JOURNAL OF BACTERIOLOGY, July 2004, p. 4613–4619 Vol. 186, No. 14
0021-9193/04/$08.00+0 DOI: 10.1128/JB.186.14.4613–4619.2004

The *Vibrio cholerae* FlgM Homologue Is an Anti-σ^{28} Factor That Is Secreted through the Sheathed Polar Flagellum

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Received 20 February 2004/Accepted 8 April 2004

Vibrio cholerae **has a single polar sheathed flagellum that propels the cells of this bacterium. Flagellar synthesis, motility, and chemotaxis have all been linked to virulence in this human pathogen. *V. cholerae* expresses flagellar genes in a hierarchy consisting of σ^{54}- and σ^{28}-dependent transcription. In other bacteria, σ^{28} transcriptional activity is controlled by an anti-σ^{28} factor, FlgM. We demonstrate that the *V. cholerae* FlgM homologue (i) physically interacts with σ^{28}, (ii) has a repressive effect on some *V. cholerae* σ^{28}-dependent flagellar promoters, and (iii) is secreted through the polar sheathed flagellum, consistent with anti-σ^{28} activity. Interestingly, FlgM does not have a uniform repressive effect on all σ^{28}-dependent promoters, as determined by measurement of σ^{28}-dependent transcription in cells either lacking FlgM (Δ*flgM*) or incapable of secretion (Δ*fliF*). Further analysis of a Δ*fliF* strain revealed that this flagellar assembly block causes a decrease in class III (FlrC- and σ^{54}-dependent) and class IV (σ^{28}-dependent), but not class II (FlrA- and σ^{54}-dependent), flagellar transcription. *V. cholerae flgM* and *fliA* (encodes σ^{28}) mutants were only modestly affected in their ability to colonize the infant mouse intestine, a measure of virulence. Our results demonstrate that *V. cholerae* FlgM functions as an anti-σ^{28} factor and that the sheathed flagellum is competent for secretion of nonstructural proteins.**

Studies of bacterial flagellar assembly have revealed that the flagellum is assembled in a stepwise fashion that begins by insertion of a type III export apparatus into the cytoplasmic membrane (reviewed in reference 27). Flagellar components are then secreted through this export machinery to be added to the growing tip of the flagellum in the specific order in which they are assembled (reviewed in reference 42). The bulk of the flagellum is composed of flagellin subunits, which are only added to the flagellum after the basal-body–hook structure is completed. Transcription of flagellar genes generally occurs in a hierarchical fashion that mirrors assembly of the nascent flagellum; i.e., the genes encoding early flagellar components are transcribed prior to the genes encoding late flagellar components, such as flagellin subunits (24; reviewed in reference 41).

In *Salmonella enterica* serovar Typhimurium, transcription of the flagellin gene is repressed until the basal-body–hook structure is completed, through the action of an anti-sigma factor, FlgM. The flagellin gene is transcribed by RNA polymerase (RNAP) containing the alternate sigma factor σ^{28} (encoded by *fliA*) (34). FlgM binds to σ^{28} and prevents its association with RNAP, preventing flagellin gene transcription (23). However, once the basal-body–hook structure is assembled, FlgM is secreted through the flagellar export apparatus to the extracellular milieu, which allows σ^{28} to associate with RNAP and transcribe the flagellin gene (15). Thus, the function of FlgM is to couple flagellar assembly to appropriate temporal flagellar gene transcription.

Flagellar gene transcription in *Vibrio cholerae*, which possesses a single polar sheathed flagellum, is also organized into a transcription hierarchy (35). However, the four-tiered transcription hierarchy has notable differences from the three-tiered hierarchy of *S. enterica* serovar Typhimurium, which possess multiple peritrichous flagella. In *V. cholerae*, the genes encoding the early structural components of the flagellum (basal-body–hook) are transcribed in two distinct temporal classes (II and III) by RNAP containing the alternate sigma factor σ^{54}; in contrast, transcription of the equivalent genes in *S. enterica* serovar Typhimurium occurs within the single "early" temporal class (II) by RNAP containing the housekeeping σ^{70} subunit. However, the last temporal class of flagellar genes in *V. cholerae* (class IV), as in *S. enterica* serovar Typhimurium (class III), is transcribed by RNAP containing σ^{28}. The main σ^{28}-dependent genes are four distinct flagellin genes, *flaB*, *flaC*, *flaD*, and *flaE* (20, 35). σ^{54}-dependent transcription of a fifth flagellin gene, *flaA*, precedes transcription of the other four flagellin genes (20). The four-tiered σ^{54}- and σ^{28}-dependent flagellar transcription hierarchy of *V. cholerae* (35) is likely to be identical to the polar hierarchy of *V. parahaemolyticus* (18), is remarkably similar to those of *Pseudomonas aeruginosa* (3) and *Campylobacter jejuni* (14), and has similarities to that of *Helicobacter pylori* (1), suggesting that this flagellar hierarchy may be common among gram-negative bacteria with polar flagella.

The σ^{54}-dependent FlaA flagellin is essential for *V. cholerae* motility and flagellar synthesis, while the σ^{28}-dependent FlaB, FlaC, FlaD, and FlaE flagellins are largely dispensable both singly and in combination (20). A *fliA* strain (lacking σ^{28}) is nonmotile and does not transcribe *flaB*, *flaC*, *flaD*, or *flaE* but produces a truncated flagellum, indicating that some FlaA subunits are assembled (35). Because FlaA transcription and assembly into the flagellum appear to precede the transcription and incorporation of the other flagellin subunits, it suggests

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that the checkpoint that controls σ^{28}-dependent transcription may differ between *V. cholerae* and *S. enterica* serovar Typhimurium, where completion of the basal-body–hook structure causes the export of FlgM and σ^{28}-dependent transcription (17). Also, the presence of a sheath enveloping the *V. cholerae* flagellum, which is contiguous with the outer membrane (7), could inhibit or prevent the secretion of nonstructural proteins, such as an anti-sigma factor, through the flagellum; *S. enterica* serovar Typhimurium flagella are not sheathed. These observations invited the question of whether *V. cholerae* FlgM functions similarly to FlgM of *S. enterica* serovar Typhimurium.

FlgM homologues have been identified and characterized in the gram-negative polar flagellates *H. pylori* (1, 16) and *P. aeruginosa* (6). These studies demonstrated that the FlgM homologues in these bacteria physically interact with σ^{28} and inhibit σ^{28}-dependent transcription, but they did not address the question of whether FlgM is secreted through the flagellum. In fact, Josenhans et al. suggested that FlgM is not secreted through the sheathed *H. pylori* flagellum (16). A report on a *flgM C. jejuni* mutant (14) demonstrated that the FlgM of that organism has only weak repressive activity on σ^{28}-dependent transcription but also did not address FlgM secretion. We report here that the *V. cholerae* FlgM homologue has the characteristics of the *S. enterica* serovar Typhimurium anti-σ^{28} factor: it physically interacts with σ^{28}, it represses transcription from some σ^{28}-dependent promoters, and it is secreted through the (polar sheathed) flagellum.

MATERIALS AND METHODS

Bacterial strains and media. *Escherichia coli* strain DH5α (11) was used for cloning manipulations, SM10λ*pir* (32) was used to transfer plasmids to *V. cholerae* by conjugation, and BL21(DE3) (Novagen) was used for protein expression. The *V. cholerae* strains used in this study are listed in Table 1. Construction of chromosomal deletions and insertions with pKEK229, a pCVD442 derivative with the *sacB* gene (4), has been described previously (2). Bacteriophage CP-T1ts-mediated transduction (12) was used to construct KKV1441(Δ*flgM*::Cm^r); the correct construction of all strains was verified by PCR and sequencing.

Luria broth (LB) was used for both liquid and agar media. Antibiotics were added when appropriate at the following concentrations: ampicillin, 50 μg/ml; chloramphenicol, 2 or 20 μg/ml (for *V. cholerae* and *E. coli*, respectively); streptomycin, 100 μg/ml. Agar plates consisting of LB with 0.3% agar were used to measure motility. For counterselection with *sacB*-containing plasmids, LB without NaCl and with 10% sucrose was used.

Plasmid construction. All of the plasmids and oligonucleotide primers used are listed in Table 1. *V. cholerae* O395 chromosomal DNA was used as the template for PCR amplification. The in-frame deletion in *flgM* (Δ*flgM*) was constructed by first amplifying the 5′ fragment with oligonucleotides FLGM DEL1 and FLGMDEL2, digesting the amplified product with EcoRI and BamHI, and then ligating it into similarly digested pWSK30 (39) to form pKEK375. The 3′ fragment was PCR amplified with oligonucleotides FLGM DEL3 and FLGMDEL4, digested with EcoRI and HindIII, and ligated into similarly digested pKEK375 to form pKEK381 (Δ*flgM*). This results in the removal of 165 nucleotides that correspond to amino acids 29 to 83 of the predicted gene product. The chloramphenicol acetyltransferase gene from pACYC184 (37) was amplified by PCR with primers CAT1 and CAT2 (22), digested with MfeI, and ligated into pKEK381 digested with EcoRI to form pKEK411 (Δ*flgM*::Cm^r). The Δ*flgM* and Δ*flgM*::Cm^r mutations were moved into plasmid pKEK229 via NotI and SalI digestion and ligation to form pKEK389 and pKEK427, respectively.

To construct a plasmid for the expression of FlgM with an amino-terminal FLAG tag (FLAG-FlgM), *flgM* was PCR amplified with primers FLAGFLGM and FLGMUBAMHI, digested with NcoI and BamHI, and ligated into similarly digested pET15b (Novagen) to form pKEK460. pKEK460 was then digested with PshAI and HindIII and ligated with a PshAI-HindIII fragment of pACYC184 (37) to generate pKEK470. This plasmid still contains the T7*lac* promoter and FLAG-FlgM, but with p15A *ori* and Cm^r from pACYC184. For expression of FLAG-FlgM in *V. cholerae*, pKEK470 was digested with NcoI and HindIII and ligated into similarly digested pBAD24 (10) to form pKEK474. The plasmid used for expression of σ^{28} with an amino-terminal His_6 tag (His-σ^{28}) was constructed by PCR amplification of *fliA* with primers FLIANDEI and FLIAUBAMHI, followed by digestion with NdeI and BamHI and ligation into similarly digested pET-15b to form pKEK462.

TABLE 1. Bacterial strains, plasmids, and oligonucleotide primers used in this study

Strain, plasmid, or primer	Relevant genotype description or sequence (5′–3′)	Source or reference
V. cholerae strains		
O395	Wild type classical Ogawa	30
KKV598	O395, Δ*lacZ*	2
KKV1113	O395 Δ*fliA* Δ*lacZ*	35
KKV1247	O395 Δ*fliF* Δ*lacZ*	This study
KKV1365	O395 Δ*fliA* Δ*flgM*::Cm Δ*lacZ*	This study
KKV1384	O395 Δ*flgM* Δ*lacZ*	This study
KKV1441	O395 Δ*flgM*::Cm Δ*lacZ*	This study
KKV1461	O395 Δ*flgM* Δ*lacZ*	This study
Plasmids		
pKEK229	R6K *ori sacB mob* Amp^r	2
pKEK389	Δ*flgM* in pKEK229	This study
pKEK424	Δ*fliF* in pKEK229	25
pKEK427	Δ*flgM*::Cm in pKEK229	This study
pET15b	ColE1 *ori* His tag T7*lac* promoter Amp^r	Novagen
pKEK460	FLAG-*flgM* in pET15b	This study
pKEK462	*fliA* in pET15b	This study
pKEK470	p15A *ori* T7*lac* promoter, FLAG-*flgM* Cm^r	This study
pBAD24	ColE1 *ori* Amp^r *araBAD* promoter	10
pKEK474	FLAG-flgM in pBAD24	This study
pRS551	Transcriptional *lacZ* fusion vector Amp^r Kan^r	38
pKEK72	*flrB* promoter-*lacZ* fusion in pRS551	21
pKEK76	*flaC* promoter-*lacZ* fusion in pRS551	20
pKEK77	*flaD* promoter-*lacZ* fusion in pRS551	20
pKEK79	*flaB* promoter-*lacZ* fusion in pRS551	20
pKEK81	*flaE* promoter-*lacZ* fusion in pRS551	20
pKEK80	*flaA* promoter-*lacZ* fusion in pRS551	20
pKEK327	*fliE* promoter-*lacZ* fusion in pRS551	35
pKEK329	*flhA* promoter-*lacZ* fusion in pRS551	35
pKEK331	*flgK* promoter-*lacZ* fusion in pRS551	35
pKEK332	*flgB* promoter-*lacZ* fusion in pRS551	35
pKEK415	*flaG* promoter-*lacZ* fusion in pRS551	35
Primers		
FLGMDEL1	GCGGATCCGTGTGGGTGATGTG ATTGAAC	
FLGMDEL2	GCGAATTCGCTGGCATCAGAAG AACGCAC	
FLGMDEL3	GCGAATTCGGCTCTTACGTGGTT GACCCT	
FLGMDEL4	GCGAAGCTTCGGATTTGACTCA CCAACTCA	
FLIANDEI	GGGGAACCTTCATATGAATAAA GCGC	
FLIAUBAMHI	GCGGATCCTTAGTCATTCCG AGTCCAAGAAC	
FLGMUBAMHI	GCGGATCCAGGTTAGAAGCCGC CCAGTTC	
FLAGFLGM	CGCGCCATGGACTACAAGGATG ACGACGACAAGCATATGGCAG GTATTGATAACATACGC	

β-Galactosidase assays. *V. cholerae* strains were transformed with the promoter-*lacZ* fusion-containing plasmids listed in Table 1, grown in LB plus antibiotic, and then harvested at an optical density at 600 nm of ~0.2 to 0.4. Bacterial

cells were permeabilized with chloroform and sodium dodecyl sulfate (SDS) and assayed for β-galactosidase activity by the method of Miller (31). All experiments were performed at least three separate times.

Electron microscopy. Strains were grown to mid-log phase in LB, centrifuged, and then resuspended in 0.15 M NaCl. Samples were allowed to adhere to a carbon-coated grid and stained with 1% uranyl acetate before microscopy with a JEOL 1230 microscope. The AMT software was used to measure the lengths of flagella on wild-type and Δ*flgM* mutant *V. cholerae* strains (25 of each), which were determined to be significantly different by Student's two-tailed *t* test.

In vivo colonization assays. The infant mouse colonization assay has been described previously (9). The inocula consisted of ~10^6 CFU for both wild-type and mutant bacteria.

Protein detection. For coprecipitation of His-σ^{28} and FLAG-FlgM, plasmids pKEK470 and/or pKEK462 were transformed into BL21(DE3) and expression of the proteins was induced by growth in LB with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 5 h at 37°C. Supernatants of sonicated cells were mixed with anti-His Dynabeads (Dynal Biotech), pelleted, and eluted in accordance with the manufacturer's instructions.

For detection of FLAG-FlgM secretion, KKV598 (wild type) and KKV1247 (Δ*fliF*) were transformed with pKEK474. These strains were grown in LB plus 0.1% arabinose at 37°C for 2 h. Samples were removed and centrifuged at 17,000 × *g* for 30 min. The supernatants were passed through a 0.2-μm syringe filter and concentrated with 10% trichloroacetic acid (TCA). Samples were then centrifuged (68,000 × *g* for 1.5 h), and the pellets were washed twice with cold acetone.

Samples were separated by SDS–15% polyacrylamide gel electrophoresis, probed by Western immunoblotting with either His-Tag (Novagen) or anti-FLAG M2 (Sigma) monoclonal antibody, and detected with ECL detection reagent (Amersham-Pharmacia).

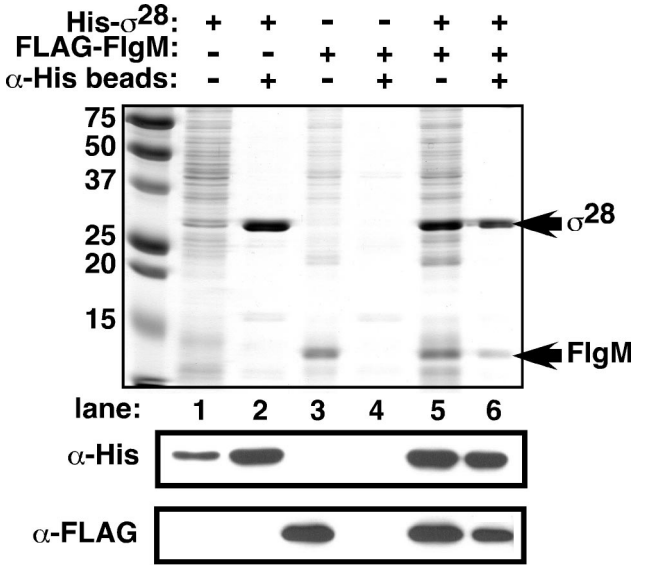

FIG. 1. Interaction of *V. cholerae* FlgM with σ^{28}. His-σ^{28} and FLAG-FlgM were expressed separately and together in *E. coli* BL21(DE3), which contains T7 polymerase, from T7 promoters in plasmids pKEK462 and pKEK470, respectively, and magnetic anti-His beads (Dynal Biotech) were used to capture His-tagged protein from cell lysates as described in Materials and Methods. In the upper panel, samples were separated on Coomassie-stained polyacrylamide gel, and the lower panels show Western immunoblots of these same samples probed with anti-His tag and anti-FLAG tag antibodies. Masses of molecular size markers are noted on the left in kilodaltons, and arrows depict migration of His-σ^{28} and FLAG-FlgM. Lane numbers correspond to BL21(DE3) cells expressing His-σ^{28} (lane 1, whole-cell lysate; lane 2, anti-His bead eluate), FLAG-FlgM (lane 3, whole-cell lysate, lane 4, anti-His bead eluate), and His-σ^{28} and FLAG-FlgM (lane 5, whole-cell lysate, lane 6, anti-His bead eluate).

RESULTS

Interaction of FlgM with σ^{28}. The gene encoding a FlgM homologue was identified in the *V. cholerae* genome sequence (VC2204) (13). The *flgM* gene is predicted to encode a protein of 11.3 kDa. As we noted previously (35), *V. cholerae* FlgM has homology with *S. enterica* serovar Typhimurium FlgM (28% identity). If *V. cholerae* FlgM functions as an anti-σ^{28} factor, it is predicted to physically interact with σ^{28} (FliA). To detect a physical interaction between FlgM and σ^{28}, His-tagged *V. cholerae* σ^{28} and FLAG-tagged *V. cholerae* FlgM were coexpressed from different compatible plasmids within *E. coli* (Fig. 1). Magnetic metal chelation beads (anti-His; Dynal Biotech) were then used to precipitate His-σ^{28} from the *E. coli* lysate. Eluates from the magnetic beads were separated by SDS-polyacrylamide gel electrophoresis and then probed with either anti-His or anti-FLAG antibodies by Western immunoblotting.

When His-σ^{28} is expressed alone, it is efficiently captured by the anti-His magnetic beads (lane 2); in contrast, when FLAG-FlgM is expressed alone, there is no detectable capture of this protein by the anti-His magnetic beads (lane 4), despite the presence of a large amount of FlgM protein in the lysate (lane 3). However, when His-σ^{28} and FLAG-FlgM are coexpressed within the same cell, capture of His-σ^{28} by magnetic beads causes the cocapture of a large amount of FLAG-FlgM (lane 6). These results demonstrate protein-protein interaction between His-σ^{28} and FLAG-FlgM, consistent with the predicted anti-σ^{28} activity of FlgM.

Secretion of FlgM through the *V. cholerae* flagellum. The *S. enterica* serovar Typhimurium FlgM protein is secreted through the flagellar export apparatus. To monitor secretion of *V. cholerae* FlgM through the flagellar export apparatus, a FLAG tag was fused to the N terminus of FlgM to facilitate detection; a FLAG tag at the N terminus of *S. enterica* serovar Typhimurium FlgM does not inhibit secretion through the flagellum (K. Hughes, personal communication). To determine if the *V. cholerae* FlgM protein is likewise secreted through the flagellum, the FLAG-FlgM protein was expressed in a wild-type (flagellated) *V. cholerae* strain. FLAG-FlgM was also expressed within a Δ*fliF V. cholerae* strain, which lacks the MS ring, the first component assembled in the flagellum, and thus this strain lacks any flagellar structure or export apparatus. Protein in the filtered supernatant was concentrated by TCA precipitation, separated by SDS-polyacrylamide gel electrophoresis, and then subjected to Western immunoblot analysis with anti-FLAG antibodies.

FLAG-FlgM could be detected both in the supernatant (S) and in the cell pellet (P) of the wild-type (flagellated) strain (WT, Fig. 2). However, FLAG-FlgM was only found within the cell pellet (P) of the Δ*fliF* (nonflagellated) strain (*fliF*) and there was no detectable FLAG-FlgM present in the supernatant (S). These results indicate that there is a requirement for the flagellum to achieve secretion of FlgM into the supernatant, consistent with FlgM secretion through the *V. cholerae* polar (sheathed) flagellum. Interestingly, the FlgM protein in the supernatant has a slightly different mobility in the gel than the FlgM protein within the cells, suggesting either some C-terminal processing or an SDS-resistant conformational change.

Construction of a *V. cholerae flgM* mutant. To study the function of *V. cholerae* FlgM, we constructed a strain with an in-frame deletion within the *flgM* gene. Construction of this

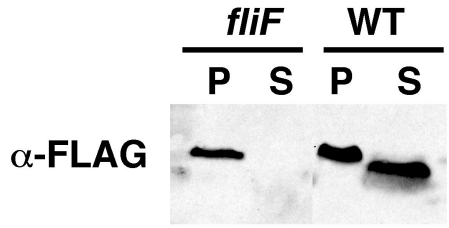

FIG. 2. FlgM is secreted through the *V. cholerae* flagellum. FLAG-FlgM was expressed from plasmid pKEK474 in *V. cholerae* strains KKV598 (wild type [WT]) and KKV1247 (*fliF* mutant). Bacterial pellets (P) and supernatants (S) were processed for Western immunoblot analysis with anti-FLAG antibodies as described in Materials and Methods.

strain was difficult; our initial attempts to introduce the Δ*flgM* mutation into the chromosome of a wild-type *V. cholerae* strain via suicide plasmid integration into the chromosome and subsequent sucrose counterselection to remove the plasmid were unsuccessful. Only wild-type (*flgM*$^+$) strains were recovered, despite multiple attempts. We reasoned that this may be due to the deleterious effect of unregulated σ^{28} activity in a *flgM* strain, so the attempt at strain construction was repeated with a Δ*fliA* strain, KKV1113 (35). In the Δ*fliA* background, the Δ*flgM* chromosomal mutation was readily obtained; approximately one-half of the sucrose-resistant colonies contained this mutation. These results demonstrate that there is selective pressure against obtaining a Δ*flgM* mutation in a *fliA*$^+$ strain background, suggesting that *flgM* exerts its effects through *fliA*.

To obtain a *fliA*$^+$ strain with the Δ*flgM* mutation, we first constructed a Δ*flgM*::Cm^r chromosomal mutation in Δ*fliA* mutant strain KKV1113 via suicide plasmid pKEK427 (strain KKV1365) and then moved the Δ*flgM*::Cm^r mutation into the wild-type (*fliA*$^+$) strain (KKV1441) via CP-T1ts-mediated transduction (12). Cm^r transductants in the *fliA*$^+$ background took 2 days to form colonies on plates, compared to Cm^r transductants in the *fliA* mutant background, which only took 1 day to form colonies. PCR analysis demonstrated that these Cm^r transductants had a chromosomal Δ*flgM*::Cm^r mutation (not shown). The Δ*flgM*::Cm^r mutation was then replaced with Δ*flgM* (without any insertion) via suicide plasmid pKEK389. In this manner we constructed Δ*flgM* mutant *V. cholerae* strain KKV1461. Interestingly, although the Δ*flgM* strain took 2 days to form colonies on selective plates, its growth curve is indistinguishable from that of the wild-type strain in liquid LB (not shown).

Motility of the Δ*flgM* mutant strain was measured in motility agar plates (Fig. 3A). The swimming pattern of this strain was slightly reduced compared to that of the wild-type strain, indicating that the Δ*flgM* mutation has a modest negative effect on the ability of the bacteria to swim from the point of inoculation in this assay. In contrast, the Δ*fliA* mutation abolished motility, as we have shown previously (35). A Δ*fliF* mutant strain, which contains a mutation in the structural gene for the MS ring, the first component of the flagellum assembled (27), is also nonmotile in this assay, as expected. A *flgM* mutant of *P. aeruginosa* showed a significant motility defect in this assay (6), suggesting possible differences between the function of *V. cholerae* FlgM and that of *P. aeruginosa* FlgM.

Δ*flgM* mutant *V. cholerae* cells were visualized by electron

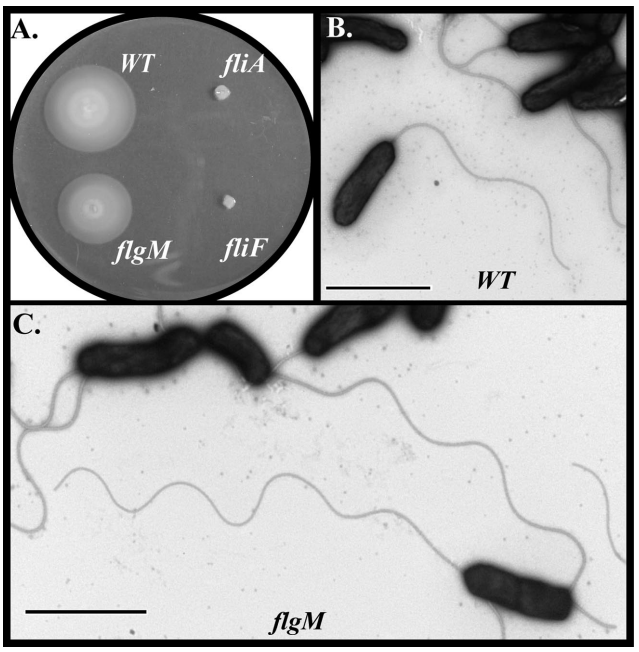

FIG. 3. Motility phenotype and electron micrograph of a *V. cholerae flgM* strain. (A) *V. cholerae* strains KKV598 (wild type [*WT*]), KKV1114 (*fliA* mutant), KKV1461 (*flgM* mutant), and KKV1247 (*fliF* mutant) were inoculated into motility agar and incubated at 30°C. Electron micrographs of *V. cholerae* strains KKV598 (wild type) (B) and KKV1461 (*flgM*) (C) are also shown. Bars are equivalent to 2 μm.

microscopy (Fig. 3C). The cells possessed single polar flagella that were noticeably longer than those of the wild-type strain (Fig. 3B). Measurement of flagella of the wild-type and Δ*flgM* mutant strains revealed a significantly longer flagellum (P = 0.001) in the Δ*flgM* strain (6.95 ± 1.18 μm) in comparison to that of the wild-type strain (5.71 ± 1.42 μm), consistent with the notion that FlgM plays a repressive role in flagellar length.

FlgM has repressive effects on σ^{28}-dependent transcription. If FlgM functions as an anti-σ^{28} factor, its absence in a Δ*flgM* mutant would be predicted to cause an increase in σ^{28}-dependent transcription. Transcription of the five *V. cholerae* flagellins was measured by promoter-*lacZ* transcriptional fusion plasmids (20) in the wild-type, Δ*fliA*, Δ*flgM*, and Δ*fliA* Δ*flgM* strains (Fig. 4). The *flaA* promoter is a σ^{54}-dependent class III promoter, while the *flaB*, *flaC*, *flaD*, and *flaE* promoters are σ^{28}-dependent class IV promoters (35).

As we have shown previously, the lack of σ^{28} (in a Δ*fliA* mutant) eliminates transcription of the *flaB*, *flaC*, *flaD*, and *flaE* promoters but has no effect on *flaA* transcription compared with transcription in a wild-type strain (35). The lack of FlgM (in the Δ*flgM* strain) had an approximately twofold stimulatory effect on *flaD* and *flaE* transcription, in comparison with transcription in the wild-type strain. Oddly, there was a decrease in *flaC* transcription in the Δ*flgM* strain, and transcription of the *flaB* promoter was unaffected by the lack of FlgM. The effect of the lack of FlgM seen at these four promoters is due to σ^{28}-dependent transcription because introduction of a Δ*fliA* mutation into the Δ*flgM* background (the Δ*fliA* Δ*flgM* strain) eliminates *flaB*, *flaC*, *flaD*, and *flaE* transcription. There was no effect of the Δ*flgM* or Δ*fliA* Δ*flgM*

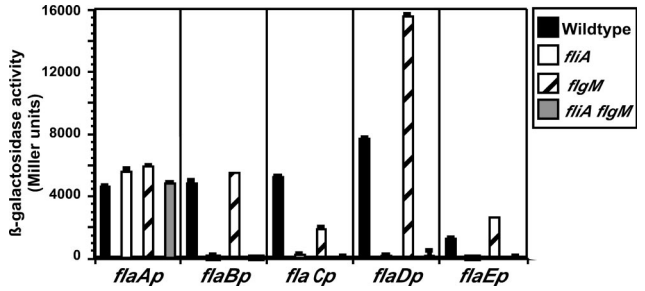

FIG. 4. Expression of flagellin promoters in *fliA* and *flgM* mutant *V. cholerae* strains. *V. cholerae* strains KKV598 (wild type), KKV113 (*fliA* mutant), KKV1461 (*flgM* mutant), and KKV1384 (*fliA flgM* mutant) carrying plasmids pKEK80 (*flaAp-lacZ*), pKEK79 (*flaBp-lacZ*), pKEK76 (*flaCp-lacZ*), pKEK77 (*flaDp-lacZ*), and pKEK81 (*flaEp-lacZ*) were assayed for β-galactosidase activity during logarithmic growth in LB. Assays were performed in triplicate, and standard deviations are shown.

mutations on *flaA* transcription. Our results demonstrate a repressive effect of FlgM on σ^{28}-dependent transcription of *flaD* and *flaE*, as would be expected of an anti-σ^{28} factor. However, σ^{28}-dependent transcription of the *flaB* and *flaC* promoters was either unaffected or increased in the presence of FlgM, indicating a lack of uniform repressive FlgM activity at all σ^{28}-dependent promoters.

Lack of the MS ring causes decreases in class III and IV and increases in class II flagellar transcription. Because the MS ring is the first structural component assembled in the nascent flagellum (27), absence of the MS ring in a Δ*fliF V. cholerae* mutant will prevent export and subsequent assembly of any flagellar component located exterior to the cytoplasm. To determine the effect on transcription of blocking flagellar assembly at the initial step, we measured the transcription of a number of class II, III, and IV promoters in the *V. cholerae* wild-type and Δ*fliF* mutant strains (Fig. 5).

The absence of the MS ring in the Δ*fliF* mutant caused an increase in transcription (up to threefold) of all three class II (FlrA- and σ^{54}-dependent) flagellar promoters, *flhA*, *fliE*, and *flrB*, in comparison with that in the wild-type strain. In contrast, transcription of the four class III (FlrC- and σ^{54}-dependent) flagellar promoters, *flgK*, *flgB*, *flaG*, and *flaA*, was decreased (up to threefold) in the Δ*fliF* mutant, compared to that in the wild-type strain. Finally, transcription of three class IV (σ^{28}-dependent) flagellar promoters, *flaC*, *flaD*, and *flaE*, was reduced more than twofold in the Δ*fliF* mutant, in comparison to that in the wild-type strain. Oddly, transcription of the class IV *flaB* promoter was unaffected by the absence of the MS ring, just as it was unaffected by the absence of FlgM (see above). In general, these results demonstrate that blocking flagellar assembly at the initial step causes an increase in class II gene transcription and decreases in class III and IV gene transcription.

***flgM* and *fliA* have little effect on intestinal colonization.** Flagellar synthesis, regulation, and chemotaxis have all been linked to *V. cholerae* colonization defects in the infant mouse (2, 26, 40), but the connection between these phenomena remains unclear. To determine the effect of *flgM* and *fliA* mutations on *V. cholerae* intestinal colonization of the infant mouse, a competition assay was performed with an inoculum that consisted of both mutant and isogenic wild-type strains (see Materials and Methods). Colonization defects are recognized by a competitive index (CI) of less than 1.

The Δ*fliA*, Δ*flgM*, and Δ*fliA* Δ*flgM* mutant strains demonstrated modest colonization defects in this assay (Fig. 6). The Δ*fliA* and Δ*fliA* Δ*flgM* mutant strains were more affected for colonization (CI, ~0.3) than the Δ*flgM* strain (CI, 0.601), indicating that loss of σ^{28} is more deleterious for colonization than is loss of FlgM. Still, these are modest colonization defects, demonstrating that σ^{28} and FlgM play little role in intestinal colonization. Control experiments showed wild-type growth rates of all strains in LB at 37°C; thus, no obvious in vitro growth defects account for the modest in vivo colonization defects of these strains.

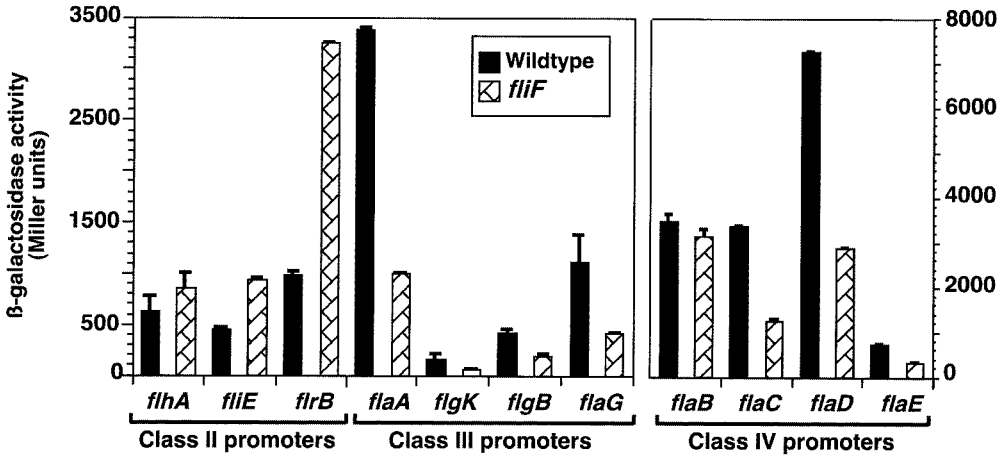

FIG. 5. Expression of class II, III, and IV flagellar promoters in a *fliF* mutant *V. cholerae* strain. *V. cholerae* strains KKV598 (wild type) and KKV1247 (*fliF* mutant) carrying plasmids pKEK72 (*flrBp-lacZ*), pKEK327 (*fliEp-lacZ*), pKEK329 (*flhAp-lacZ*), pKEK332 (*flgBp-lacZ*), pKEK331 (*flgKp-lacZ*), pKEK80 (*flaAp-lacZ*), pKEK415 (*flaGp-lacZ*), pKEK79 (*flaBp-lacZ*), pKEK76 (*flaCp-lacZ*), pKEK77 (*flaDp-lacZ*), and pKEK81 (*flaEp-lacZ*) were assayed for β-galactosidase activity during logarithmic growth in LB. Assays were performed in triplicate, and standard deviations are shown.

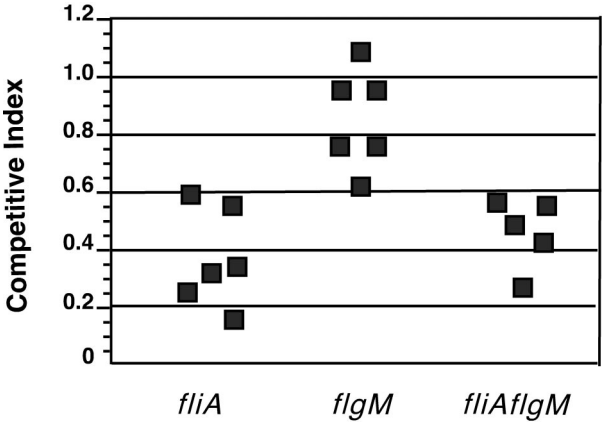

FIG. 6. Intestinal colonization of *fliA* and *flgM* mutant *V. cholerae* strains. Strains KKV1113 (*fliA* mutant), KKV1461 (*flgM* mutant), and KKV1384 (*fliA flgM* mutant) were coinoculated with O395 perorally into infant mice at a ratio of ~1:1; intestinal homogenates were recovered at 24 h postinoculation, and CFU of wild-type and mutant strains were counted. The CI is given as the output mutant/wild-type ratio divided by the input mutant/wild-type ratio; each value shown is from an individual mouse.

DISCUSSION

The control of σ^{28}-dependent transcription via anti-σ^{28} factor FlgM is a fascinating example of how temporal transcription within the bacterial cytoplasm can be controlled by the assembly of an external organelle. In *S. enterica* serovar Typhimurium, FlgM binds to σ^{28} and prevents its association with RNAP until completion of the basal-body–hook structure, at which point FlgM is secreted through the nascent flagellum (15). This allows σ^{28} holoenzyme to transcribe class III flagellar genes, the products of which are then assembled into the flagellar filament. The bulk of the filament is composed of flagellin subunits, and these are not needed until completion of the basal-body–hook structure; thus, FlgM prevents their expression until an appropriate point in flagellar assembly.

V. cholerae possesses a single polar sheathed flagellum that contains five distinct flagellin subunits; four of the flagellin genes are transcribed by σ^{28} holoenzyme, while a fifth flagellin gene is transcribed by σ^{54} holoenzyme (20). Transcription and assembly of the σ^{54}-dependent flagellin, FlaA, appear to precede transcription and assembly of the σ^{28}-dependent flagellins (35). This situation differs from that in *S. enterica* serovar Typhimurium, where there is a single flagellin gene transcribed by σ^{28} holoenzyme upon completion of the basal-body–hook structure, suggesting that control of σ^{28}-dependent transcription differs between *V. cholerae* and *S. enterica* serovar Typhimurium. Also, the *V. cholerae* flagellum, unlike those of *S. enterica* serovar Typhimurium, is covered by a sheath that may impede the secretion of nonstructural proteins, such as FlgM.

Our results demonstrate that *V. cholerae* FlgM interacts with σ^{28}, is secreted through the flagellum, can inhibit transcription of some σ^{28}-dependent promoters, and represses flagellar length; all of these functions are consistent with anti-σ^{28} activity, as initially characterized in *S. enterica* serovar Typhimurium. One would therefore anticipate that the absence of FlgM would result in increased σ^{28}-dependent transcription at all of the σ^{28}-dependent flagellin promoters, and in fact *flaD* and *flaE* transcription increased in the Δ*flgM* strain. However, *flaC* transcription actually decreased in a Δ*flgM* strain while *flaB* transcription was unaffected. Perhaps unregulated σ^{28} activity causes sequestration of a limited amount of σ^{28} holoenzyme at certain promoters (*flaD*, *flaE*), causing a decrease in transcription at other promoters (*flaC*).

It is difficult to rationalize the unresponsiveness of the *flaB* promoter to both the absence of FlgM and the absence of the MS ring. This promoter is clearly σ^{28} dependent, yet its activity is not diminished in the absence of secretion (Δ*fliF* mutant) nor increased in the absence of FlgM; thus, this promoter behaves differently than other σ^{28}- and FlgM-controlled promoters. These differences in transcriptional responsiveness of the individual flagellin genes undoubtedly lead to differences in the composition of the flagellin subunits within the flagellum under different conditions, and we predict that this leads to altered swimming behavior. Complex flagella (i.e., composed of multiple distinct flagellin subunits) are present in many bacteria with polar flagella (e.g., *Vibrio*, *Pseudomonas*, and *Campylobacter* spp., etc.), but no studies have been performed yet that link the flagellin composition under different conditions to specific attributes of flagellar performance, such as rotation rate, speed, etc.

The lack of the MS ring in *V. cholerae* (Δ*fliF* mutant) led to general decreases in class III and IV flagellar transcription. We have already shown that transcription of class III genes is dependent on phosphorylation of FlrC (encoded by a class II gene) (2, 21), which suggests that the lack of secretion and/or lack of the MS ring inhibits phosphorylation of FlrC. Although FlrB, the kinase that phosphorylates FlrC, has been identified (2), it is not clear what modulates FlrB activity; these results suggest that it may be either an early structural component or a functional secretion apparatus. Transcription of the analogous *C. jejuni* class III genes, which are regulated by a FlrC homologue, FlgR, have been shown to depend upon the formation of the flagellar secretory apparatus (14), so we favor a similar functional, rather than structural, checkpoint in *V. cholerae*.

Although a number of bacteria possess sheathed flagella (e.g., *Vibrio* and *Helicobacter* spp.), very little is known about the sheath itself. The sheath is hypothesized to mask the proinflammatory flagellin subunits from the host immune system (28). Limited studies of *V. cholerae* demonstrated that the sheath possesses lipopolysaccharide, indicating that it is contiguous with the outer membrane (7). Additional studies of *V. alginolyticus* and *V. anguillarum* have identified a specific protein and lipopolysaccharide associated with the flagellar sheath in these species (8, 33). Analysis of mutations in hook-associated proteins that connect the flagellin filaments to the basal body in *V. parahaemolyticus* suggested that the sheath acts as a barrier to the secretion of flagellin subunits to the extracellular milieu (29), raising the question of the competence of the sheathed *Vibrio* flagellum to secrete FlgM (28).

Our studies demonstrate that *V. cholerae* FlgM is secreted through the sheathed flagellum, thus opening up the possibility that other nonstructural proteins could also be secreted through the flagellar export machinery. Type III secretion systems (TTSS) are frequently used by pathogenic bacteria to alter host physiology during infection, but the only TTSS present in the *V. cholerae* genome is the flagellar export appa-

ratus (13). However, *Yersinia enterocolitica* is known to use the flagellar TTSS to secrete a virulence-associated phospholipase (43). Various studies have linked motility and flagellar synthesis to aspects of *V. cholerae* virulence (2, 5, 9, 26, 36, 40), but the phenomena are frequently strain and animal model dependent so it has been difficult to elucidate the contribution of the flagellum to cholera pathogenesis. The studies presented here demonstrate that the lack of σ^{28} and/or FlgM has a modest impact on the ability of a classical *V. cholerae* strain (O395) to colonize the infant mouse intestine; this animal model has been useful in predicting colonization behavior in humans (19). The effect of these mutations in other *V. cholerae* strains and on other aspects of virulence (intoxication, dissemination, reactogenicity, etc.) may be more profound.

ACKNOWLEDGMENTS

We thank Kelly Hughes for helpful discussions.
This work was supported by NIH AI43486 to K.E.K.

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