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Molecular Characterization of *flgM*, a Gene Encoding a Negative Regulator of Flagellin Synthesis in *Salmonella typhimurium*

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The expression of flagellin in *Salmonella typhimurium* is coupled to the assembly of complete flagella. Mutations which disrupt this coupling define a gene, *flgM*, which represses the expression of the flagellin genes in strains with mutations in the basal body, switch, or hook flagellar gene (K. L. Gillen and K. T. Hughes, J. Bacteriol. 173:2301–2310, 1991). Complementation studies demonstrated that the *flgM* gene is contained within a 600-bp cloned DNA fragment. Sequence analysis revealed that this fragment carries a small open reading frame corresponding to a 97-amino-acid protein. The FlgM protein observed in a T7-mediated expression system showed an apparent molecular mass of 9.5 kDa, similar to the predicted size of 10.6 kDa. Upstream of the *flgM* coding region is a putative promoter sequence which shows strong homology to that thought to be recognized by the flagellin-specific sigma factor (FliA). Consistent with the use of this promoter in vivo, promoter mapping by primer extension demonstrated a transcriptional start site 11 bases downstream from the center of the putative −10 promoter element, which was dependent on functional FliA for full expression.

Motility and chemotaxis in peritrichously flagellated *Salmonella typhimurium* require the expression of over 50 genes and subsequent ordered assembly of these gene products into about 7 to 10 complete flagellar structures (for a review, see references 27 and 28). The flagellum is a complicated structure consisting of a filament, the external element composed of flagellin which is rotated for motility and chemotaxis; the basal body, a group of membrane-bound rod and ring proteins of the flagellar motor; and the hook, a flexible joint linking the filament and the basal body. Additional associated components include the switch complex and chemotaxis proteins, which change the direction of filament rotation (20, 45, 46); three hook-associated proteins incorporated into the external structure (16); and proteins required for energy transduction (2, 8, 19).

Expression of flagellar components is highly regulated (21, 22, 24, 25). Cells defective for basal body, hook, or switch proteins do not transcribe either of the flagellin genes, *fliC* or *fljB*, alternately expressed by *S. typhimurium* (22, 25, 40). In addition, cells defective for either of two positive activator proteins, encoded by the *flhC* and *flhD* genes (23, 38), will not transcribe any flagellar genes (22, 25). A regulatory hierarchy composed of three main classes has been described by Kutsukake et al. for *S. typhimurium* (25). In this model, the genes at the top of the hierarchy or in the early class include the *flhC* and *flhD* genes. The second or middle class includes those genes which encode components of the basal body, hook, and switch complex as well as the *fliA* gene. The *fliA* gene encodes an alternate sigma factor (29) that directs the transcription of the flagellin genes, which are included in the third or late class of the hierarchy along with the genes for chemotaxis proteins.

We recently identified a negative regulatory locus which is evidently responsible for the coupling of flagellin expression to flagellar assembly by preventing expression of the flagellin genes when a component of the middle class of proteins is defective (11). Insertional inactivation of this locus, designated *flgM* (previously designated *flgR*; see Materials and Methods), allowed expression of the flagellin genes in all flagellar mutant backgrounds studied except in those strains with mutations in the *flhC*, *flhD*, or *fliA* gene. In this article we present a further characterization of the *flgM* gene. By complementation assays and DNA sequence analysis with transposon Tn*1000* insertions, we have determined that the *flgM* gene lies within a 600-bp *Ssp*I-*Bsm*I cloned fragment of DNA. FlgM activity was localized to an open reading frame corresponding to a 97-amino-acid protein, confirmed by the construction of a frameshifted *flgM* mutant. Further evidence to support the identification of the reading frame came from primer extension assays to locate the start of transcription and T7-mediated expression of the protein product of the *flgM* gene. The significance of these results for FlgM-mediated regulation of flagellin is discussed.

MATERIALS AND METHODS

Media. Nutrient broth (Difco) supplemented with NaCl (5 g/liter) was used as a rich medium for growing strains, and Bacto-agar (Difco) was added to a final concentration of 1.5% for solid medium. LB (9) supplemented with E-salts (43) and 0.2% glucose was used for growth of P22 phage. MacConkey-lactose (Difco) and TTC-lactose plates were used as indicator media. TTC-lactose agar (26, 44) contained tryptone (10 g/liter), yeast extract (1 g/liter), NaCl (5 g/liter), lactose (10 g/liter), Bacto-agar (15 g/liter), and triphenyl tetrazolium chloride (TTC; 50 μg/ml). The same medium without TTC, lactose, or agar was used for growth of strains in primer extension assays. E-medium (43) was used as a minimal medium and contained 0.2% glucose; thiamine (20 μg/ml) and to 18 amino acids (no methionine or cysteine) at concentrations from 0.005 to 0.01% were added for T7 experiments. Motility agar (37) was used for complementation assays of flagellar defects and contained tryptone (10 g/liter), NaCl (5 g/liter), and Bacto-agar (3 g/liter). Antibiotics were added as appropriate to growth media to the following final concentrations: ampicillin, 100 μg/ml; tetracycline, 15 μg/ml; chloramphenicol, 12.5 μg/ml; and kanamycin, 50 μg/ml. X-gal (5-bromo-4-chloro-3-indolyl-β-D-ga-

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TABLE 1. Strains used

Strain	Relevant genotype	Source or reference
E. coli		
DPWC	*supE42 recA* Δ(*Sst*II-*Eco*RI) Δ*srl*::Tn*10* (Tc^s) F$^+$	39
JGM	*F$^-$ araD139* Δ(*ara-leu*)*7696* Δ(*lac*)*X74 galU galK hsdR2* (r_K^- m_K^+) *mcrB1 rpsL* (Str^r) Tn*5 seq-1* (Kan^r)	39
DH5α	*endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argF*)*U169* [ϕ80d *lac* Δ(*lacZ*)M15]	13
S. typhimurium		
MS1868	*leuA414*(Am) su^0 *hsSB* (r^-m^+) Fels$^-$	M. Susskind
TH1077	*fliC5001*::MudJ	11
TH1983	*flgM5001*::Tn*10*dCm Δ*flgG-L* (*fla-2157*) *fljB5001*::MudJ	This study
TH1984	*flgM5001*::Tn*10*dCm Δ*flgG-L* (*fla-2157*) *fljB5001*::MudJ *fliA2087*	This study
TH1985	*flgM5001*::Tn*10*dCm *fliC5001*::MudJ	This study
TH1986	*flgA2085 fljB5001*::MudJ	This study

lactopyranoside) was added when appropriate to a final concentration of 100 μg/ml.

Bacterial strains and plasmids. The strains used in this study are listed in Table 1. *S. typhimurium* strains were derived from strain LT2. Plasmids constructed in the course of this work are derivatives of pBR322, BlueScript (Stratagene), or pTZ18R (Pharmacia).

Plasmid pMH71 (17) was obtained from M. Carsiotis (4). It contains a 6.5-kb chromosomal insert from *S. typhimurium* and was the parent plasmid for subcloning the *flgM* gene (Fig. 1). Plasmid pKG3 was derived from pMH71 by deleting a 1.4-kb *Cla*I-*Pvu*II fragment. In this and subsequent subcloning steps, DNA fragments were separated by agarose gel electrophoresis and gel purified by using GeneClean II (Bio 101). The 3′ recessed end of the *Cla*I site was filled in with T4 DNA polymerase (Pharmacia) at 15°C (30), and the free ends were ligated with T4 DNA ligase (New England BioLabs). *Escherichia coli* DH5α (13) was transformed and plated on nutrient agar containing ampicillin. Other clones were obtained in a similar manner. Plasmid pKG4 was derived from pKG3 by deleting the 2.4-kb *Bsm*I fragment. Subclone pKG5 was obtained by cutting a 2.2-kb *Hin*dIII fragment out of pKG3 and inserting it into pBR322. Plasmid pKG7 was obtained by deleting pKG5 from the *Ssp*I to the *Pvu*II site. Plasmid pKG8 was constructed by deleting pKG5 for the 0.5-kb *Bsm*I fragment. Subclone pKG11 (not shown) was obtained by insertion of the 0.6-kb *Ssp*I-*Bsm*I fragment of pMH71 into the *Sma*I site of pBS. Subclone pKG12 was derived from pKG11 by insertion of the 0.6-kb *Bam*HI-*Kpn*I fragment into pTZ18R.

Plasmid pKG13 (not shown) was derived from pKG12 by partial *Ase*I digestion to obtain linearized fragments. This was followed by filling in the 2-base 5′ overhang with the Klenow fragment of DNA polymerase I (New England BioLabs) and subsequent religation and transformation. This resulted in loss of the *Ase*I restriction site and the inability of this clone to complement a chromosomal *flgM* mutation.

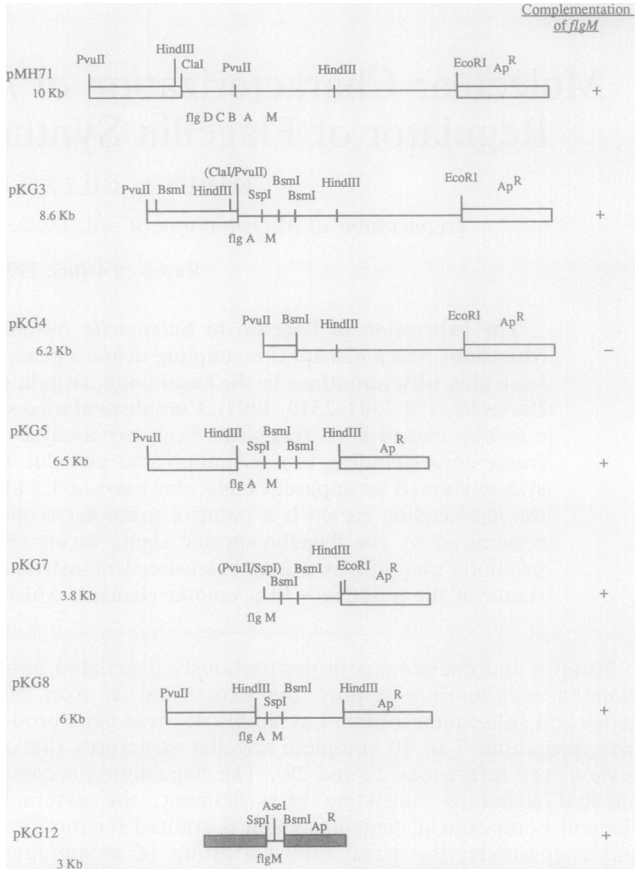


FIG. 1. Complementation of *flgM* by pMH71 and its derivatives. The open box indicates pBR322 sequence; the shaded box indicates pTZ18R sequence. The approximate location of the β-lactamase gene of pBR322 is indicated by Ap^r. Complementation of an *flgM* chromosomal mutation is indicated by + (*flgM*$^+$) or −(*flgM*) and was monitored by expression of *fljB*.

Complementation assays. Plasmid pMH71 and its derivatives were checked for complementation of chromosomal *flgM* and *flgA* mutations. In the presence of flagellar mutations, no expression of the *fljB* and *fliC* flagellin genes occurred (11, 25), as monitored by *lac* expression from MudJ (5, 6) operon fusions to the flagellin genes. In *flgM* mutant strains, the flagellin genes are expressed despite the presence of flagellar mutations (11). Complementation of *flgM* mutant strains was observed as restoration of repression of the flagellin genes (Lac$^-$ phenotype [red colonies on TTC-lactose medium]). Complementation of a chromosomal *flgA* mutation was checked by the strain's ability to swarm on motility agar.

Genetic manipulations of *S. typhimurium*. Transductions in *S. typhimurium* were carried out with generalized transducing phage P22 *HT105/1 int-201* (31). Transductants were purified on green indicator plates (7). Plasmid DNA isolated from *E. coli* strains by a plasmid miniprep procedure (15) was introduced by electroporation into *S. typhimurium* with a Bio-Rad Gene Pulser equipped with a pulse controller per the manufacturer's instructions.

Tn*1000* insertions. Tn*1000* (γδ) is a member of the Tn*3* family of transposons, which replicate via a nonconservative or replicative mechanism of transposition (35). A stable intermediate of transposition, known as a cointegrate, is

formed during the transposition process and consists of two copies of the transposon uniting the donor and recipient plasmids. If F-mediated conjugation occurs before the cointegrate is resolved, both the F-factor and the recipient (target) plasmid are transferred to the recipient cells (12). Subsequent resolution results in a plasmid which contains a Tn*1000* insertion.

Tn*1000* insertions were obtained essentially as described by Strathmann et al. (39). Briefly, F-factor-containing *E. coli* DPWC carrying target plasmid pMH71 was used as the donor strain. The donor was allowed to conjugate with the recipient *E. coli* strain JGM (Km^r). Portions (0.1 ml) of overnight cultures were used to inoculate 2 ml of LB, and these cultures were incubated for 4 h at 37°C on a roller drum (about 30 rpm). Cultures were diluted to 10^{-2} to 10^{-3}, and 0.1-ml portions were plated on nutrient agar plates containing kanamycin and ampicillin, which selects for recipient cells (Km^r) carrying the target plasmid (Ap^r) but against the donor cells (Km^s) which contain that plasmid. At least 50 colonies from each experiment were pooled, and the DNA was isolated by a plasmid miniprep procedure (15). Portions (1 μl) of DNA were electroporated into *S. typhimurium* MS1868. Transformants were recovered on nutrient agar plates containing ampicillin. Colonies were pooled, and generalized transducing phage P22 was grown on the pooled cells. The resulting phage lysate was used to transduce TH1983 to Ap^r on TTC-lactose indicator plates. Plasmid-containing colonies which complemented the chromosomal *flgM* mutation were Lac^-, while those which did not complement it were Lac^+ and harbored Tn*1000* insertions in the plasmid-borne *flgM* gene.

Sequencing. DNA sequence analysis was performed by the dideoxy sequencing method of Sanger et al. (32) and commercial kits (Sequenase; USB) according to the manufacturer's instructions, with ^{35}S-labeled α-dATP or ^{32}P-labeled α-dATP (New England Nuclear). Double-stranded DNA was denatured with NaOH. Primers GD1 and GD2 (39) to the ends of Tn*1000* were used to sequence in both directions from the point of insertion. Primers complementary to the multiple cloning site in BlueScript (M13 $^{-}$20 and KS) were used to complete sequencing of both strands of the 0.6-kb *Ssp*I-*Bsm*I clone (pKG11).

Overproduction of the FlgM protein. BlueScript- or pTZ18R-derived plasmids containing the presumed *flgM* open reading frame adjacent to the T7 promoter were transformed into DH5α cells carrying plasmid pGP1-2 (41). pGP1-2 carries the gene for the T7 polymerase under the control of the temperature-sensitive λ*c*I857 repressor. Strains were grown in E-medium, and expression of the T7 polymerase gene was induced by growth at 42°C with the subsequent addition of rifampin (final concentration, 200 μg/ml) to prevent function of the cellular RNA polymerase, essentially as described before (41). After 1 h at 30°C, [^{35}S]methionine/cysteine (New England Nuclear) was used to label proteins made from T7 RNA polymerase-induced transcripts for 5 min. Extracts of these cultures were electrophoresed in a Hoeffer Mighty-Small gel apparatus with the tricine-sodium dodecyl sulfate (SDS)-polyacrylamide gel system described by Schagger and Von Jagow (34) with 16.5% acrylamide. To prevent cracking of this type of gel, the following drying conditions were used: the gel was placed on wet 3MM filter paper, covered with plastic wrap, and placed under vacuum for 10 min before the slow cycle (Bio-Rad model 583 Gel Drier) was used to a maximum temperature of 50°C for 75 min.

Primer extension. RNA was isolated as described before (42). RNase inhibitor (Boehringer Mannheim) and DNase (Bethesda Research Laboratories) were added to the RNA in RNase-free water (sterile water for irrigation; Baxter Healthcare Corp.). ^{32}P labeling of primers GD1 and GD2 was done with T4 polynucleotide kinase and [γ-^{32}P]ATP (New England Nuclear) (1). Primer extension reactions were carried out (1) with Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) and buffer supplied by the manufacturer at 42°C for 30 min.

Nomenclature. The work in this article characterizes the gene *flgM*, which was originally named *flgR* (11). This new nomenclature conforms to guidelines established for the flagellar genes of *S. typhimurium* and *E. coli* (18).

Nucleotide sequence accession number. The nucleotide sequence data shown in this article have been assigned GenBank accession number M74222.

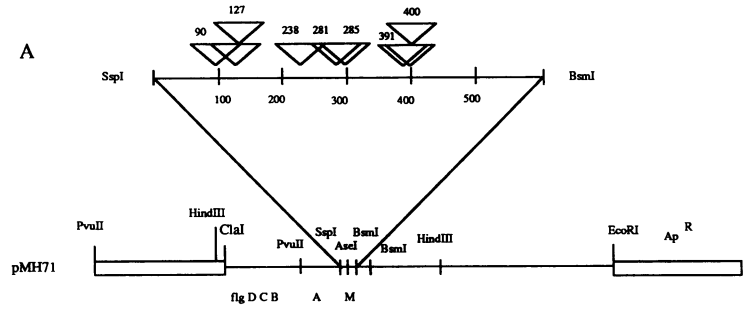
RESULTS

Isolation of the *flgM* gene by complementation. Repression of flagellin expression in flagellar-mutant backgrounds is alleviated when the *flgM* locus is insertionally inactivated or deleted (11). These mutations mapped the *flgM* locus to the *flg* region of the chromosome. To clone the *flgM* locus, plasmid pMH71 (17) was obtained from M. Carsiotis. This plasmid contains a 6.5-kb chromosomal insert which extends from *flgD* through *flgA* and about 4 kb beyond *flgA* (Fig. 1). The ability to complement a chromosomal *flgM* defect in *S. typhimurium* TH1983 was used to identify clones carrying the *flgM* locus. This strain is deleted for flagellar genes *flgG,-H,-I,-J,-K*, and *-L* and has a *lacZ* reporter gene transcriptionally fused to the *fljB* flagellin gene. The presence of a transposon insertion in the *flgM* gene allows expression of the flagellin genes (and hence *lacZ*) despite the presence of flagellar defects.

Introduction of pMH71 into TH1983 resulted in colonies which were Lac^-, indicating complementation of the chromosomal *flgM* mutation (Fig. 1). Several derivatives of pMH71 were constructed in order to define the borders of the *flgM* locus. The results shown in Fig. 1 demonstrate that in all cases, strains carrying plasmids which contain a 600-bp region of DNA between the *Ssp*I and *Bsm*I sites restore an $FlgM^+$ phenotype. Plasmids containing the 0.6-kb *Ssp*I-*Bsm*I region in a strain which has no flagellar defects also cause reduced expression of an *fliC*::MudJ *lac* transcriptional fusion on lactose indicator plates (data not shown). Also, clones which are missing the fragment between the *Ssp*I and *Pvu*II restriction sites no longer complement a chromosomal *flgA* mutation (data not shown), demonstrating that the *flgA* gene lies adjacent to the *flgM* gene.

Obtaining Tn*1000* insertions in the *flgM* gene. As described in Materials and Methods, Tn*1000* insertion mutations were generated in plasmid pMH71. These plasmids were transferred to the *S. typhimurium* test strain TH1983 (*flgM* Δ*flg fljB-lac*) and screened for the loss of *flgM*-complementing activity. Cells receiving a plasmid in which a Tn*1000* insertion disrupted the *flgM* gene did not show complementation of the chromosomal *flgM* defect and remained Lac^+. Nine independent insertions which gave an $FlgM^-$ phenotype were obtained and are shown in Fig. 2A. Sequence data (see below) showed that all of the insertions mapped within the same 0.6-kb *Ssp*I-*Bsm*I fragment shown by complementation to contain the *flgM* locus.

Sequencing the *flgM* region. Once the *flgM* region had been defined, the DNA sequence was obtained by using primers complementary to the ends of Tn*1000*. This method gener-



B

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1                                                        AATATTCTTATTAACCTATAATTGTGTAAAGAT
                                                                                    -35
34   TTTGTCGCGGCTGCCGATGAGATATTCAACCATGATGGTAGCTGGCCGCTACAACGTAACCCTCGATGAGGATAAATAA
                      -10          +1
113  ATG AGC ATT GAC CGT ACC TCA CCT TTG AAA CCC GTT AGC ACT GTC CAG ACG CGC GAA ACC
1    met ser ile asp arg thr ser pro leu lys pro val ser thr val gln thr arg glu thr

173  AGC GAC ACG CCG GTA CAA AAA ACG CGT CAG GAA AAA ACG TCC GCC GCG ACG AGC GCC AGC
21   ser asp thr pro val gln lys thr arg gln glu lys thr ser ala ala thr ser ala ser

233  GTA ACG TTA AGC GAC GCG CAA GCG AAG CTC ATG CAG CCA GGC GTC AGC GAC ATT AAT ATG
41   val thr leu ser asp ala gln ala lys leu met gln pro gly val ser asp ile asn met

293  GAA CGC GTC GAA GCA TTA AAA ACG GCT ATC CGT AAC GGT GAG TTA AAA ATG GAT ACG GGA
61   glu arg val glu ala leu lys thr ala ile arg asn gly glu leu lys met asp thr gly

353  AAA ATA GCA GAC TCG CTC ATT CGC GAG GCG CAG AGC TAC TTA CAG AGT AAA TAA GCGTATG
81   lys ile ala asp ser leu ile arg glu ala gln ser tyr leu gln ser lys STOP

413  ACTCGTTTGTCAGAAATACTTGACCAGATGACCACCGTCCTGAATGACCTGAAGACGGTGATGGACGCCGAGCAACAA
491  CAGCTTTCCGTAGGCCAGATTAACGGCAGCCAGCTACAGCGTATTACAGAAGAAAAAAGCTCGTTGCTGGCGACGCTG
569  GATTATCTGGAACAACAGCGCCGTCTGGAGCAGAATGC
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FIG. 2. Location and sequence of the *flgM* gene. (A) Locations of Tn*1000* inserts which resulted in an FlgM$^-$ phenotype. These insertions were used to generate the nucleotide sequence shown in panel B. The sequence shown is for the region between the *Ssp*I and *Bsm*I restriction sites. The −35 and −10 promoter regions are shown in boldface letters, the start of transcription is boxed, and the putative ribosome-binding site is underlined in boldface. The location of the *Ase*I restriction site is underlined.

ated about 900 bases of sequence, 600 bases of which included the region between the *Ssp*I and *Bsm*I restriction sites. This region is shown in Fig. 2B. This sequence contains two consecutive open reading frames in an apparent operon, one which would encode a 97-amino-acid protein, and a second one which continues beyond the *Bsm*I restriction site by at least 24 codons. The second open reading frame is unlikely to be the *flgM* gene because of the complementation results obtained above. There were no long open reading frames in the opposite direction.

The 97-amino-acid open reading frame is preceded by a putative ribosome-binding site (36) located 7 bases upstream of an ATG start codon; a protein beginning at that methionine would have a predicted molecular mass of 10,556 Da. Upstream of this region lies a sequence, 5′-TAAA-N_{15}-GCCGATGA-3′, which is a close match to the published consensus for FliA-dependent promoter sequences (5′-TAAA-N_{15}-GCCGATAA-3′ [14, 25, 29]) and may be the promoter for the putative *flgM* gene.

Mapping the start of transcription of the *flgM* gene. If the presumed FliA-dependent promoter sequence described above were responsible for promoting transcription of the putative *flgM* gene, then transcripts beginning from that region would extend into a Tn*1000* insert located downstream. Primer extension with a primer complementary to the promoter-proximal end of the Tn*1000* element can therefore be used to map the transcriptional start site by reverse transcriptase-mediated extension of a complementary DNA product back to the start of the mRNA.

RNA samples isolated from the following strains of *S. typhimurium* were used to determine the start of transcription: TH1983, the test strain for *flgM* complementation (*flgM* Δ*flg fljB-lac*); TH1984, a *fliA* derivative of TH1983; and TH1985, which is *flgM fljB-lac* (Fla$^+$). These test strains carried either the control plasmid pMH71 (*flgM*$^+$) or a Tn*1000*-containing derivative of pMH71. The particular Tn*1000* insertion used was located 127 bases downstream of the putative promoter for the *flgM* gene, within the coding region of the gene.

The results of primer extension analysis are shown in Fig. 3 and demonstrate a single transcriptional start site in the three strains which carry the Tn*1000* insert plasmid. No

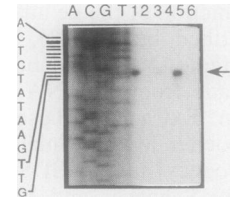

FIG. 3. Transcriptional mapping of the *flgM* promoter by primer extension. Lanes A, C, G, T, sequencing ladder. Primer extensions are shown in lanes 1 to 6. Lanes 1 and 2, TH1983 with pMH71/Tn*1000* and with pMH71, respectively; lanes 3 and 4, TH1984 (TH1983 *fliA*) with pMH71/Tn*1000* and with pMH71, respectively; lanes 5 and 6, TH1985 with pMH71/Tn*1000* and with pMH71, respectively. The arrow indicates the primer extension products. The base shown in boldface is the transcriptional start site.

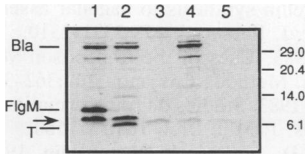

FIG. 4. T7 RNA polymerase-mediated expression of the FlgM protein. Proteins produced from pKG12 are shown in lane 1, while those made from pKG13 are shown in lane 2. Controls pKG11 (which contains the *flgM* gene in Bluescript in the opposite orientation from the T7 promoter), pTZ18R, and BlueScript are shown in lanes 3, 4, and 5, respectively. The FlgM and β-lactamase (Bla) proteins are indicated. T, truncated FlgM product. Arrow, second protein made from constructs. Positions of molecular mass markers are shown (in kilodaltons).

primer extension product was observed in any of the control strains which carry the wild-type plasmid. The observed start site maps to an adenine nucleotide positioned 11 bases downstream of the center of the putative σ^{FliA} promoter −10 region (boxed nucleotide in Fig. 2). This result supports the hypothesis that this is the promoter used for transcription of the *flgM* gene.

T7 RNA polymerase-mediated expression of the FlgM protein. To verify that the 97-amino-acid open reading frame is expressed and is responsible for the complementation of a chromosomal *flgM* defect, in vivo production of this protein was assayed. Plasmid pKG12 contains the putative *flgM*$^+$ open reading frame in pTZ18R in the correct orientation for transcription from the inducible T7 promoter. This plasmid and control plasmids were transformed into *E. coli* DH5α carrying pGP1-2, which encodes the inducible T7 RNA polymerase. Transcription from the T7 promoter was promoted by induction of T7 RNA polymerase synthesis. Protein products were labeled in vivo and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) analysis.

^{35}S-labeled protein products obtained from pKG12 are shown in lane 1 of Fig. 4. Two small proteins with apparent molecular masses of about 9.5 and 8.0 kDa appear in this lane. We believe that the 9.5-kDa protein corresponds to the *flgM* gene product. This conclusion is based on the results shown in lane 2, which displays protein products obtained from pKG13. In plasmid pKG13, an *Ase*I site internal to the *flgM* gene was digested and filled in, resulting in a 2-bp frameshift. The frameshift causes the addition of eight incorrect amino acids after codon 58 of the open reading frame, followed by termination after 66 amino acids (31 amino acids early). This construct no longer complemented a chromosomal *flgM* mutation. This appears in lane 2 as a loss of the 9.5-kDa gene product and the appearance of a smaller protein product with an apparent molecular mass of 6.8 kDa, corresponding to the predicted molecular mass of the frameshift product. The 8.0-kDa protein product remains the same. This protein may correspond to the open reading frame downstream from the *flgM* gene. Proteins made from pKG11 (lane 3), which contains the *Ssp*I-*Bsm*I fragment in the opposite orientation with respect to the T7 promoter in BlueScript, also appear in the BlueScript vector control lane (lane 5). In pTZ18R, the *bla* gene is also in the correct orientation for transcription from the T7 promoter. A band corresponding to about 30 kDa in lanes 1 and 2 is probably the β-lactamase protein, since it also appears in lane 4, the pTZ18R vector control.

DISCUSSION

When basal body, hook, or switch proteins are defective, no transcription occurs from the flagellin genes, an effect mediated by the *flgM* locus. When the *flgM* locus is insertionally inactivated, the negative feedback regulation is uncoupled, and expression of the flagellin genes resumes in flagellar mutant strains (11). In this study we have determined the primary structure of the *flgM* gene.

Plasmids carrying the intact *flgM* gene were able to complement a chromosomal *flgM* mutation and restore a repressed condition of the flagellin structural genes in the presence of chromosomal flagellar defects, suggesting that *flgM* encodes a diffusible protein product. We obtained nine independent Tn*1000* insertions, in a 10-kb plasmid containing the *flgM* gene, which resulted in an FlgM$^-$ phenotype. All of these insertions were located within a 320-bp region. We have sequenced this region and have found an open reading frame which encodes a 97-amino-acid protein with a predicted molecular mass of 10.6 kDa. In the T7-based expression system, the protein responsible for complementation of an *flgM* chromosomal defect was expressed and observed by SDS-PAGE analysis to have an apparent molecular mass of 9.5 kDa. Upon introduction of a frameshift mutation into the plasmid-borne clone of the *flgM* gene at an *Ase*I site internal to the 97-amino-acid open reading frame, the plasmid no longer complemented an *flgM* chromosomal mutant and the 9.5-kDa protein band was lost.

The start of a second open reading frame lies just 5 bp downstream of the *flgM* stop codon. A total of 90 codons of this open reading frame were determined in our DNA sequence analysis. This open reading frame may be part of an operon that includes the *flgM* gene. Further characterization of this open reading frame is needed to determine whether this putative gene has a role in the assembly or regulation of functional flagella.

The FlgM protein sequence was compared with known sequences in the GenBank 66 and EMBL 25 sequence data banks by using the FastDB sequence comparison software (Intelligenetics, Inc.) (3). The only significant homology obtained was to an open reading frame downstream of the gene for *Yersinia enterocolitica* invasin, the *invA* gene (47), for which only a portion of the DNA sequence has been determined. The FlgM protein has 57% identity with 47 amino acids from that open reading frame (data not shown).

Upstream of the open reading frame for the *flgM* gene lies a sequence which closely matches the promoter sequence for the gene encoding the flagellin-specific alternate sigma factor FliA. When the FliA sigma factor was removed by mutation, a low-level transcription of *flgM* was observed. It is not clear why there was residual expression of *flgM* in the *fliA* mutant background. One possibility is that the promoter for *flgM* is not completely dependent on a FliA-containing RNA polymerase for transcription. Another explanation is that low-level expression is peculiar to the particular *fliA* point mutation that we used in our assays.

The mechanism of action of the *flgM* gene product remains to be determined. The action of the FlgM protein theoretically requires two functions: repression of flagellin genes and recognition that a component of the flagellum is missing or defective. Recently, *fliA* mutants insensitive to *flgM* regulation have been isolated (33), and these results indicate that the FlgM protein acts to prevent FliA function or *fliA* expression. This would suggest a regulatory circuit in which *flgM*, transcribed by a FliA-containing RNA polymerase,

negatively regulates FliA function in flagellar-mutant backgrounds.

Possible hypotheses for the mechanism of recognition of flagellar defects by the FlgM protein include sequestering of the protein product until it is needed; modification, such as phosphorylation, of a constitutive protein product; and regulated transcription of the *flgM* gene. Our results indicate that the *flgM* gene is transcribed in both the presence and absence of flagellar defects. However, since there was no wild-type FlgM present in the strains assayed, it may be that FlgM negatively regulates its own transcription in wild-type but not mutant backgrounds. Also, high-copy-number plasmids containing the *flgM*$^+$ gene in a wild-type strain carrying a *lac* fusion to the *fliC* gene cause a decrease in expression of the flagellin gene despite the absence of any flagellar defects. This result suggests that overexpression of the *flgM* gene causes a superrepressor phenotype and that FlgM is limiting in wild-type cells. In addition, some flagellar mutants become weakly motile when the *flgM* gene is inactivated (10), suggesting that in some cases the cells are nonmotile due as much to the action of the FlgM gene product as to the particular mutation. None of these results can solidly eliminate any of the hypotheses proposed. Additional characterization of the *flgM* locus must be done in order to fully understand the function and mechanism of action of FlgM.

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