0840. Phone: (801) 585-6950. Fax: (801) 585-9735. E-mail: hughes@biology.utah.edu.

† Supplemental material for this article may be found at http://jb.asm.org/.

‡ Present address: Department of Biology, University of Utah, Salt Lake City, UT 84112.

▽ Published ahead of print on 16 July 2010.

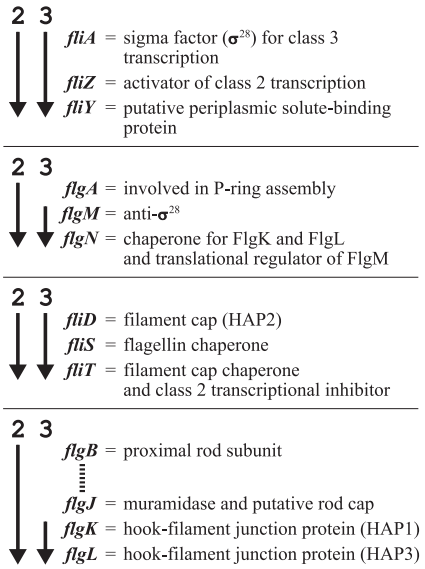


FIG. 1. Four operons in *Salmonella* are expressed from both class 2 and class 3 promoters. The arrows represent the transcripts that are generated. Additionally, *fliY* is transcribed from its own nonflagellar promoter and has no known flagellar function.

were defective in either class 2 transcription or class 3 transcription for each operon. The mutants were tested for their ability to swim through liquids and swarm over surfaces. Our results indicated that the class 3 promoters for the HAPs are important during swarming and may be involved in the repair of broken flagella. Changes in flagellar gene expression were also examined using transcriptional reporters. The *fliAZY* and *flgMN* promoter helped to coordinate class 3 transcription with the stage of assembly of the HBBs.

MATERIALS AND METHODS

Bacterial strains and general techniques. The strains used in this study were derived from *Salmonella enterica* serovar Typhimurium strain LT2. Many of these strains are listed in Table 1. Additional strains were constructed from the alleles in Table 1 and are listed in Table S1 of the supplemental material. Cultures of bacterial strains and phage P22 lysates were prepared as described previously (9), except that LB (with, per liter, 10 g Bacto tryptone, 5 g Bacto yeast extract, 5 g NaCl) was used as a rich medium for growing bacteria, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was added to plates at a concentration of 40 μg/ml. Chlortetracycline (50 μg/ml, autoclaved) or tetracycline (15 μg/ml) was used to induce transcription from the *tetA* promoter within the T-POP transposon, and 0.2% arabinose was used to induce transcription from the *araBAD* promoter. Transductions and tetracycline-sensitive (Tc^s) selections were performed as described previously (27, 28), except that LB was used instead of nutrient broth and Tc^s selection plates were incubated at 42°C. Strain constructions utilizing λ-Red recombination, including targeted *tetRA* insertions and replacements, were performed using plasmid pKD46, as described previously (38). Primers were synthesized by Integrated DNA Technologies (Coralville, IA) and are listed in Table S2 of the supplemental material. PCR products were sequenced at the DNA Sequencing Facility (Department of Biochemistry) at the University of Washington or at the DNA Sequencing Core Facility at the University of Utah.

Allele construction. Specific mutations in flagellar promoters were generated using primers containing the desired mutations. These primers were designed with about 40 bp of homology to the promoter (for recombination), then the mutation, and about 20 bp of homology to the promoter (for amplification; ~55°C melting temperature). Either wild-type promoters or mutant promoters that were generated in an earlier study (38) were used as templates for amplification. λ-Red recombination was used to move these mutations into the chromosome to replace tetracycline resistance markers (6, 15). The *fliDST* class 3 promoter was mutagenized by using a primer that had a random mix of bases for four positions in the −10 region of the promoter. After recombination, colonies were checked for Lac activity on lactose indicator plates (LB–X-Gal, MacConkey-lactose, and triphenyl tetrazolium chloride-lactose plates). A colony that had wild-type class 2 transcription but was defective for class 3 transcription was chosen (see Results). To insert transcriptional terminators immediately after the stop codons of *flgA* and *flgJ*, λ-Red recombination was used to move in 250 bp of *E. coli* DNA containing the strong *rrnB* transcriptional terminators.

Genes were placed under the control of the arabinose promoter at the *ara* locus by a procedure described in an earlier study (39). These constructs replace the *araB* start codon through the *araD* stop codon with the start codon through the stop codon of the cloned gene.

The *fliZ6591*::MudJ allele (38) was moved to 10 bp after the *fliZ* stop codon. λ-Red recombinations were used to insert *fliZ* DNA before MudJ and delete DNA after MudJ. There is no duplication of DNA at the ends of the resulting *fliZ7082*::MudJ insertion.

RNA isolation and quantitative real-time PCR. For each strain, total RNA was isolated using an RNeasy kit (Qiagen) from each of three independent cultures grown to mid-log phase in LB medium. Genomic DNA was then removed by incubation with DNase I (ZymoResearch) for 30 min at 37°C. Reverse transcription (RT) was performed using the RevertAid first-strand cDNA synthesis kit (Fermentas) with the included random hexamers. Quantitative PCR mixtures consisted of the EvaGreen qPCR master mix (Bio-Rad) and the primers that are listed in Table S2 of the supplemental material. Reactions were run on a CFX96 real-time PCR instrument (Bio-Rad). Relative changes in mRNA levels were calculated by the $2^{-\Delta\Delta CT}$ method (25) with normalization to control for the genes *gyrB* and *gapA* (34).

Swimming and swarming plates. Swimming plates were prepared as described previously (9). Single colonies from overnight streak plates were poked into the swimming plates by using toothpicks and incubated for 6 h. At least eight independent colonies were checked for each strain assayed.

Swarming plates were used to assay the ability of cells to spread over surfaces. For swarming plates, swarming agar (with, per liter, 10 g Bacto tryptone, 5 g Bacto yeast extract, 10 g NaCl, 6 g Bacto agar) in a flask was autoclaved in a pan of water to limit evaporation. Glucose was added to a final concentration of 0.5%. A 25-ml volume of the swarming agar was pipetted into each plate. The plates were spread in a single layer for even evaporation. After sitting at room temperature for 16 h, 5 μl of an overnight culture grown in LB was spotted in the center of each plate and allowed to dry for 10 min with the lid removed. Plates were incubated in a single layer in a 37°C incubator for 9 h before pictures were taken. To induce genes from the arabinose promoter, overnight cultures grown in LB-Ara were spotted on swarming plates containing arabinose. The magnetic lasso tool in Adobe Photoshop was used to trace around the irregular border of each swarm, and the number of pixels in the selected area was used to calculate an average diameter. The average diameter of a nonmotile strain (TH3930 *cheY5458*::MudJ) was subtracted from the average diameter of each strain tested. This distance was then normalized to the distance moved by the wild-type strain. For each strain, the assays were performed on three different days with three independent cultures spotted onto three swarming plates each day. To increase the contrast between the bacterial growth and the background, Adobe Photoshop was used to adjust the brightness levels for pictures of both swimming and swarming plates.

Flagellar staining. For each sample, a 7-mm-wide chamber with about 12-μl capacity was formed from a glass slide cleaned with ethanol, a coverslip wiped with a Kimwipe, and two pieces of double-sided tape for the sides. Fifteen microliters of poly-L-lysine was pipetted into each chamber, and then chambers were inverted for about 3 min. When liquids were applied to the top of the chamber, the slide was placed at about a 45° angle. During incubations, the slides were placed upside down onto wooden sticks in a 15-cm-diameter petri dish that had wet Kimwipes around the inside perimeter to prevent the chambers from drying out. For the staining of swimming cells, an overnight culture was diluted 1:200 in 2 ml of LB in a test tube and incubated at 37°C with shaking for 2.5 h. Fifteen microliters of culture was applied twice to the chamber, and the slide was inverted for about 3 min. For the staining of swarming cells, the swarming assay was performed as described earlier. After 8 h of incubation at 37°C, 10 μl of phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM Na_2HPO_4, 3 mM KH_2PO_4; pH 7.5) was spotted halfway onto the edge of the swarm. After about 10 s, 5 μl of liquid was pipetted from the surface and transferred to 1 ml of PBS in a microcentrifuge tube. The tube was gently inverted twice to dilute the cells. A new pipette tip was used to transfer 10 μl to the chamber. The same procedure was then followed for both swimming and swarming cells.

First, 8 μl of 18% formaldehyde in PBS was added, and the slide was inverted

TABLE 1. *S. enterica* serovar Typhimurium strains used in this study

Strain	Genotype	Reference or source[a]
TH437	LT2	J. Roth
TH714	*fljB5001*::MudJ	9
TH2100	Δ*flgHI958 fljB*^{e,n,x} *vh2*	S. Yamaguchi
TH2779	*flgM5222*::MudJ	10
TH3730	P*flhDC5451*::Tn*10d*Tc[Δ*25*]	18
TH3930	*cheY5458*::MudJ	Lab collection
TH3933	*motA5461*::MudJ	Lab collection
TH4212	*fliS5480*::MudK	H. Bonifield
TH4702	pKD46 (λ-Red recombinase plasmid, Ap^r, temperature-sensitive replication)	6
TH4721	*flgK5396*::MudJ	1
TH5139	Δ*flgM5628*::FRT	2
TH5238	Δ*fliD5630*::FKF	Lab collection
TH5504	Δ*fliA5647*::FRT	2
TH5712	Δ*fliZ5738*::FCF	J. Karlinsey
TH5737	Δ*fliS5720*::FKF	8
TH5794	P*fliD5744*::Tn*10d*Tc Δ*flgHI958 fljB*^{e,n,x} *vh2*	Lab collection
TH5832	Δ*fliT5769*::FKF	J. Karlinsey
TH5861	Δ*hin-5717*::FCF (*fliC*^{ON})	H. Bonifield
TH6233	Δ*hin-5718*::FRT (*fliC*^{OFF})	H. Bonifield
TH6701	Δ*araBAD925*::*tetRA*	P. Aldridge
TH6706	pKD46/Δ*araBAD925*::*tetRA*	P. Aldridge
TH7270	*flgJ5964*::*tetRA* (inserted after stop codon)	38
TH7278	pMC147 (pBAD24-*fliA*)	16
TH7395	Δ*flgM5628*::FRT *motA5461*::MudJ Δ*araBAD923*::*flgM*-FKF P*araB935*	2
TH8091	*flgA6093*::*tetRA* (inserted after stop codon)	38
TH8241	*flgA6066* (terminator after stop codon; *flgMN* P2$^-$)	
TH8242	*flgJ6094* (terminator after stop codon; *flgKL* P2$^-$)	
TH8927	CRR4107[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^s)] Δ*araBAD956*::*fliA* Δ*fliA5647*::FRT	38
TH9250	*fliA6399*::*tetRA* (replaces bp −79 to −44 with *tetRA*)	38
TH9300	CRR4107[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^s)] Δ*araBAD956*::*fliA* Δ*fliA5647*::FRT *flgM5222*::MudJ *flgM6441* (C-65T; *flgMN* P3$^-$)	38
TH9442	CRR4107[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^s)] Δ*araBAD956*::*fliA* Δ*fliA5647*::FRT *flgK5396*::MudJ *flgK6443* (T-59C)	38
TH9576	*flgM6441* (C-65T; *flgMN* P3$^-$)	
TH9588	CRR4107[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^s)] Δ*araBAD956*::*fliA* Δ*fliA5647*::FRT *fliS5480*::MudK *fliD6476* (T-95C)	38
TH9602	CRR4107[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^s)] Δ*araBAD956*::*fliA* Δ*fliA5647*::FRT *ataA*::P22[*sieA′*-Km6-P*fliA*(−583 to +1)-*lacZYA′-′9*] *fliA6488* (T-52C)	38
TH10049	*fliZ6591*::MudJ	38
TH10113	*fliA6602* (C-31T, T-52C; *fliAZY* P3$^-$)	
TH10128	*flgK6607* (A-36G, T-59C; *flgKL* P3$^-$)	
TH10214	*fliD6561* (T-42G, A-43T, G-44A, C-45T; *fliDST* P3$^-$)	
TH10215	*fliD6566* (T-95C, T-102A, A-107G, A-120G, G-121T; *fliDST* P2$^-$)	
TH10271	CRR4107[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^s)] Δ*araBAD956*::*fliA* Δ*fliA5647*::*FRT* *fliZ6591*::MudJ *fliA6609* (T-62C, C-90T)	38
TH10826	*fliA6785*::*tetRA* (inserted −43 bp from GTG)	38
TH11108	*fliZ6823*::*tetRA* (inserted after *fliZ* stop codon)	
TH11428	*fliA7081* (A-38G, A-41G, T-62C, C-90T; *fliAZY* P2$^-$)	
TH11429	*fliZ7082*::MudJ (inserted 10 bp after stop codon, no duplication)	
TH11495	Δ*araBAD975*::*fliT*$^+$ Δ*fliT5769*::FKF	
TH11498	Δ*araBAD976*::*fliS*$^+$ Δ*fliS5720*::FKF	
TH11864	Δ*araBAD980*::*fliD*$^+$ Δ*fliD5630*::FKF	
TH13991	CRR4107[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^s)] P*araB935* Δ*araBAD956*::*fliA*$^+$ Δ*fliA5647*::FRT	

[a] Strains for which no reference or source is reported were constructed for this study.

for 10 min. Next, 20 μl of PBS, 20 μl of 10% bovine serum albumin (BSA) in PBS, and 10 μl of rabbit anti-FliC antibodies diluted 1:1,000 in PBS were sequentially applied to the chamber. The slide was incubated inverted at room temperature for 1 h. Then, 20 μl of PBS, 20 μl of 10% BSA, and 10 μl of anti-rabbit antibodies conjugated to Alexa Fluor 488 diluted 1:1,000 in PBS were sequentially added to the chamber. The slide was incubated inverted for 30 min at room temperature in the dark. Finally, 20 μl of PBS and 15 μl of a solution containing three parts poly-L-lysine to 1 part 5-μg/ml FM4-64 in PBS were sequentially added. The chamber was sealed with clear nail polish to prevent evaporation.

Slides were examined on an Applied Precision optical sectioning microscope. Slides were prepared for three independent biological replicates for each strain for both swimming and swarming cells. After focusing the microscope on the sample, the slide was moved 100 μm over and 100 μm down. A series of six 65-μm square panels were then taken with four sections in the *z*-stack to span the flagella. Before analysis, swimming panels were randomly reordered and renamed to reduce bias. Each panel was subjectively evaluated for the average number of flagella per cell. Swarming panels were similarly examined, except that the percentage of highly flagellated cells was also subjectively evaluated.

Shearing and time-to-motility experiments. For the shearing experiment shown in Fig. 5 below, each overnight culture was diluted 1:200 in 25 ml of LB in a 125-ml flask. Cultures were incubated at 37°C with shaking for 140 min until

an optical density at 600 nm (OD_{600}) of about 0.6 was reached. Each culture was placed on ice, poured into a centrifuge tube, and centrifuged for 10 min at 270 × *g*, and the supernatants were poured off. To minimize foaming during blending, the pellets were resuspended in 10 ml cold saline by gentle swirling. Each culture was poured into a 25-ml blending cup and blended for 30 s using a Waring blender. A 0.75-ml aliquot of blended culture was added to 1.5 ml of LB in a test tube and incubated at 37°C with shaking. At each time point, culture was added to a slide and immediately examined under phase contrast using a Zeiss Axioskop 2 Mot plus microscope. Five-second-long movies for three different locations on the slide were taken at each time point. Three independent cultures were blended for each strain.

For the time-to-motility experiment shown in Fig. 7B below, overnight cultures were diluted 1:200 in 2 ml of LB in a test tube and incubated at 37°C with shaking. At 90 min, 200 μl of 165 μg/ml tetracycline in LB was added to induce the *tetA* promoter controlling the *flhDC* genes. At each time point, 5-s movies were taken at two or three different locations on the slide. Three independent cultures were induced for each strain.

All the cells present on the first frame of a 5-s movie were analyzed. Cells that made at least one 360° rotation during the movie were considered to be tumblers. Cells that made significant forward progress were subjectively identified as swimmers. Some cells near the edges of the frame were not counted if they drifted or moved out of view before the type of motility could be determined. For each time point, the motilities of between 58 and 138 cells were determined. An average of 97 cells were counted at each time point.

β-Galactosidase assays. β-Galactosidase assays were performed as described previously (39) except that the cells for the experiments shown below in Fig. 3 and 6 were grown to a density of 70 Klett units, which corresponds to an OD_{600} of about 0.7. For the experiments summarized below in Fig. 7A and 8 and Table 2, overnight cultures were diluted in 30 ml of LB in 125-ml flasks to an OD_{600} of 0.01. The cultures were incubated at 37°C with shaking. Samples (1 ml) were removed periodically to follow the growth of the cultures with a spectrophotometer, and samples of culture (between 0.25 ml and 10 ml) were put on ice for the β-galactosidase assays. Prewarmed LB or LB-tetracycline was added back to the cultures to maintain at least a 15-ml volume. The tetracycline-inducible promoter included in the experiment shown below in Fig. 7A was induced after 80 min of growth with 5 ml of 84 μg/ml tetracycline. For the experiment shown below in Fig. 3, class 2 transcription was assayed in the presence of a chlortetracycline inducer in a TH8927-type background that expresses $FlhD_4C_2$ from a tetracycline-dependent promoter and σ^{28} (FliA) from the wild-type arabinose promoter. Class 3 transcription was assayed in the presence of an arabinose inducer in a TH13991-type background. TH13991 differs from TH8927 in that it also contains a mutated arabinose promoter (P*ara935*), which lowers class 3 transcription to near-wild-type levels (38). For the experiment shown below in Fig. 8B, average new LacZ synthesis was calculated for each sampling period according to the following equation: [ending activity − (starting activity/fold change in OD_{600})]/elapsed time.

RESULTS

Construction of promoter mutants. Four operons in *Salmonella* (*fliAZY*, *flgMN*, *fliDST*, and *flgKL*) are transcribed from both class 2 and class 3 promoters (Fig. 1) (13, 14). To explore the purposes for expressing these operons from both promoter classes, mutations were introduced into these promoter regions to greatly reduce either class 2 or class 3 transcription. Since the class 2 and class 3 promoters are overlapping in the *fliAZY* and *fliDST* operons, point mutations were used to selectively knock out each promoter. The point mutations introduced into the promoters were chosen based upon an earlier mutagenesis study (38). The mutations that were used are located in the conserved −10 and −35 promoter regions or are located in or near the FlhDC binding sites. Typically, several mutations had to be combined to reduce promoter activity to less than 3% of the wild-type level.

For the *flgMN* and *flgKL* operons, the class 2 promoter is one or more genes upstream of the class 3 promoter (Fig. 1). To prevent transcription of these genes from the class 2 promoters, rRNA transcriptional terminators were inserted upstream

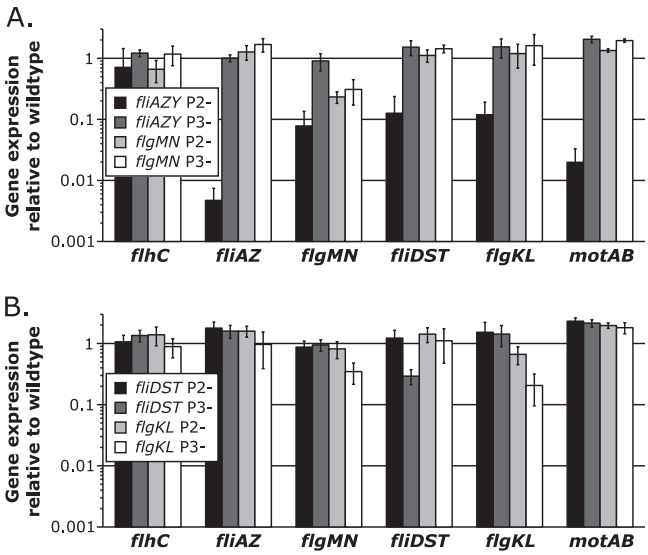


FIG. 2. mRNA levels of flagellar genes were determined using quantitative RT-PCR. RNA was extracted from three independent cultures for each promoter mutant, and the mRNA levels of each gene were normalized to the mRNA levels in the wild-type background. The mRNA levels for different genes in the same operon were averaged to give the overall transcript levels for those genes. For the *motA* and *motB* genes, however, only one RT-PCR was performed in which one primer was in *motA* and one primer was in *motB*. For the genes measured, *flhC* is expressed from the class 1 promoter, *fliAZ*, *flgMN*, *fliDST*, and *flgKL* are expressed from both class 2 and class 3 promoters, and *motAB* is expressed from a class 3 promoter.

of each class 3 promoter. These terminators block transcription of *flgMN* and *flgKL* from the class 2 promoters while leaving transcription of the upstream genes intact (*flgA* and *flgB* to *flgJ*, respectively). The class 3 promoters for the *flgMN* and *flgKL* operons were separately mutagenized using point mutations.

In the case of the *fliDST* class 3 promoter, four positions in the −10 region were randomized, and mutants were screened for low class 3 transcription and wild-type class 2 transcription. This approach was taken because all of the *fliDST* class 3 promoter is overlapped by the class 2 promoter or its mRNA, which made it difficult to isolate mutations that only affected class 3 transcription.

To assay class 2 transcription independent of class 3 transcription, a strain that expresses *flhDC* from a tetracycline-inducible promoter and *fliA* (σ^{28}) from an arabinose-inducible promoter was used (38). When this strain is grown in the presence of tetracycline, *flhDC* is transcribed and only class 2 promoters are activated. When the strain is grown in medium containing arabinose, *fliA* (σ^{28}) is transcribed and only class 3 promoters are activated. Insertions of *lac* fusions in the first or second gene of an operon were used to quantify any changes in transcription. The introduced mutations and transcriptional terminators reduced transcription by at least 20-fold. The activities of the nonmutated promoters remained near the wild-type level.

mRNA levels in the promoter mutants. Quantitative RT-PCR was used to determine the overall levels of mRNA transcripts (class 2 and class 3 transcripts combined) for the oper-

ons with mutated promoters (Fig. 2). The *fliAZY* class 2 promoter mutation knocked down *fliAZ* transcript levels to 0.5% of the wild-type level (Fig. 2A), which was a much larger decrease than observed for any of the other mutants. This dramatic reduction is likely due to the loss of class 3 transcription. Without the *fliAZY* class 2 promoter, no σ^{28} (FliA) would be expressed from the *fliAZY* operon and no class 3 promoters would then be transcribed. With no class 2 transcription of *fliAZY* due to the mutation and no class 3 transcription due to the lack of σ^{28}, the mRNA levels were accordingly very low. In the *fliAZY* class 2 promoter mutant, the mRNA levels of all other operons expressed from class 2 or class 3 promoters were also decreased, which is probably due to the reduction in both σ^{28} and the class 2 transcriptional activator FliZ (Fig. 2A).

Surprisingly, mutating the class 3 promoter for the *fliAZY* operon had no effect on *fliAZ* mRNA levels (Fig. 2A). It had been proposed that the σ^{28} produced from the *fliAZY* operon would feed back through the *fliAZY* class 3 promoter to increase *fliAZY* mRNA levels (18). In both of the *flgMN* promoter mutants, mRNA levels of the *flgMN* genes were reduced to about a quarter of the wild-type level (Fig. 2A). This suggests that both promoters contribute significantly to the overall *flgMN* transcript level. For the *fliDST* and *flgKL* operons, which express the genes for the hook-associated proteins, the class 3 promoter contributed most of the activity for each operon (Fig. 2B). Transcripts for these two operons were reduced much more in the class 3 promoter mutants (71% and 79% decreases) than in the class 2 promoter mutants (22% increase and 34% decrease). Finally, the *flgKL* class 3 promoter mutation not only decreased *flgKL* transcripts by 79% but also reduced *flgMN* transcripts by 65% (Fig. 2B). FlgK and FlgL may be affecting *flgMN* transcript levels through FlgN, which is the secretion chaperone for FlgK and FlgL. In a previous study, FlgN was found to increase FlgM expression posttranscriptionally (17). If lower FlgKL levels in the *flgKL* class 3 promoter mutant decreased FlgN stability, less FlgN could then result in the fewer observed *flgMN* transcripts.

Effects of promoter mutations on swimming, swarming, and flagellation. Each promoter mutant was checked for defects in two types of motility: swimming through liquids and swarming across surfaces. In the swimming assay, bacteria are poked with a toothpick into a 0.3% agar plate. As the bacteria grow and divide, individual cells swim away from the poke through liquid channels in pursuit of additional nutrients. In swarming plates with 0.6% agar, there are not enough liquid channels for the *Salmonella* cells to swim through, but there is enough moisture to swarm across the surface. Swarming cells have more flagella per cell and secrete wetting agents to increase the hydration on the surface. Swarming cells stay in close contact with each other and move rapidly past each other in random directions to expand the edge of the swarm (11, 36).

The flagella on swimming and swarming cells were fluorescently labeled to look for changes in flagellation. The wild-type strain typically had about six flagella on a swimming cell (Fig. 3). In contrast, swarming cells were less evenly flagellated. About 40% of swarmer cells were highly flagellated, with an average of nine flagella per cell. The number of flagella on the remaining 60% of cells typically varied from zero to a few per cell.

Not surprisingly, the *fliAZY* class 2 promoter mutant was most defective for motility (Fig. 3). Because the class 2 promoter was not present to transcribe *fliA* in the mutant, no FliA (σ^{28}) would have been available to initiate transcription from the class 3 promoters. The cells for the *fliAZY* class 2 promoter mutant lacked flagella. The *fliAZY* class 3 promoter mutant, in contrast, was indistinguishable from the wild-type strain for swimming, swarming, and flagellation, which is consistent with the wild-type mRNA levels observed earlier (Fig. 2A).

The *flgMN* class 2 promoter mutant, the *flgMN* class 3 promoter mutant, and the *fliDST* class 2 promoter mutant all had slightly larger (~20%) swimming diameters than the wild-type strain but had wild-type flagellation (Fig. 3). Both operons contain negative regulators of flagellar transcription (FlgM and FliT). Smaller amounts of these regulators could result in more flagellar components that could slightly improve swimming motility. During swarming, the *flgMN* promoter mutants had a larger proportion of highly flagellated cells. About two-thirds of the *flgMN* mutant swarming cells were highly flagellated in comparison with about 40% of the wild-type swarming cells. This increased flagellation did not translate into a more rapid spread over the swarming plates, however. In fact, the *flgMN* class 2 promoter mutant swarmed 31% less than the wild-type strain. As described later, this reduced swarming ability may result from slower flagellar assembly.

The *fliDST* and *flgKL* class 3 promoter mutants exhibited close to wild-type swimming diameters and a 53% and 29% decrease in swarming distance, respectively (Fig. 3). The *fliDST* class 3 promoter mutant had two-thirds the wild-type number of flagella on both swimming and swarming cells, whereas the *flgKL* class 3 promoter mutant had a wild-type level of flagellation. These two operons express the genes for the HAPs (FliD, FlgK, and FlgL) that assemble at the end of the hook before the filament subunits are added to the structure. This seems to be a class 3-specific effect, since the *fliDST* and *flgKL* class 2 promoter mutants were not defective in swarming or flagellation and exhibited near-wild-type swimming diameters. These data are consistent with the much larger decrease in mRNA levels in the class 3 promoter mutants (Fig. 2B). This pattern was more pronounced when double mutants were constructed. When the *fliDST* and *flgKL* class 3 promoter mutations were combined into a single strain, the result was a nearly wild-type swimming diameter, a further defect in swarming motility, and about two-thirds the wild-type number of flagella in both swimming and swarming cells. In contrast, a class 2 *fliDST flgKL* double mutant looked like the wild-type strain for swimming and swarming motility. Like the *flgMN* mutants, the class 2 *fliDST flgKL* double mutant also had about two-thirds of swarming cells highly flagellated.

Complementation of the *fliDST* class 3 promoter mutant. Complementation was used to determine which gene in the *fliDST* operon was responsible for the defect in swarming motility. Each of the three genes in the operon were individually placed under the control of the arabinose promoter at the *ara* locus. The cloned genes were able to complement deletions of *fliD*, *fliS*, or *fliT* in swimming plates (data not shown). These alleles were tested for their ability to complement the swarming defect of the *fliDST* class 3 promoter mutant (Fig. 4). Higher expression of the filament cap gene *fliD* restored the wild-type swarming diameter. Swarming motility was not significantly increased when the *fliS* gene, which encodes the

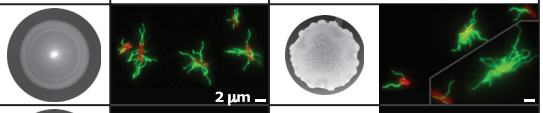
Strain		Promoter activity		Total mRNA levels	Swimming		Swarming	
		class 2	class 3		0.3% agar	liquid	0.6% agar	
wt		100	100	100				
fliAZY	**class 2 -** A-38G, A-41G, T-62C, C-90T	2.5	91	0.5				
	class 3 - C-31T, T-52C	115	1.1	100				
flgMN	**class 2 -** terminator after *flgA* stop codon	4.8	84	23				
	class 3 - C-65T	94	1.3	31				
fliDST	**class 2 -** T-95C, T-102A, A-107G, A-120G, G-121T	0.8	107	122				
	class 3 - T-42G, A-43T, G-44A, C-45T	104	0.6	29				
flgKL	**class 2 -** terminator after *flgJ* stop codon	1.6	82	66				
	class 3 - A-36G, T-59C	88	1.1	21				
fliDST flgKL	**class 2 -**	N.A.	N.A.	N.A.				
	class 3 -	N.A.	N.A.	N.A.				

FIG. 3. Promoter activity, mRNA levels, swimming, swarming, and flagellation were characterized for each promoter mutant. The positions of point mutations are given in relation to the distance from the start codon. Promoter activity was measured in backgrounds that express only class 2 transcription (TH8927) or class 3 transcription (TH13991). The activity from each class 2 promoter or class 3 promoter was quantified using *lac* fusions to *fliZ*, *flgM*, *fliS*, or *flgK* and normalized to the activity of the wild-type promoter. Standard deviations were on average 9% of the promoter activities. Total mRNA levels for each mutant were taken from Fig. 2 and determined using quantitative RT-PCR in an otherwise-wild-type background. Pictures of representative swimming and swarming plates are shown. The wild-type strain spread over 69% of the swarming plate at 9 h after inoculation and moved through 32% of the swimming plate at 6 h after inoculation. In order to facilitate comparisons, the pictures were resized so that the swimming and swarming diameters of the wild-type strain were equal to each other. Pictures of flagella were taken for the mutants in a background (TH5861) that is phase locked for one of the two flagellin genes (*fliC*) in *S.* Typhimurium. Representative flagellated cells are shown. The cell membranes are in red and were labeled with FM4-64. Flagella are green and were labeled with antiflagellin antibody and secondary antibody conjugated to Alexa Fluor 488. N.A., not applicable.

chaperone for the filament subunits, was overexpressed. Finally, overexpressed FliT further inhibited motility. FliT is a negative regulator for class 2 transcription and is the secretion chaperone for the filament cap protein FliD (7, 23, 40).

The ability of FliD to restore a wild-type swarming diameter is consistent with the swarming defect observed for the *flgKL* class 3 promoter mutant. FliD, FlgK, and FlgL are all HAPs that assemble at the end of the hook and are needed for efficient polymerization of the filament subunits. However, FliD could also increase swarming by binding to FliT to prevent FliT from inhibiting class 2 transcription. To separate FliD's structural role as a HAP from its ability to bind FliT,

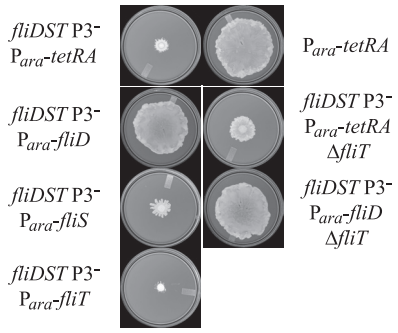


FIG. 4. FliD, FliS, and FliT were tested for their abilities to complement the *fliDST* class 3 promoter mutant in swarming plates containing arabinose. In the control strain, the arabinose genes that are normally expressed from the arabinose promoter were replaced by the *tetRA* cassette, which provided tetracycline resistance.

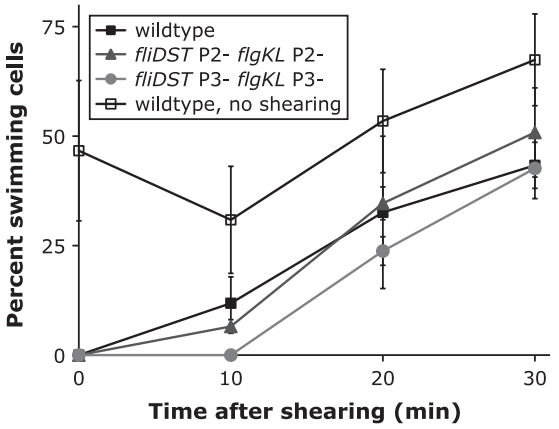


FIG. 5. The percentage of swimming cells was followed after flagella were sheared off in a blender. The cultures for the no-shearing control were put on ice, centrifuged, and resuspended in cold saline, i.e., the same as for the cultures that were blended.

complementation was performed in the absence of FliT (Fig. 4). By itself, a *fliT* deletion increased the swarming diameter of the *fliDST* class 3 promoter mutant. This suggests that the increased numbers of flagella in a Δ*fliT* mutant (41) promote swarming. However, complementation with FliD still restored the wild-type swarming diameter in the absence of FliT. These complementation data support the hypothesis that higher levels of HAPs are needed during swarming.

Recovery of motility after flagellar shearing. The need for more HAPs during swarming could reflect an increased need to repair broken flagella. Greater tension on the flagella during swarming has been found to result in increased breakage (3). Because swarming cells are in close contact with a surface and with each other, there may be more opportunities for a flagellum to stick or get tangled and then break off. If this breakage occurs after the hook, a flagellum could potentially be repaired by reassembly of the needed HAPs and then repolymerization of the filament (12, 30). Twofold increases in the mRNA levels of *fliD* and the filament subunit genes were observed in swarming cells (35) and may occur to promote this filament repair.

To see if class 3 expression of the HAPs is needed for flagellar repair, strains were tested for their ability to recover motility after their flagella were sheared off in a blender. The blender has previously been used as a nonlethal means for breaking off flagella (30, 33). The proportion of swimming bacteria was then quantified from movies taken of the cells by phase-contrast microscopy (Fig. 5). Immediately after shearing, 96% of the cells were nonmotile, 4% were slowly tumbling, and no cells were swimming. The proportion of swimming cells steadily increased over the next 30 min for the wild-type strain and the class 2 *fliDST flgKL* double mutant. The recovery of the class 3 *fliDST flgKL* double mutant appeared to lag behind the wild-type strain by about 5 min and had significantly fewer swimming cells at the 10-min time point ($P = 2 \times 10^{-7}$; all reported *P* values are two-tailed). This 5-min delay in recovery indicates a possible deficiency in flagellar repair and could significantly impair motility if flagella are repeatedly broken during swarming. However, such a short delay could also result from the lower level of flagellation of the class 3 double mutant. Fewer flagella to repair could delay the time when enough flagella are long enough for swimming.

Effects of *flgMN* and *fliAZY* promoter mutations on class 3 transcription. Because FlgM helps control the level of class 3 transcription by binding to and inhibiting σ^{28}, the *flgMN* promoter mutants were checked for their ability to inhibit class 3 transcription. The *motA* class 3 promoter fused to a *lac* transcriptional fusion was used to quantify class 3 transcription levels. In both the class 2 and class 3 *flgMN* promoter mutants, there was about a 46% increase in class 3 transcription (Fig. 6A). These increases in class 3 transcription were not as high as in a *flgM* deletion strain, which indicates that both *flgMN* promoters contribute to the inhibition of σ^{28}. In a Δ*flgHI* background, which lacks the periplasmic and outer membrane rings needed for completion of the HBBs, the *motA* class 3 promoter was shut off (Fig. 6A). This inhibition of class 3 transcription occurs because the HBBs do not change secretion specificity to filament-type substrates like FlgM until HBBs are completed. Without FlgM secretion, intracellular FlgM levels rise and prevent σ^{28} from initiating transcription at class 3 promoters (12, 20). The class 3 *flgMN* promoter mutant maintained this inhibition in the Δ*flgHI* background, but the class 2 *flgMN* promoter mutant exhibited high levels of class 3 transcription (Fig. 6A). Apparently, not enough FlgM is produced from the class 3 *flgMN* promoter to tightly control class 3 transcription before completion of the HBBs. These results are consistent with other studies identifying FlgM expressed from the class 2 promoter as responsible for inhibition before completion of the HBBs (10, 17).

To determine the role of the *fliAZY* class 3 promoter in controlling class 3 transcription, the *fliAZY* class 3 promoter mutations were introduced into the backgrounds tested in Fig. 6A. The same pattern of class 3 transcriptional activity was observed with or without the *fliAZY* class 3 promoter mutation (Fig. 6B), which suggests that the *fliAZY* class 3 promoter is not involved in regulating class 3 transcription levels. FliA expressed from the class 2 promoter and FlgM expressed from the class 2 and class 3 promoters appear to be sufficient to control class 3 transcription levels.

Class 3 transcription after induction of *flhDC*. To further define the roles of the *flgMN* promoters and the *fliAZY* class 3

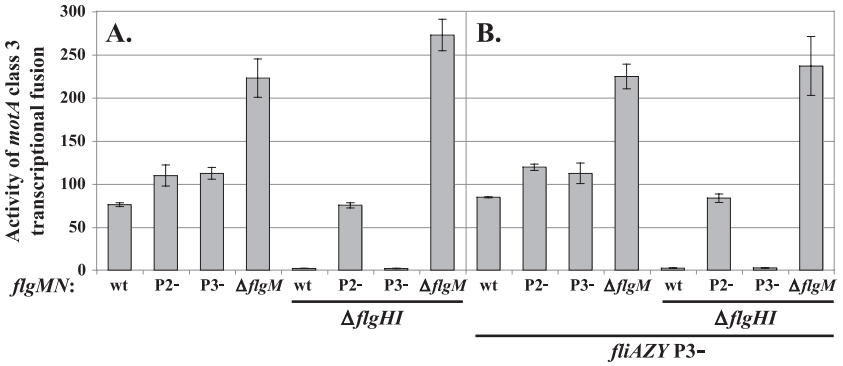


FIG. 6. The level of transcription from the *motA* class 3 promoter was quantified in *flgMN* and *fliAZY* promoter mutant backgrounds. The activities from a *lac* reporter fused to the *motA* class 3 promoter were measured at an OD_{600} of about 0.7 and are expressed in units of nmol/min/OD_{650}/ml. The Δ*flgHI* allele prevents secretion of FlgM because FlgH and FlgI are needed for completion of HBBs.

promoter, class 3 transcription was followed after induction of the *flhDC* operon. The *flhDC* genes are at the top of the flagellar transcriptional hierarchy and encode the activator of class 2 promoters. The *flhDC* operon was placed under the control of a tetracycline-inducible promoter (18) in order to synchronize induction of the flagellar regulon. A highly expressed transcriptional fusion to the *fljB* class 3 promoter was used to determine at what point class 3 transcription was turned on in wild-type and mutant backgrounds (Fig. 7A). As had been seen in the earlier study (18), class 3 transcription in the wild-type background turned on at about 30 min. In the *flgMN* class 2 promoter mutant, class 3 transcription turned on 15 min earlier. In contrast, class 3 transcription in the *flgMN* class 3 promoter mutant turned on at the same time as the wild-type strain but reached significantly higher levels than in the wild-type strain much later, at 90 min ($P = 0.06$) and 150 min ($P = 0.009$) after induction. These data are consistent with the hypothesis that the *flgMN* class 2 promoter is needed to control class 3 transcription before completion of the HBBs, and the *flgMN* class 3 promoter is needed after completion of the HBBs.

In a separate experiment, the proportion of swimming cells was followed after induction of the *flhDC* operon (Fig. 7B). Nearly half of the cells for the wild-type strain were swimming by 50 min after *flhDC* induction, which was consistent with the results of the earlier study (18). The *flgMN* class 2 promoter mutant had significantly fewer swimming cells than the wild-type strain at 50 min ($P = 4 \times 10^{-6}$) and 60 min ($P = 0.05$). This less-than-10-min delay in swimming motility is short but potentially significant. The tens of thousands of filament subunits expressed prematurely may slow construction of the HBBs by competing with HBB subunits for interaction with the secretion system. This delay in assembly may have contributed to the defect in swarming observed earlier for the *flgMN* class 2 promoter mutant (Fig. 3).

Class 3 transcription was also followed in strains that had the class 1 promoter intact (Fig. 8A). In this wild-type background, class 3 transcription is turned on when the cells begin to grow exponentially and is shut off in stationary phase, as seen previously (4). Because LacZ is a stable reporter protein, average new synthesis of LacZ was calculated and plotted for each sampling interval (Fig. 8B). Class 3 transcription in both

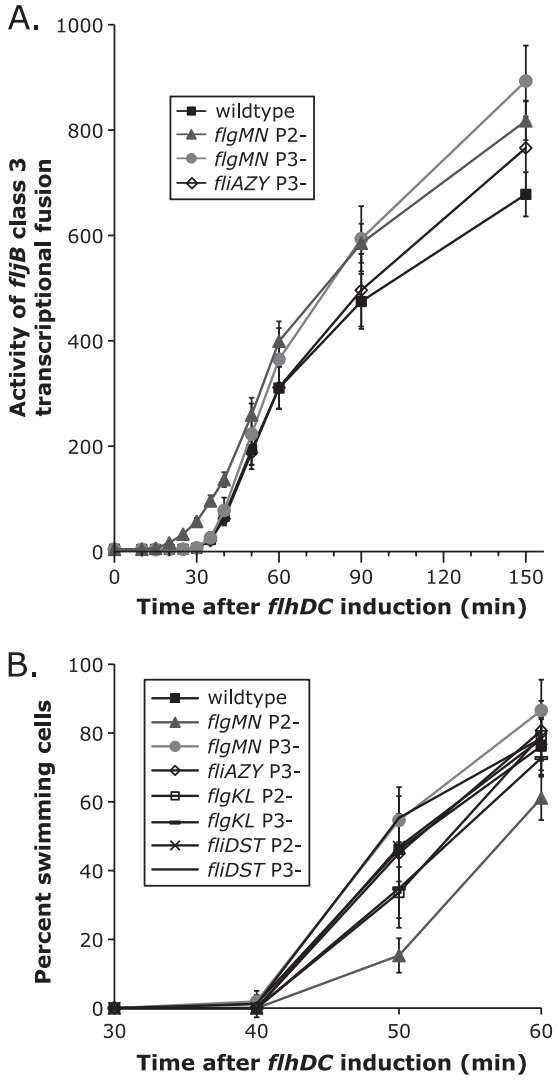


FIG. 7. Transcription from the *fljB* class 3 promoter (A) and the percentage of swimming cells (B) were followed after induction of the *flhDC* genes. The *flhDC* genes were put under the control of a tetracycline-inducible promoter and induced in early exponential phase. The activities from a *lac* reporter fused to the *fljB* class 3 promoter are expressed in units of nmol/min/OD_{650}/ml.

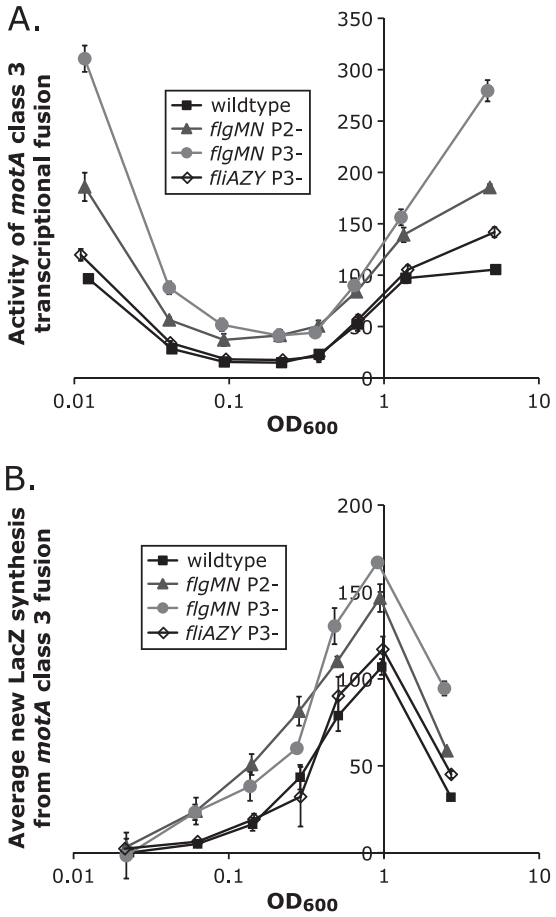


FIG. 8. Transcription from the *motA* class 3 promoter was followed in *flgMN* and *fliAZY* promoter mutants. Overnight cultures were diluted to an OD_{600} of 0.01 in fresh LB to start the experiment. The wild-type strain reached a peak doubling time of 24 min during exponential growth, and no growth defects were observed in any of the mutants. (A) Activities from a *lac* reporter fused to the *motA* class 3 promoter are expressed in units of nmol/min/OD_{650}/ml. (B) Average new LacZ synthesis was calculated from the activities in panel A, and these were plotted at the midpoint of each sampling period. Average new LacZ synthesis is expressed in units of nmol/min/OD_{650}/ml/h.

flgMN promoter mutants turned on to a higher level and probably earlier than in the wild-type background. Class 3 transcription may turn on earlier in the *flgMN* class 3 promoter mutant because the cells have preexisting flagella in addition to the HBBs being newly synthesized. When the cultures entered stationary phase, class 3 transcription was 165% higher in the *flgMN* class 3 promoter mutant than in the wild-type strain (Fig. 8A). Presumably, secretion of FlgM through completed HBBs would make class 3 expression of FlgM even more important in stationary phase. Finally, there was a 34% increase in class 3 transcription in the *fliAZY* class 3 promoter mutant in stationary phase. This is a counterintuitive result in that by removing a promoter for *fliA* (σ^{28}), there should be less σ^{28} produced and less class 3 transcription. However, a higher level of class 3 transcription was observed here.

The *fliAZY* class 3 promoter helps inhibit class 3 transcription. Transcriptional fusions to the *fliZ* and *motA* genes were utilized to investigate the phenomenon of higher class 3 transcription levels in the *fliAZY* class 3 promoter mutant. In Table 2, it can be seen that the *fliAZY* class 3 promoter mutant exhibited a 41% decrease in *fliAZ* transcription in stationary phase but not in exponential phase. This makes sense in that removing a promoter for *fliA* should decrease its transcription. Less *fliA* transcription should then result in less σ^{28} and less class 3 transcription. Instead, a 37% increase in class 3 transcription was observed from a transcriptional fusion to the *motA* class 3 promoter (Table 2). To determine whether the increase was due to FlgM or FliZ, the *flgMN* promoter mutations and a *fliZ* deletion were moved into these backgrounds. The *flgMN* class 2 promoter mutation and a *fliZ* deletion did not significantly alter the class 3 transcription ratio when they were introduced. When the *flgMN* class 3 promoter mutation was moved in, the 37% increase in transcription disappeared. These data suggest that higher σ^{28} levels act through FlgM's class 3 promoter to decrease transcription of *motA* in stationary phase. Higher levels of FlgM produced from a class 3 promoter during exponential growth may persist into stationary phase to more quickly shut off overall class 3 transcription.

DISCUSSION

This study explored the purposes for expressing four flagellar operons from both class 2 and class 3 promoters. As expected, the class 2 promoter for the *fliAZY* operon was needed to express FliA (σ^{28}) so that class 3 promoters could be activated. Contrary to expectations, the class 3 promoter for the *fliAZY* operon was found to lower class 3 transcription in stationary phase. Both *flgMN* promoters were involved in controlling class 3 transcription levels (Fig. 9A). The *flgMN* class 2 promoter was needed to shut off class 3 transcription before completion of the HBBs, and the *flgMN* class 3 promoter inhibited class 3 transcription after completion of the HBBs. The expression of the HAPs (FliD, FlgK, and FlgL) from class 3 promoters was important for swarming motility (Fig. 9B). Finally, mutating the class 2 promoters for the *flgKL* and *fliDST* operons had no effect on swimming or swarming motility. These mutants have provided insights into the functions

TABLE 2. Effects of the *fliAZY* promoter mutant on transcription

OD group and construct	Transcription activity of[a]:		Ratio[b]
	Wild type	*fliAZY* P3$^-$	
OD_{600} of 0.6			
fliZ::MudJ (*fliZ*$^+$)[c]	27 ± 5	24 ± 2	0.91
motA::MudJ	55 ± 3	60 ± 1	1.09
motA::MudJ *flgMN* P2$^-$	87 ± 5	91 ± 7	1.05
motA::MudJ *flgMN* P3$^-$	77 ± 5	79 ± 8	1.03
motA::MudJ Δ*fliZ*	53 ± 2	57 ± 1	1.08
OD_{600} = 5			
fliZ::MudJ (*fliZ*$^+$)	138 ± 6	81 ± 4	0.59
motA::MudJ	130 ± 6	178 ± 5	1.37
motA::MudJ *flgMN* P2$^-$	229 ± 11	291 ± 17	1.27
motA::MudJ *flgMN* P3$^-$	350 ± 11	374 ± 2	1.07
motA::MudJ Δ*fliZ*	58 ± 1	79 ± 1	1.37

[a] β-Galactosidase activities (means ± standard deviations) are in units of nmol/min/OD_{650}/ml.
[b] The ratio *fliAZY* P3$^-$ activity to wild-type activity.
[c] *fliZ*::MudJ was inserted 10 bp after the stop codon of *fliZ*.

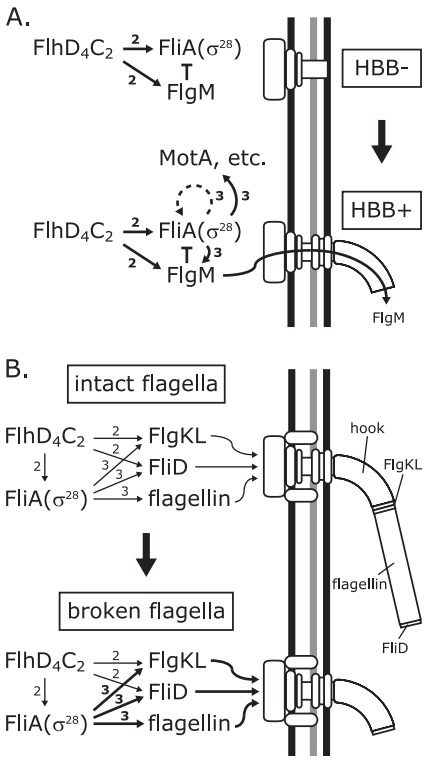


FIG. 9. The role of flagellar promoters in coordinating class 3 transcription with HBB assembly (A) and in repairing broken flagella during swarming (B). (A) While HBBs are being assembled, class 2-expressed FliA (σ^{28}) is inhibited by class 2-expressed FlgM. After HBBs are completed and FlgM secretion begins, the level of class 3 transcription is maintained by FliA expressed from a class 2 promoter and FlgM expressed from both class 2 and class 3 promoters. A dashed arrow is used for class 3 expression of FliA (σ^{28}) because of its contradictory effects in both increasing *fliA* transcription and decreasing class 3 transcription in stationary phase. (B) Expression of FlgKL and FliD from either the class 2 promoters or the class 3 promoters was sufficient to construct flagella for swimming. Additional expression of FlgKL, FliD, and flagellin from class 3 promoters could be important for repairing broken flagella during swarming.

of some of the promoters, but much further characterization is needed.

The most surprising result of this study was the involvement of the *fliAZY* class 3 promoter in inhibiting class 3 transcription. By stationary phase, the *fliAZY* class 3 promoter accounted for 41% of *fliAZY* transcription (Table 2). Transcription of the *fliAZY* operon from the class 3 promoter should lead to higher levels of σ^{28} and higher levels of class 3 transcription. However, a 27% decrease in class 3 *motA* transcription was observed (Table 2). One possible explanation is that the class 3 promoters for σ^{28} and FlgM result in larger pools of both regulators during exponential growth. Overall, class 3 transcription may not increase during exponential growth because the higher levels of σ^{28} would be balanced by higher levels of FlgM. If FlgM is then more stably maintained into stationary phase than σ^{28}, the higher FlgM levels could more effectively shut off class 3 promoters.

The characterization of the *flgMN* promoter mutants further supports the idea that FlgM coordinates flagellar transcription with assembly (12, 20). The advantage to this coordination had not been determined, however. One hypothesis is that coordination enables HBB components to be synthesized, secreted, and assembled without competition from tens of thousands of filament subunits (12). The *flgMN* class 2 promoter mutant provided some support for this hypothesis since the mutant was slower to become motile after induction of the flagellar regulon (Fig. 7B). The *flgMN* class 3 promoter mutant, on the other hand, did not exhibit any defects in motility. The advantage to having a class 3 *flgMN* promoter could be to prevent extra subunits from being synthesized when class 3 transcription levels are high. More efficient use of resources may not affect motility but may be particularly important during stationary phase. This efficiency argument could explain why the class 2 promoter mutants for operons expressing the HAPs had little effect on motility or mRNA levels. A little class 2 expression of the HAPs could give the HAPs time to assemble before the filament subunits are produced (17, 21). Without assembly of the HAPs, filament subunits are secreted into the extracellular medium because they are unable to polymerize.

It is noteworthy that the RT-PCR assays showed about equal levels of *flgMN* transcription when either the class 2 or class 3 *flgMN* promoter was mutated. FlgM is an autoregulatory protein. Loss of FlgM results in the derepression of both class 2 and class 3 *flgMN* promoters (10, 22). Previous work utilizing a *flgM*::Mud-*lac* reporter fusion to investigate the effects demonstrated an 80% reduction in P_{flgMN} transcription when class 3 *flgMN* transcription was inhibited and only a 20% reduction when class 2 *flgMN* transcription was inhibited (10). Since the Mud-*lac* insertion also disrupted the *flgM* gene, this had the added benefit of removing FlgM autoregulation from the final result. Thus, the measurement of *flgM* transcription from its class 2 and class 3 promoters in the *flgM*::Mud-*lac* background provides a more accurate measurement of actual levels of transcription from its respective promoters than using RT-PCR on wild-type cells.

In summary, our data suggest that there are advantages to expressing flagellar operons from both class 2 and class 3 promoters. The swarming data indicate that at least part of this regulation exists to respond to specific environmental conditions. The time-to-motility experiment provides weak evidence that such complex regulation is needed for fast and efficient assembly of flagella. Further characterization of the mutants constructed in this study should provide additional insights into the important roles of these flagellar promoters.

ACKNOWLEDGMENTS

This work was supported by PHS grant GM056141 from the National Institutes of Health.

We thank the members of the Hughes lab for helpful discussions and critical reading of the manuscript. We also thank Rasika Harshey and members of her lab for help in formulating our swarming procedures.

REFERENCES

1. **Aldridge, P., J. Karlinsey, and K. T. Hughes.** 2003. The type III secretion chaperone FlgN regulates flagellar assembly via a negative feedback loop containing its chaperone substrates FlgK and FlgL. Mol. Microbiol. **49:**1333–1345.
2. **Aldridge, P. D., J. E. Karlinsey, C. Aldridge, C. Birchall, D. Thompson, J. Yagasaki, and K. T. Hughes.** 2006. The flagellar-specific transcription factor, σ^{28}, is the type III secretion chaperone for the flagellar-specific anti-sigma28 factor FlgM. Genes Dev. **20:**2315–2326.
3. **Attmannspacher, U., B. E. Scharf, and R. M. Harshey.** 2008. FliL is essential for swarming: motor rotation in absence of FliL fractures the flagellar rod in swarmer cells of *Salmonella enterica*. Mol. Microbiol. **68:**328–341.

4. **Barembruch, C., and R. Hengge.** 2007. Cellular levels and activity of the flagellar sigma factor FliA of *Escherichia coli* are controlled by FlgM-modulated proteolysis. Mol. Microbiol. **65:**76–89.
5. **Chilcott, G. S., and K. T. Hughes.** 2000. Coupling of flagellar gene expression to flagellar assembly in *Salmonella enterica* serovar Typhimurium and *Escherichia coli*. Microbiol. Mol. Biol. Rev. **64:**694–708.
6. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. U. S. A. **97:**6640–6645.
7. **Fraser, G. M., J. C. Bennett, and C. Hughes.** 1999. Substrate-specific binding of hook-associated proteins by FlgN and FliT, putative chaperones for flagellum assembly. Mol. Microbiol. **32:**569–580.
8. **Frye, J., J. E. Karlinsey, H. R. Felise, B. Marzolf, N. Dowidar, M. McClelland, and K. T. Hughes.** 2006. Identification of new flagellar genes of *Salmonella enterica* serovar Typhimurium. J. Bacteriol. **188:**2233–2243.
9. **Gillen, K. L., and K. T. Hughes.** 1991. Negative regulatory loci coupling flagellin synthesis to flagellar assembly in *Salmonella typhimurium*. J. Bacteriol. **173:**2301–2310.
10. **Gillen, K. L., and K. T. Hughes.** 1993. Transcription from two promoters and autoregulation contribute to the control of expression of the *Salmonella typhimurium* flagellar regulatory gene *flgM*. J. Bacteriol. **175:**7006–7015.
11. **Harshey, R. M., and T. Matsuyama.** 1994. Dimorphic transition in *Escherichia coli* and *Salmonella typhimurium*: surface-induced differentiation into hyperflagellate swarmer cells. Proc. Natl. Acad. Sci. U. S. A. **91:**8631–8635.
12. **Hughes, K. T., K. L. Gillen, M. J. Semon, and J. E. Karlinsey.** 1993. Sensing structural intermediates in bacterial flagellar assembly by export of a negative regulator. Science **262:**1277–1280.
13. **Ide, N., T. Ikebe, and K. Kutsukake.** 1999. Reevaluation of the promoter structure of the class 3 flagellar operons of *Escherichia coli* and *Salmonella*. Genes Genet. Syst. **74:**113–116.
14. **Ikebe, T., S. Iyoda, and K. Kutsukake.** 1999. Promoter analysis of the class 2 flagellar operons of *Salmonella*. Genes Genet. Syst. **74:**179–183.
15. **Karlinsey, J. E.** 2007. λ-Red genetic engineering in *Salmonella enterica* serovar Typhimurium. Methods Enzymol. **421:**199–209.
16. **Karlinsey, J. E., and K. T. Hughes.** 2006. Genetic transplantation: *Salmonella enterica* serovar Typhimurium as a host to study sigma factor and anti-sigma factor interactions in genetically intractable systems. J. Bacteriol. **188:**103–114.
17. **Karlinsey, J. E., J. Lonner, K. L. Brown, and K. T. Hughes.** 2000. Translation/secretion coupling by type III secretion systems. Cell **102:**487–497.
18. **Karlinsey, J. E., S. Tanaka, V. Bettenworth, S. Yamaguchi, W. Boos, S. I. Aizawa, and K. T. Hughes.** 2000. Completion of the hook-basal body complex of the *Salmonella typhimurium* flagellum is coupled to FlgM secretion and *fliC* transcription. Mol. Microbiol. **37:**1220–1231.
19. **Komeda, Y., and T. Iino.** 1979. Regulation of expression of the flagellin gene (*hag*) in *Escherichia coli* K-12: analysis of *hag-lac* gene fusions. J. Bacteriol. **139:**721–729.
20. **Kutsukake, K.** 1994. Excretion of the anti-sigma factor through a flagellar substructure couples flagellar gene expression with flagellar assembly in *Salmonella typhimurium*. Mol. Gen. Genet. **243:**605–612.
21. **Kutsukake, K., and N. Ide.** 1995. Transcriptional analysis of the *flgK* and *fliD* operons of *Salmonella typhimurium* which encode flagellar hook-associated proteins. Mol. Gen. Genet. **247:**275–281.
22. **Kutsukake, K., and T. Iino.** 1994. Role of the FliA-FlgM regulatory system on the transcriptional control of the flagellar regulon and flagellar formation in *Salmonella typhimurium*. J. Bacteriol. **176:**3598–3605.
23. **Kutsukake, K., T. Ikebe, and S. Yamamoto.** 1999. Two novel regulatory genes, *fliT* and *fliZ*, in the flagellar regulon of *Salmonella*. Genes Genet. Syst. **74:**287–292.
24. **Kutsukake, K., Y. Ohya, and T. Iino.** 1990. Transcriptional analysis of the flagellar regulon of *Salmonella typhimurium*. J. Bacteriol. **172:**741–747.
25. **Livak, K. J., and T. D. Schmittgen.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods **25:**402–408.
26. **Macnab, R. M.** 2003. How bacteria assemble flagella. Annu. Rev. Microbiol. **57:**77–100.
27. **Maloy, S. R.** 1990. Experimental techniques in bacterial genetics. Jones and Bartlett, Boston, MA.
28. **Maloy, S. R., and W. D. Nunn.** 1981. Selection for loss of tetracycline resistance by *Escherichia coli*. J. Bacteriol. **145:**1110–1112.
29. **Ohnishi, K., K. Kutsukake, H. Suzuki, and T. Iino.** 1990. Gene *fliA* encodes an alternative sigma factor specific for flagellar operons in *Salmonella typhimurium*. Mol. Gen. Genet. **221:**139–147.
30. **Rosu, V., and K. T. Hughes.** 2006. σ^{28}-Dependent transcription in *Salmonella enterica* is independent of flagellar shearing. J. Bacteriol. **188:**5196–5203.
31. **Samatey, F. A., H. Matsunami, K. Imada, S. Nagashima, T. R. Shaikh, D. R. Thomas, J. Z. Chen, D. J. DeRosier, A. Kitao, and K. Namba.** 2004. Structure of the bacterial flagellar hook and implication for the molecular universal joint mechanism. Nature **431:**1062–1068.
32. **Soutourina, O. A., and P. N. Bertin.** 2003. Regulation cascade of flagellar expression in Gram-negative bacteria. FEMS Microbiol. Rev. **27:**505–523.
33. **Stocker, B. A. D., and J. C. Campbell.** 1959. The effect of non-lethal deflagellation on bacterial motility and observations on flagellar regeneration. J. Gen. Microbiol. **20:**670–685.
34. **Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman.** 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. **3:**research0034.
35. **Wang, Q., J. G. Frye, M. McClelland, and R. M. Harshey.** 2004. Gene expression patterns during swarming in *Salmonella typhimurium*: genes specific to surface growth and putative new motility and pathogenicity genes. Mol. Microbiol. **52:**169–187.
36. **Wang, Q., A. Suzuki, S. Mariconda, S. Porwollik, and R. M. Harshey.** 2005. Sensing wetness: a new role for the bacterial flagellum. EMBO J. **24:**2034–2042.
37. **Wang, S., R. T. Fleming, E. M. Westbrook, P. Matsumura, and D. B. McKay.** 2006. Structure of the *Escherichia coli* FlhDC complex, a prokaryotic heteromeric regulator of transcription. J. Mol. Biol. **355:**798–808.
38. **Wozniak, C. E., and K. T. Hughes.** 2008. Genetic dissection of the consensus sequence for the class 2 and class 3 flagellar promoters. J. Mol. Biol. **379:**936–952.
39. **Wozniak, C. E., C. Lee, and K. T. Hughes.** 2009. T-POP array identifies EcnR and PefI-SrgD as novel regulators of flagellar gene expression. J. Bacteriol. **191:**1498–1508.
40. **Yamamoto, S., and K. Kutsukake.** 2006. FliT acts as an anti-FlhD2C2 factor in the transcriptional control of the flagellar regulon in *Salmonella enterica* serovar Typhimurium. J. Bacteriol. **188:**6703–6708.
41. **Yokoseki, T., T. Iino, and K. Kutsukake.** 1996. Negative regulation by FliD, FliS, and FliT of the export of the flagellum-specific anti-sigma factor, FlgM, in *Salmonella typhimurium*. J. Bacteriol. **178:**899–901.