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Differential Regulation of Multiple Flagellins in *Vibrio cholerae*

KARL E. KLOSE[1]† AND JOHN J. MEKALANOS[1,2]*

Department of Microbiology and Molecular Genetics[1] and Shipley Institute of Medicine,[2] Harvard Medical School, Boston, Massachusetts 02115

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***Vibrio cholerae*, the causative agent of the human diarrheal disease cholera, is a motile bacterium with a single polar flagellum. Motility has been implicated as a virulence determinant in some animal models of cholera, but the relationship between motility and virulence has not yet been clearly defined. We have begun to define the regulatory circuitry controlling motility. We have identified five *V. cholerae* flagellin genes, arranged in two chromosomal loci, *flaAC* and *flaEDB*; all five genes have their own promoters. The predicted gene products have a high degree of homology to each other. A strain containing a single mutation in *flaA* is nonmotile and lacks a flagellum, while strains containing multiple mutations in the other four flagellin genes, including a *flaCEDB* strain, remain motile. Measurement of *fla* promoter-*lacZ* fusions reveals that all five flagellin promoters are transcribed at high levels in both wild-type and *flaA* strains. Measurement of the flagellin promoter-*lacZ* fusions in *Salmonella typhimurium* indicates that the promoter for *flaA* is transcribed by the σ^{54} holoenzyme form of RNA polymerase while the *flaE*, *flaD*, and *flaB* promoters are transcribed by the σ^{28} holoenzyme. These results reveal that the *V. cholerae* flagellum is a complex structure with multiple flagellin subunits including FlaA, which is essential for flagellar synthesis and is differentially regulated from the other flagellins.**

Cholera is a life-threatening diarrheal disease caused by *Vibrio cholerae*, a gram-negative curved rod that is highly motile by means of a single sheathed polar flagellum. The organism enters the host through the ingestion of contaminated food or water. Once in the intestine, *V. cholerae* swims toward and penetrates the mucus gel lining of the small intestine, eventually adhering to the apical surface of the intestinal epithelial cells (19). Adherent bacteria produce cholera toxin (CT), which activates adenylate cyclase in host epithelial cells, which in turn leads to the profuse watery diarrhea that is the hallmark of this disease (30, 37).

A number of virulence factors are coordinately regulated by the action of ToxR, a transcriptional regulatory protein implicated in the control of CT expression (40, 41). ToxR is known to activate expression of ToxT, a second transcriptional regulator (8), which activates the expression of both CT and the toxin-coregulated pilus (TCP), the primary intestinal colonization factor of *V. cholerae* (54). Laboratory conditions that stimulate ToxR-dependent expression of CT and TCP have been elucidated, but the true in vivo environmental conditions that influence virulence factor production are not known. Clearly, environmental cues present during the course of infection stimulate virulence factor production.

Motility has been identified as a virulence determinant of *V. cholerae*. Nonmotile mutants have been shown to be defective for adherence to isolated rabbit brush borders (13) and to cause less fluid accumulation in rabbit ligated ileal loops and less disease in the rabbit RITARD model (47); however, these observations are greatly influenced by changes in the particular biotype or mutant strain of *V. cholerae* evaluated and also by the animal model used. Interestingly, compared to isogenic motile strains, nonmotile mutants of live attenuated *V. cholerae* vaccines show reduced reactogenicity in humans while maintaining their ability to colonize the intestine (7, 24). Further, nonmotile mutants show no significant defect in their ability to colonize the infant mouse small intestine in competition assays (14, 47), a widely used model system that has accurately predicted the colonization properties of live attenuated cholera vaccines. Recently, genetic studies have suggested that virulence factor production and motility phenotypes are related. For example, some nonmotile mutants express higher levels of CT and TCP than wild-type strains do under noninducing laboratory conditions, while other "hyperswarming" mutants express little or no CT or TCP under inducing laboratory conditions (14) (the nature of hyperswarming, which is characterized by large swarm sizes in motility agar, remains to be determined). A *toxR* mutant has a similar hyperswarmer phenotype, perhaps indicative of a negative regulatory role for ToxR in motility. Bile has been shown to stimulate *V. cholerae* motility while simultaneously decreasing CT production in a ToxR-independent manner (16), indicating that other factors may contribute to the relationship of virulence and motility. Mutations affecting motility can also alter *V. cholerae* protease production and adherence to cultured cells (14). However, with the exception of *motB* mutants, these studies were performed with *V. cholerae* strains carrying unidentified motility mutations. Thus, the exact connection between motility and virulence gene expression has remained elusive.

Studies of the motility of two closely related *Vibrio* species, the human pathogen *V. parahaemolyticus* and the fish pathogen *V. anguillarum*, have revealed that these organisms have a polar flagellum composed of multiple flagellin subunits (33, 34). Mutations in several of the flagellin genes of *V. anguillarum*, although not causing significant motility defects, lead to significant defects in virulence, even after direct inoculation into the host (34, 42). This indicates that some of the flagellins may play additional roles in virulence not directly related to motility.

In the present study, we have identified and characterized multiple flagellin genes in *V. cholerae*. Our results reveal that the flagellum of *V. cholerae*, like those of *V. parahaemolyticus*

* Corresponding author. Mailing address: Department of Microbiology and Molecular Genetics, Harvard Medical School, 200 Longwood Ave., Boston, MA 02115. Phone: (617) 432-2263. Fax: (617) 738-7664. E-mail: jmekalan@warren.med.harvard.edu.

† Present address: Department of Microbiology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78284-7758.

and *V. anguillarum*, is composed of multiple flagellin subunits. We have identified a "core" flagellin essential for flagellar synthesis and have found that it is differentially regulated from the other flagellins; namely, its expression is controlled by RNA polymerase containing the alternate sigma factor σ^{54}.

MATERIALS AND METHODS

Media. Luria broth (LB) in both liquid medium and agar plates was routinely supplemented with 2 mM glutamine and supplemented with antibiotics when appropriate. Agar plates consisting of LB with 0.3% agar and 2 mM glutamine were used to measure motility. Evans blue-uranine indicator plates (31) supplemented with 2 mM glutamine were used to purify all constructed *Salmonella typhimurium* strains free of P22 phage. LB agar, made without NaCl and supplemented with 10% sucrose, was used to select for second recombinational events during construction of chromosomal deletions and insertions with vectors containing the *sacB* gene (see below).

Oligonucleotides and PCR. Degenerate oligonucleotide primers based on conserved amino acid sequences of flagellin genes from multiple bacteria were used for PCR amplification of *V. cholerae* flagellin genes. The primers used were FLAX1 (GCGGATCCTCNATGGARCGNYTNTCNTC) and FLAX2 (GCGAATTCRTTNATRTANGTNGCNARCT), where N represents any nucleotide, R represents any purine, Y represents any pyrimidine, corresponding to the conserved amino acid sequences SMERLSS and ELATYIN, respectively; the underlined nucleotides represent restriction sites for *Bam*HI and *Eco*RI. Degenerate oligonucleotide primers based on conserved amino acid sequences of putative GTP-binding proteins homologous to ORF1 from *V. anguillarum* (34) were used for PCR amplification of an internal fragment of a putative *V. cholerae* GTP-binding protein. The primers used were ORF1-1 (GCGAAGCTTTTRACNTTRAARCCNACNATG) and ORF1-2 (GCGAATTCTTTNCCNGCYTCYTTNGCNCC), corresponding to the conserved amino acid sequences LTLKPTM and GAKEAGK, respectively; the underlined nucleotides represent restriction sites for *Hin*dIII and *Eco*RI. PCR with degenerate primers was performed for 30 cycles of 45 s at 92°C, 1 min at 42°C, and 2 min 30 s at 72°C with TaqPlus DNA polymerase (Stratagene). Two fragments of approximately 600 bp and 2 kb in length were produced from *V. cholerae* Classical O1 strain O395 with the FLAX primer pair; the smaller fragment corresponded to the coding sequence for amino acids 28 to 227 of FlaA, and the larger fragment corresponded to the coding sequences of amino acids 28 to 377 of FlaD, the *flaD-flaB* intergenic region, and amino acids 1 to 226 of FlaB. One fragment of approximately 450 bp was produced with the ORF1 primer pair corresponding to the equivalent coding sequence of amino acids 197 to 341 in the *Haemophilus influenzae* homolog HI0393 (12).

We cloned the *flaAC* locus by amplifying overlapping PCR fragments. The oligonucleotides used to amplify the carboxyl-terminal coding region of *flgL*, the *flgL-flaA* intergenic region, and the amino-terminal coding region of *flaA*, were FLGLD1 (GCTCTAGAGGCTATCAGTTAGAGCGTAA) (based on the *V. cholerae flgL* sequence kindly provided by S. Mel; this primes immediately upstream of the *flaAC* locus sequence reported here) and FLAAU1 (GCGAATTCCATCGCACCTTCTGCGGTTTG) (corresponding to amino acids 76 to 82 of FlaA); the underlined nucleotides correspond to restriction sites for *Xba*I and *Eco*RI, respectively. The oligonucleotides used to amplify the carboxyl-terminal coding region of *flaA*, the *flaA-flaC* intergenic region, and the amino-terminal coding region for *flaB* were FLAAD1 (GCGAATTCCGCATGGGTGGCCAATCCTTT) (corresponding to amino acids 170 to 176 of FlaA) and FLAX2 (see above; this primed to the ELATYIN coding sequence in FlaC); the underlined sequence represents a restriction site for *Eco*RI. The oligonucleotides used to amplify the carboxyl-terminal coding region of *flaC* were FLACD1 (GCGAATTCGCTGACCGTGTTGCGATTCAAG) (corresponding to amino acids 107 to 113 of FlaC) and ORF1D1 (GCGAAGCTTTACAATGACTACATCCAATTC) (based on the deduced sequence of the *V. cholerae* putative GTP-binding protein ORF1 sequence [see above]); the underlined nucleotides correspond to restriction sites for *Eco*RI and *Hin*dIII, respectively. This oligonucleotide spuriously primed to a sequence downstream of IS*1004*.

The oligonucleotide primer pairs used to amplify internal fragments of *flaB*, *flaC*, *flaD*, and *flaE* were FLAB1 (GCGAATTCCGCGCGATTCAAGAAGAAGTG) and FLAB2 (GCAAGCTTTAAGTCGTCACCTTGTTTGGC) (amplifying a fragment corresponding to amino acids 110 to 219 of FlaB with restriction sites for *Eco*RI and *Hin*dIII, respectively), FLAC1 (GCGGATCCATGGCGGTGAATGTAAACAC) and FLAC2 (GCGAATTCACGATTACGCTCATCATTCAA) (amplifying a fragment corresponding to amino acids 1 to 125 of FlaC with restriction sites for *Bam*HI and *Eco*RI, respectively), FLAD1 (GCGGATTCTCAATGGAGCGTCTATCTTCA) and FLAD2 (GCGAATTCGTCAGCACCGATTTGGAACGA) (amplifying a fragment corresponding to amino acids 28 to 152 of FlaD with restriction sites for *Bam*HI and *Eco*RI, respectively), and FLAE1 (GCGGATCCTGGTGAAGCCGCACAGCTTT) and FLAE2 (GCGAATTCTCAATCACCGAGACGGCGCG) (amplifying a fragment corresponding to amino acids 154 to 297 of FlaE with restriction sites for *Bam*HI and *Eco*RI, respectively). The oligonucleotides used to amplify a second internal fragment of *flaD* were FLAD3 (GCGAATTCACCAATGCACAACAAACTTCA) and FLAD4 (GCAAGCTTGTCAGCACCGATTTGGAACGA) (amplifying a fragment corresponding to amino acids 22 to 152 of FlaD with restriction sites for *Eco*RI and *Hin*dIII, respectively). The oligonucleotides used to amplify the entire *flaA* gene were FLGL1 (see above) and FLAAU1 (GCGGATCCGTACCGTGAACTACTGCAATAAC) (amplifying a fragment corresponding to nucleotides 1 through 2010 of the reported *flaAC* locus sequence flanked with restriction sites for *Xba*I and *Bam*HI). The oligonucleotides used to amplify the carboxyl terminus of FlaA as well as the *flaA* to *flaC* intergenic region for the construction of the Δ*flaA1* allele were FLAA1 (GCGAATTCTCTGCAATCTCGTTATTGC) and FLAC3 (GCGGTACCTGCAAAAGAGGTGGTTTCAG), corresponding to nucleotides 1977 to 2953 of the *flaAC* locus sequence with restriction sites for *Eco*RI and *Kpn*I, respectively. The oligonucleotides used to amplify the aminoglycoside 3′-phosphotransferase (Kan^r) gene from pACYC177 (48) were KAN2 (GCCAATTGCAACTCAGCAAAAGTTCGAT) and KAN3 (GCCAATTGAACGGTCTGCGTTGTCGGGA), corresponding to nucleotides 1783 to 2872 of the published pACYC177 sequence; the underlined nucleotides represent restriction sites for *Mfe*I.

The promoter region of each flagellin gene was PCR amplified with oligonucleotide primer pairs which contained *Xba*I and *Bgl*II sites to orient the promoters with respect to the *lacZYA* genes in the fusion vector. The oligonucleotide pairs used were FLGL1 (see above) and FLAAP1 (GCAGATCTCGATTCATTCATCGCACCT) for *flaA*p (corresponding to nucleotides 1 to 1115 of the *flaAC* locus sequence), FLACP1 (GCTCTAGACAGTTGCCAAACTCTGCAAT) and FLACP2 (GCAGATCTGTGTTTACATTCACCGCCAT) for *flaC*p (corresponding to nucleotides 1965 to 2573 of the *flaAC* locus sequence), FLAEP1 (GCTCTAGAAAGCTTGATTCCGTCGAGCT) and FLAEP2 (GCAGATCTCTCCAGAGACTGATTGAGCAT) for *flaE*p (corresponding to nucleotides 1 to 579 of the *flaEDB* locus sequence), FLADP1 (GCTCTAGATCTGTGCTCGCTCAAGCGAA) and FLADP2 (GCAGATCTACTATTGATTTTAAAGCCTG) for *flaD*p (corresponding to nucleotides 1567 to 2035 of the *flaEDB* locus sequence), and FLABP1 (GCTCTAGAATCAAGGACACCGATTTCGCG) and FLABP2 (GCAGATCTCGTGTTTACATTAATTGCCAT) for *flaB*p (corresponding to nucleotides 2921 to 3305 of the *flaEDB* locus sequence).

We have identified a *V. cholerae* gene encoding a σ^{54} activator, *flrA*; the sequence and characterization of this gene will be presented elsewhere (25). For the purposes of the present study, we wished to control overexpression of this protein by a translational fusion to the arabinose-inducible promoter, P_{BAD}. PCR amplification of the *flrA* gene was performed with oligonucleotides FLRAMET (ATGCAGAGTTTAGCGAAACTA) (the underlined nucleotides are the initiating methionine codon) and FLRAU1 (GCGAAGCTTTGGGTTGGCTTCACGCACTA) (the underlined sequence represents a *Hin*dIII restriction site); these primers amplify a 1.7-kbp fragment which contains the entire *flrA* gene and extends partially into the downstream gene, *flrB*.

PCR with specific primers and *V. cholerae* O395 chromosomal DNA was performed for 30 cycles of 45 s at 92°C, 1 min at 50°C, and 1 min 30 s at 72°C with Vent DNA polymerase (New England Biolabs). For some reactions, the extension time was increased to 2 min 30 s at 72°C. For amplification with FLACD1 and ORF1D1 (see above), the annealing temperature was reduced to 42°C.

Plasmid construction. PCR-amplified fragments obtained from the FLAX12 primers containing flagellin genes from the *V. cholerae* Classical strain O395 (see above) were digested with *Bam*HI and *Eco*RI and ligated into pBR322 (56) that had been similarly digested, giving plasmids pKEK23 (internal fragment of *flaA*) and pKEK24 (*′flaD-flaB′* fragment). These were subsequently used for sequencing (see below).

The Δ(*flaD-B*)*1*::Cm^r mutation was constructed in several steps. The PCR-amplified internal fragment from *flaD* (FLAD12 [see above]) was digested with *Eco*RI and *Bam*HI and ligated into pWSK30 (55) that had been similarly digested to form pKEK30. The PCR-amplified internal fragment from *flaB* (FLAB12 [see above]) was digested with *Hin*dIII and *Eco*RI and ligated into pKEK30 that had been similarly digested, to form pKEK31, which thus forms the Δ(*flaD-B*)*1* deletion, which removes the coding sequences corresponding to amino acids 153 to 377 of FlaD and 1 to 109 of FlaB, as well as the *flaD-flaB* intergenic region. pKEK31 was then digested with *Eco*RI and ligated with a 1.05-kbp *Mfe*I-digested PCR-derived chloramphenicol acetyltransferase (Cm^r) gene fragment from pACYC184 (49), which has been described previously (CAT12) (26), to produce pKEK32, which carries Δ(*flaD-B*)*1*::Cm^r. This mutation was PCR amplified with primers FLAD1 and FLAB2, and the resulting fragment was ligated into pCVD422 (9) that had been digested with *Sma*I, resulting in pKEK33. This mutation was integrated into the *V. cholerae* O395 chromosome as described below. Chromosomal DNA from the resultant strain KKV23 was digested to completion with *Hin*dIII, and ligated into the *Hin*dIII site of pWSK30 (55); selection for a Cm^r transformant resulted in the isolation of pKEK52, which contains a ~15-kbp chromosomal fragment that carries Δ(*flaD-B*)*1*::Cm^r. This chromosomal fragment was digested with *Eco*RI and *Hin*dIII, and the resulting subclones were ligated into pBR322 (56) that had been digested with *Eco*RI and/or *Hin*dIII. One of the resulting plasmids, pKEK65, contains a 4-kbp *Hin*dIII-*Eco*RI fragment that encodes the complete *flaE* gene, the 5′ coding region of *flaD*, and a portion of Cm^r (the Cm^r gene contains a restriction site for *Eco*RI [49]). Another resulting plasmid was pKEK66, which contains a 2.5-kbp *Eco*RI fragment that includes the other portion of Cm^r and the 3′ coding region of *flaB* as well as the coding sequence for *flaG*. These plasmids were used to sequence the *flaEDB* locus (see below).

The Δ(*flaE-D*)*1*::Kan^r mutation was constructed in several steps. The PCR-

TABLE 1. Strains used in this study

Strain	Genotype	Source or reference
V. cholerae		
O395	Wild type (Classical Ogawa)	37
CG842	O395, Δ*lacZ*	14
KKV6	*flaE1*::pGP704	This study
KKV7	*flaD1*::pGP704	This study
KKV8	Δ(*flaE-D*)*1*::Kan^r	This study
KKV12	*flaA1*::pGP704	This study
KKV22	*flaB1*::pGP704	This study
KKV23	Δ(*flaD-B*)*1*::Cm^r	This study
KKV34	Δ(*flaE-D*)*1*::Kan^r, Δ(*flaD-B*)*1*::Cm^r	This study
KKV62	Δ*toxR1*, Δ*lacZ*	This study
KKV90	Δ*flaA1*::Cm^r; Δ*lacZ*	This study
KKV171	*flaC1*::pGP704	This study
KKV172	*flaC1*::pGP704, Δ(*flaE-D*)*1*::Kan^r	This study
KKV173	*flaC1*::pGP704, Δ(*flaD-B*)*1*::Cm^r	This study
KKV174	*flaC1*::pGP704, Δ(*flaE-D*)*1*::Kan^r, Δ(*flaD-B*)*1*::Cm^r	This study
S. typhimurium		
14028	Wild type	American Type Culture Collection
KK1	*ntrA209*::Tn*10*	26
KK80	Δ(*ntrB-C*)*1*	26
KK105	*fliA5059*::Tn*10*dTc	P22.TH1479 × 14028
KK140	*putPA1303*[Kan^r-'*lacZYA*]	26
KK156	*putPA1303*[Kan^r-*flaD*p-'*lacZYA*]	This study
KK157	*ntrA209*::Tn*10*, *putPA1303*[Kan^r-*flaD*p-'*lacZYA*]	P22.KK156 × KK1
KK158	*fliA5059*::Tn*10*dTc, *putPA1303* [Kan^r-*flaD*p-'*lacZYA*]	P22.KK156 × KK105
KK159	*putPA1303*[Kan^r-*flaB*p-'*lacZYA*]	This study
KK160	*ntrA209*::Tn*10*, *putPA1303*[Kan^r-*flaB*p-'*lacZYA*]	P22.KK159 × KK1
KK161	*fliA5059*::Tn*10*dTc, *putPA1303* [Kan^r-*flaD*p-'*lacZYA*]	P22.KK159 × KK105
KK164	*putPA1303*[Kan^r-*flaA*p-'*lacZYA*]	This study
KK165	*ntrA209*::Tn*10*, *putPA1303*[Kan^r-*flaA*p-'*lacZYA*]	P22.KK164 × KK1
KK166	*fliA5059*::Tn*10*dTc, *putPA1303* [Kan^r-*flaA*p-'*lacZYA*]	P22.KK164 × KK105
KK167	*putPA1303*[Kan^r-*flaE*p-'*lacZYA*]	This study
KK168	*ntrA209*::Tn*10*, *putPA1303*[Kan^r-*flaE*p-'*lacZYA*]	P22.KK167 × KK1
KK169	*fliA5059*::Tn*10*dTc, *putPA1303* [Kan^r-*flaE*p-'*lacZYA*]	P22.KK167 × KK105
KK173	*putPA1303*[Kan^r-*flaC*p-'*lacZYA*]	This study
KK174	*ntrA209*::Tn*10*, *putPA1303*[Kan^r-*flaC*p-'*lacZYA*]	P22.KK173 × KK1
KK175	*fliA5059*::Tn*10*dTc, *putPA1303*[Kan^r-*flaC*p-'*lacZYA*]	P22.KK173 × KK105
KK188	Δ(*ntrB-C*)*1*, *putPA1303*[Kan^r-*glnA*p-'*lacZYA*]	P22.SK3041 × KK80
KK189	Δ(*ntrB-C*)*1*; *ntrA209*::Tn*10*, *putPA1303*[Kan^r-*glnA*p-'*lacZYA*]	P22.SK284 × KK188
SK284	*ntrA209*::Tn*10*, *hisF645*	27
SK3041	*putPA1303*[Kan^r-*glnA*p-'*lacZYA*]	23
TH1479	*fliA5059*::Tn*10*dTc	K. Hughes

derived internal fragment of *flaE* (FLAE12 [see above]) was digested with *Eco*RI and *Bam*HI and ligated into pWSK30 (55) that had been similarly digested, to give pKEK19. The PCR-derived internal fragment of *flaD* (FLAD34 [see above]) was digested with *Hin*dIII and *Eco*RI and then ligated into pKEK19 that had been similarly digested, resulting in pKEK20, which thus contains Δ(*flaE-D*)*1*; this mutation removes coding sequences corresponding to amino acids 296 to 378 of FlaE and 1 to 21 of FlaD, as well as the *flaED* intergenic region. The Kan^r PCR-derived fragment (KAN23 [see above]) was digested with *Mfe*I and ligated into pKEK20 which had been digested with *Eco*RI, resulting in pKEK21 which carries Δ(*flaE-D*)*1*::Kan^r. This mutation was PCR amplified with FLAE1 and FLAD4 and ligated into the *Sma*I site of pCVD422 (9), resulting in pKEK22. This mutation was integrated into the *V. cholerae* chromosome as described below.

To make the Δ*flaA1*::Cm^r mutation, the PCR-derived FLAA1-FLAC3 fragment (see above) was digested with *Eco*RI and *Kpn*I and ligated into pWSK30 (55) that had been similarly digested, to form pKEK90. Then the PCR-derived internal fragment of *flaA* (pKEK23 [see above]) was digested with *Bam*HI and *Eco*RI and ligated into pKEK90 that had been similarly digested, resulting in pKEK91, which thus contains Δ*flaA1*, a deletion of amino acids 228 to 372 of FlaA. pKEK91 was then digested with *Eco*RI and ligated with the *Mfe*I-digested Cm^r fragment (CAT12 [26]) to give pKEK92, which contains Δ*flaA1*::Cm^r. pKEK92 was digested with *Bss*HII, which removes the entire Δ*flaA1*::Cm^r fragment; this fragment was made blunt ended with the Klenow fragment of DNA polymerase and ligated into pCVD442 (9) that had been digested with *Sma*I to form pKEK93, which was used to cross the mutation back onto the *V. cholerae* chromosome (see below).

Promoter-*lacZ* fusions to the five flagellin promoters were constructed by first modifying the *lacZ* fusion vector pRS551 (53) by creating unique *Xba*I and *Bgl*II restriction sites between the unique *Eco*RI and *Bam*HI restriction sites (25) to form pKEK75. The PCR-derived flagellin promoter fragments (see above) FLGL1FLAAP1 (*flaA*p), FLABP12 (*flaB*p), FLACP12 (*flaC*p), FLADP12 (*flaD*p), and FLAEP12 (*flaE*p) were digested with *Xba*I and *Bgl*II and ligated into pKEK75 that had been similarly digested, resulting in pKEK80, pKEK79, pKEK76, pKEK77, and pKEK81, respectively.

To construct the suicide vectors containing internal gene fragments, the PCR-derived internal fragments of *flaA* (FLAX12), *flaB* (FLAB12), *flaC* (FLAC12), *flaD* (FLAD12), and *flaE* (FLAE12) were digested with *Eco*RI and ligated into pGP704 (39) that had been digested with *Eco*RV and *Eco*RI, to form pKEK27, pKEK29, pKEK81, pKEK28, and pKEK88, respectively; these plasmids were used to create insertional mutations in these respective genes.

The PCR-derived fragment FLGL1-FLAAU1 (see above) containing the complete *flaA* gene was digested with *Xba*I and *Bam*HI and ligated into pACYC177 (48) that had been digested with *Nhe*I and *Bam*HI, to form pKEK89, which was used to complement a *flaA* strain (see below). The PCR fragment containing the complete *flrA* gene which was amplified with FLRAMET and FLRAU1 (see above) was digested with *Hin*dIII and ligated into pBAD24 (17) that had been digested with *Nco*I, made blunt ended with the Klenow fragment of DNA polymerase, and digested with *Hin*dIII. The resulting plasmid, pKEK94, is an in-frame translational fusion of *flrA* to an initiating methionine codon under the control of the P_{BAD} promoter.

Bacterial strains. *Escherichia coli* DH5α (18) was used for all cloning manipulations, unless the vector being used was a derivative of pGP704 (39) or pCVD442 (9), which contain the R6K origin of replication and therefore require the product of the *pir* gene for replication, in which case *E. coli* DH5αλ*pir* or SM10λ*pir* (39) was used. For construction of the flagellin promoter-*lacZ* chromosomal fusions in *S. typhimurium*, *E. coli* TE2680 and TE1335 (10) were used in intermediate steps (see below).

The *V. cholerae* and *S. typhimurium* strains used in this study are listed in Table 1. All *V. cholerae* strains used are isogenic with the O1 Classical strain O395, referred to as wild type. To construct strains containing mutations in single flagellin genes, plasmids pKEK27 (*flaA*), pKEK29 (*flaB*), pKEK81 (*flaC*), pKEK28 (*flaD*), and pKEK88 (*flaE*) were mated by conjugation from *E. coli*

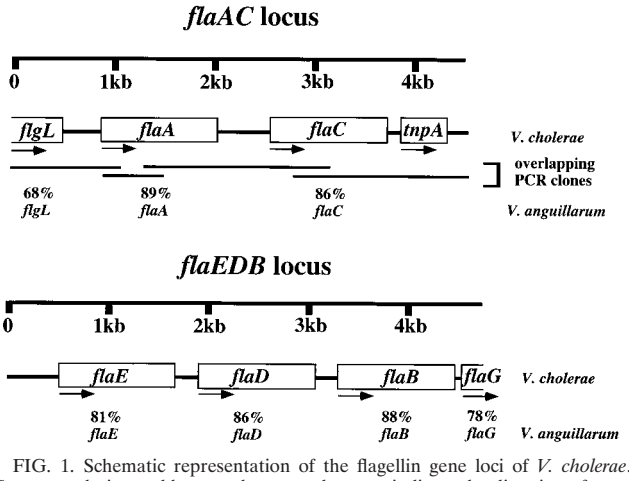

FIG. 1. Schematic representation of the flagellin gene loci of *V. cholerae*. Genes are designated by open boxes, and arrows indicate the direction of transcription. PCR-derived fragments used to sequence the *flaAC* locus (see Materials and Methods) are indicated by lines. Below each gene, the percent amino acid identity to the corresponding gene of *V. anguillarum* is indicated. The *tnpA* gene lies within an insertion sequence element, IS*1004*, which has been described only in *V. cholerae* (4).

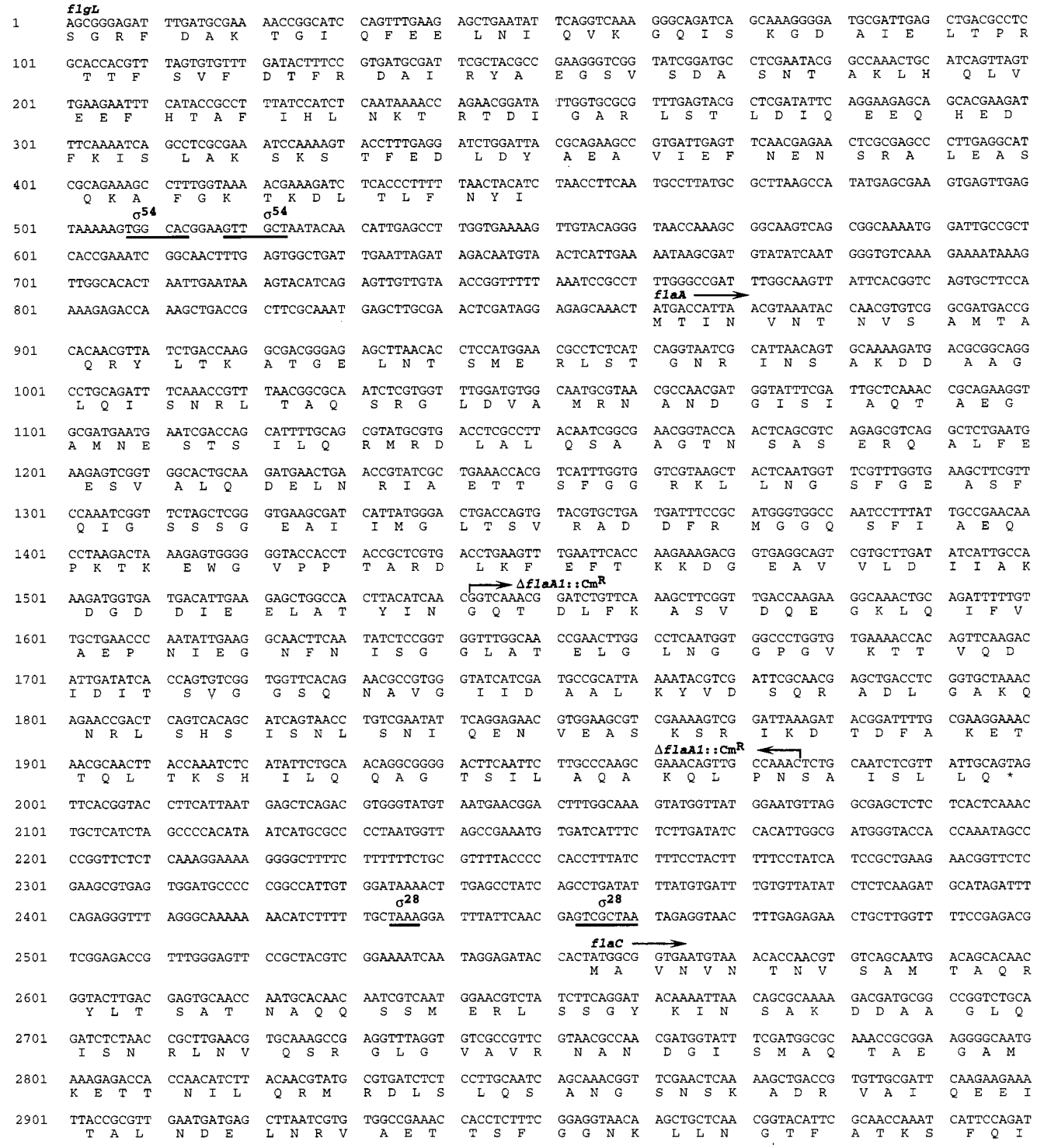

FIG. 2. Nucleotide sequences of the *flaA* and *flaC* genes. Deduced amino acid sequences encoded by the ORFs are indicated. Also included are the partial coding sequence for the *flgL* gene upstream of *flaA* and the complete coding sequence of the *tnpA* gene downstream of *flaC*; *tnpA* lies within insertion sequence element IS*1004* (4), whose boundaries are indicated by underlining of the first and last 10 bp. The putative σ^{54} promoter element in the *flaA* promoter and the putative σ^{28} promoter element in the *flaC* promoter are underlined. The boundaries of the deleted sequence of the Δ*flaA1* mutation are shown by arrows.

SM10λ*pir* into *V. cholerae* O395, selecting for streptomycin and ampicillin resistance. Since these plasmids contain internal gene fragments cloned into a suicide vector which requires the *pir* gene product for replication, the resulting strains have chromosomal insertions caused by the integration of the plasmids through homologous recombination (39). The strains formed were KKV12 [*flaA1*::pGP704 (Amp^r)], KKV22 [*flaB1*::pGP704(Amp^r)], KKV171 [*flaC1*::pGP704 (Amp^r)], KKV7 [*flaD1*::pGP704(Amp^r)], and KKV6 [*flaE1*::pGP704(Amp^r)]. pKEK81 was conjugated in a similar manner into strains KKV8, KKV23, and KKV34 to form strains KKV172, KKV173, and KKV174, respectively. Correct insertion into the target flagellin gene was confirmed by Southern blot analysis.

V. cholerae strains containing chromosomal deletions and insertions were made by the following steps: plasmids pKEK22 [Δ(*flaE-D*)*1*::Kan^r], pKEK33

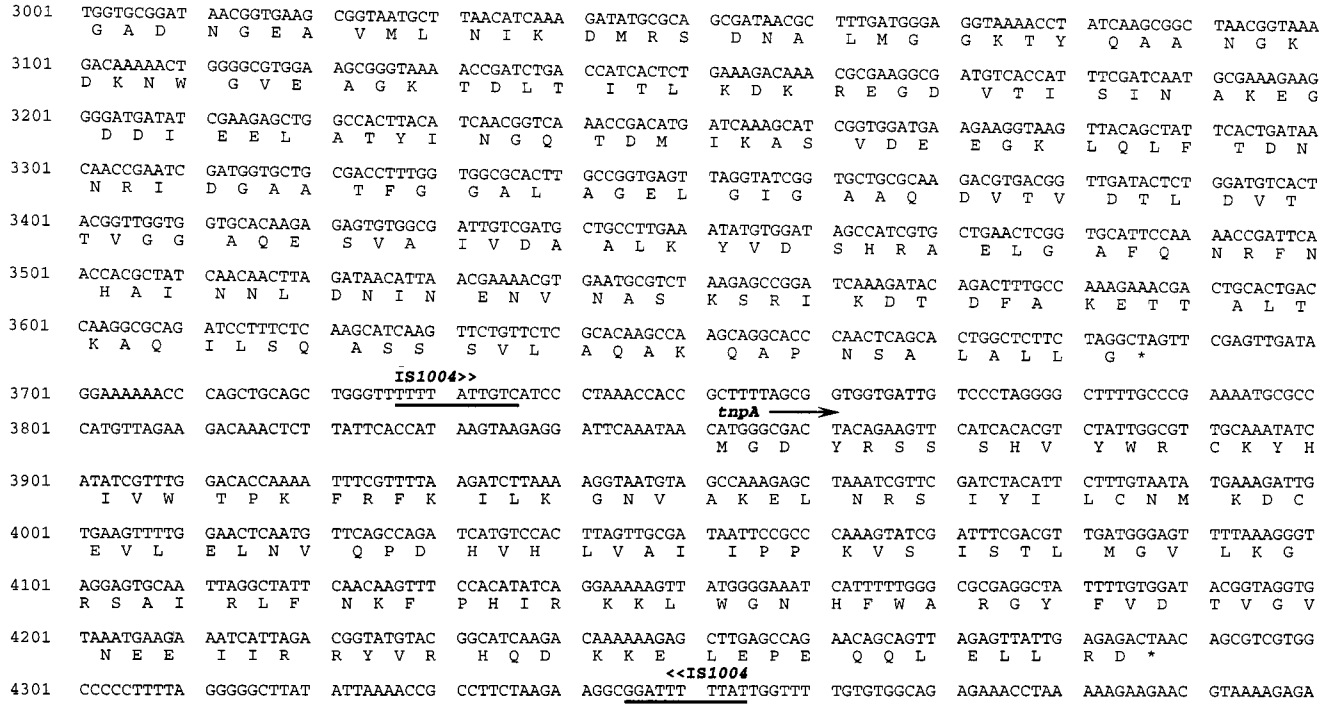

FIG. 2—*Continued.*

[Δ(*flaD-B*)*1*::Cm^r], and pKEK93 (Δ*flaA1*::Cm^r) were mated into strains O395, CG842, KKV23, or KKV34 from *E. coli* SM10λ*pir* (39) by selecting for streptomycin and ampicillin resistance. Single colonies were grown for successive generations in LB with no antibiotic selection and then plated on LB plus 10% sucrose at 30°C. The integrated plasmid contains the *sacB* gene (9), whose expression is lethal on this medium and thus selects for a second recombinational event. Sucrose-resistant colonies were tested for antibiotic resistance; Cm^r or Kan^r strains that were also Amp^s were chosen. Confirmation of correct chromosomal integration for all resultant strains was obtained either by sequencing the flanking DNA (see below) or by Southern blotting and PCR. The same procedure was used to obtain KKV62 (Δ*toxR1*); the donor plasmid pMD60 contains an in-frame deletion of *toxR* in pCVD442 (a kind gift of M. Dziejman, this mutation was derived from pVM16 [41] and removes coding sequences for amino acids 55 to 206 of ToxR [9]).

The *S. typhimurium* strains used are isogenic with ATCC 14028, also referred to as wild type. Mutant *S. typhimurium* strains were constructed with the high-transducing phage P22 HT *int*. (51), and their construction is outlined in Table 1, listing first the paternal donor upon which the P22 lysate was made and then the recipient. The integration of the *fla*p-*lacZ* chromosomal fusion cassettes inserted into the *putPA* locus has been described previously (10, 23, 26). pKEK94 was transformed into the appropriate *S. typhimurium* strains by electroporation.

Sequencing. Cycle dideoxynucleotide sequencing was carried out with an ABI sequencing kit and the ABI sequencer model 373AStretch. Both strands were sequenced for all sequences reported here. The complete nucleotide sequence of the *flaAC* locus was obtained with specific oligonucleotide primers, pKEK23, and the amplified *flaAC* PCR products (see above) (Fig. 1). The complete nucleotide sequence of the *flaEDB* locus was obtained with specific oligonucleotide primers on pKEK65, pKEK66, and pKEK24. The partial sequence of the ORF1 homolog was obtained by cloning into pTZ19U (35) and using M13 primers.

β-Galactosidase assays. *V. cholerae* strains were grown in LB supplemented with 2 mM glutamine at 37°C. *S. typhimurium* strains were grown similarly, with the addition of 0.05% arabinose. The samples were assayed at an optical density at 600 nm of approximately 0.2 to 0.4, permeabilized with chloroform and sodium dodecyl sulfate, and assayed for β-galactosidase activity by the method of Miller (38).

Electron microscopy. Strains were grown to the mid-log phase in LB 2 mM glutamine, centrifuged, and resuspended in 0.15 M NaCl. The samples were adhered to a carbon-coated grid and stained with 1% uranyl acetate before being subjected to microscopy.

Nucleotide sequence accession numbers. The sequences described above were deposited into GenBank under accession no. AF007121, AF007122, and AF007294.

RESULTS

***V. cholerae* has five flagellin genes arranged into two loci, *flaAC* and *flaEDB*.** We used degenerate oligonucleotide primers designed to recognize flagellin gene sequences to PCR-amplify two *V. cholerae* chromosomal fragments encoding partial coding sequences for the *flaA* gene and the *flaDB* genes. We constructed a deletion-insertion of the *flaDB* locus [Δ(*flaD-B*)::Cm^r] in vitro, recombined this mutation back into the *V. cholerae* chromosome (strain KKV23), and then isolated a large chromosomal fragment from this strain that conferred chloramphenicol resistance to obtain the entire *flaEDB* locus. Given the high homology of this locus to the equivalent genes from *V. anguillarum* (Fig. 1) (34), we reasoned that the *flaA* locus may be similarly homologous to that from *V. anguillarum* and were able to amplify sequences both upstream and downstream of the internal *flaA* sequence by overlapping PCR-derived fragments with primers designed to recognize a *flgL* homolog upstream of *flaA* and another flagellin gene downstream of *flaA*. Interestingly, we were able to amplify the sequence downstream of the *flaC* gene with an oligonucleotide primer specific to a *V. cholerae* putative GTP-binding protein (ORF1), which in *V. anguillarum* lies downstream of the *flaC* gene, but we amplified the corresponding fragment only with a reduced annealing temperature during PCR; the corresponding fragment revealed that the ORF1 primer annealed spuriously, and the gene immediately downstream of *flaC* is a transposase gene, *tnpA*, which lies within an insertion element IS*1004* (4).

Complete sequencing of both strands of both loci revealed open reading frames (ORFs) for five flagellin genes (Fig. 1). As stated above, these loci have significant homology to the equivalent loci from *V. anguillarum* (34), and so we have named the flagellin genes according to their counterparts in *V. anguillarum*. The complete nucleotide sequence of the *flaAC*

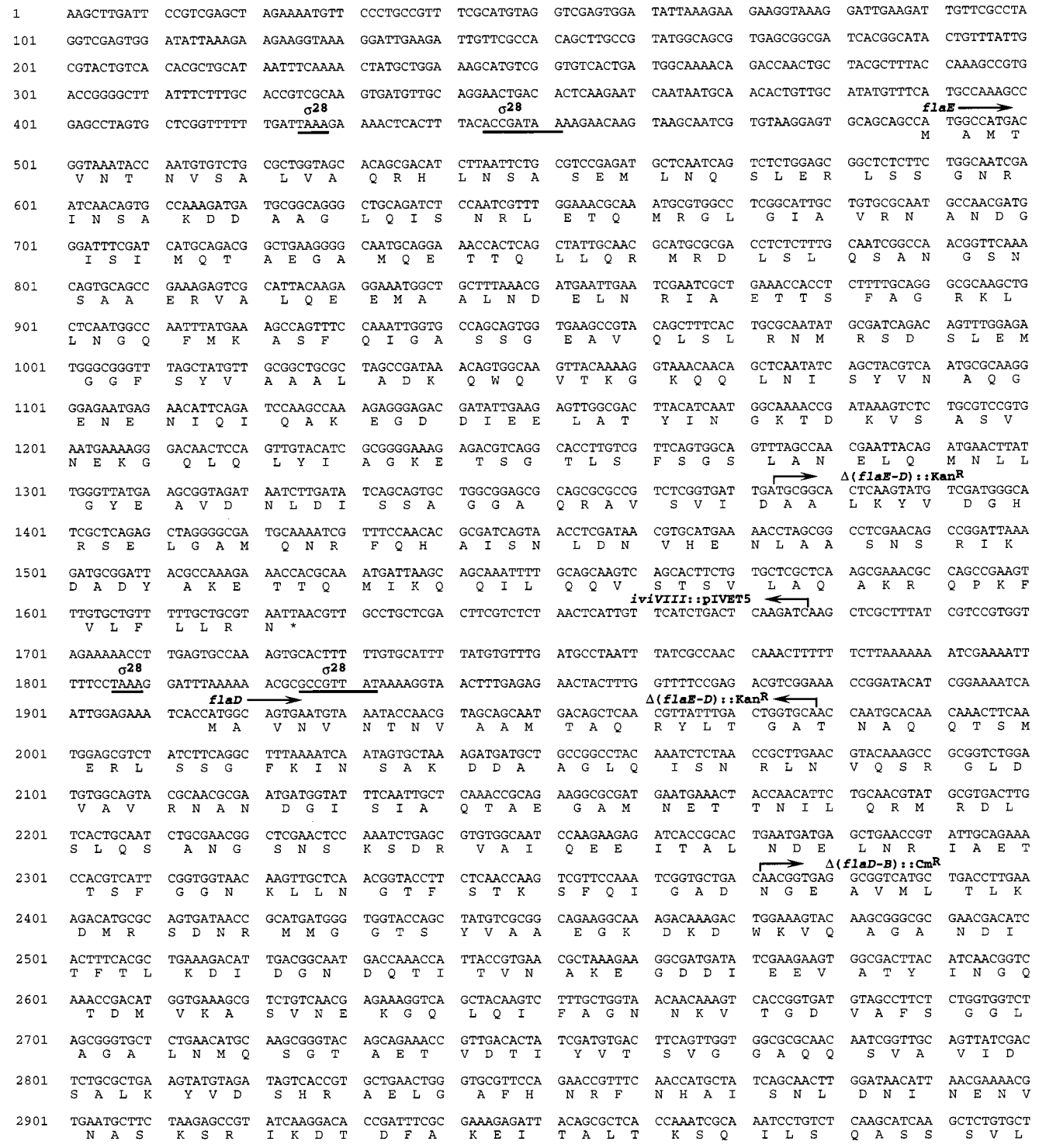

FIG. 3. Nucleotide sequences of the *flaE*, *flaD*, and *flaB* genes. Deduced amino acid sequences encoded by the ORFs are indicated. Also included is the partial coding sequence for the *flaG* gene downstream of *flaB*. The putative σ^{28} promoter elements in the *flaE*, *flaD*, and *flaB* promoters are underlined. The boundaries of the deleted sequences of the Δ(*flaE-D*)*1* and the Δ(*flaD-B*)*1* mutations are shown by arrows. The site of fusion *iviVIII*::pIVET5 (6), which detected an antisense transcript induced during infection, is shown by an arrow.

locus is shown in Fig. 2. It encodes two flagellin genes arranged in tandem. Upstream of *flaA*, a gene coding for a hook-associated protein (*flgL*) is located; the predicted protein product of the portion we sequenced shows 68% identity to *flgL* of *V. anguillarum* (which is similarly situated upstream of *flaA* in this organism) and 26% identity to that of *E. coli*. The predicted *flaA* and *flaC* gene products have homology to a large number of bacterial flagellin genes and are most homologous to the

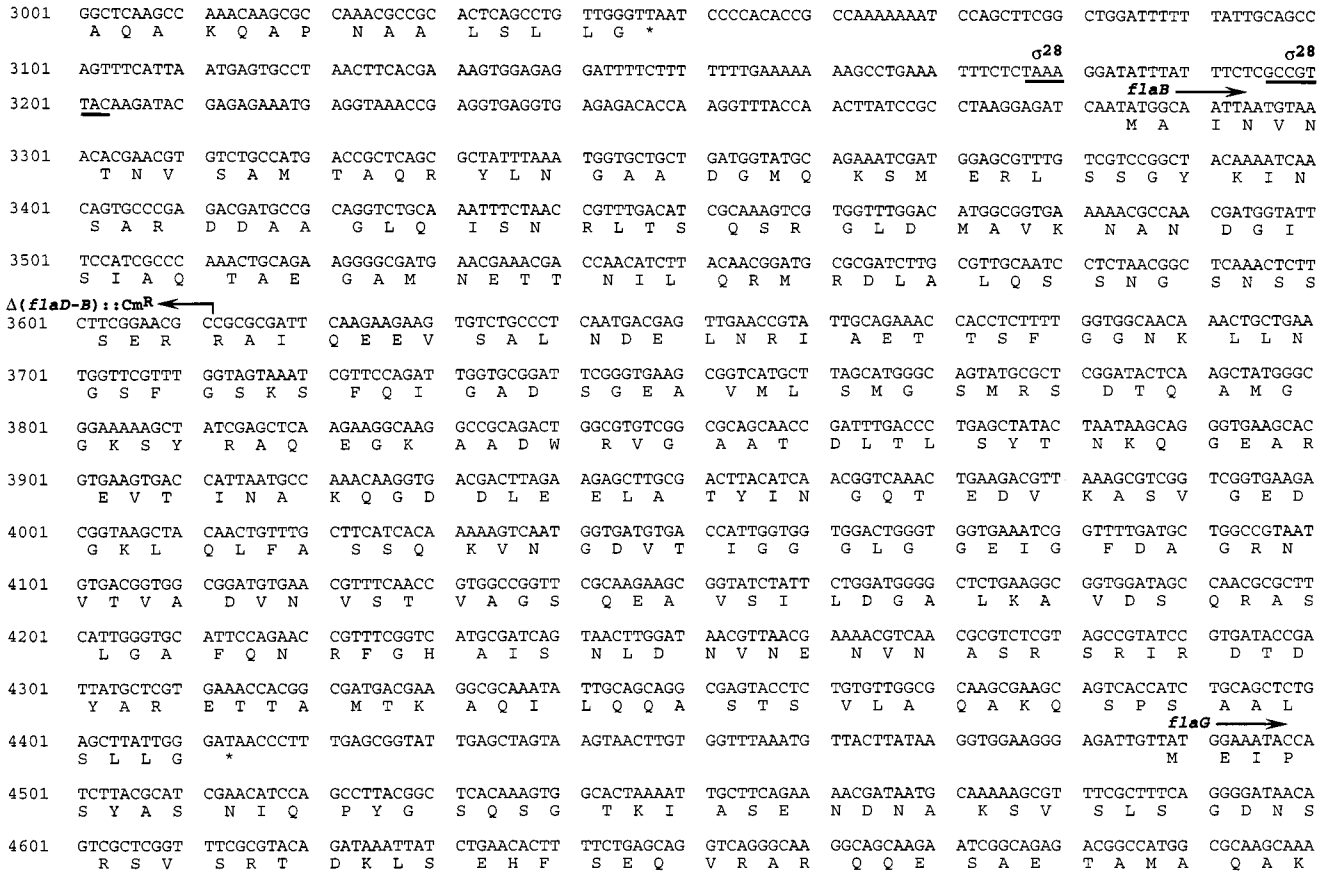

FIG. 3—*Continued.*

corresponding *flaA* and *flaC* gene products from *V. anguillarum* (89 and 86% identity, respectively). As stated above, downstream of *flaC* lies an insertion element, IS*1004*, which contains a transposase gene, *tnpA*. IS*1004* has been detected and characterized only in *V. cholerae* (4); the gene encoding the putative GTP-binding protein is located in the corresponding location in *V. anguillarum*.

The complete nucleotide sequence of the *flaEDB* locus is given in Fig. 3. This locus contains three flagellin genes arranged in tandem. Our sequence upstream of *flaE* extends to the *Hin*dIII site used to clone this fragment, which lies approximately 490 bp upstream of the start of translation, and this portion of the sequence did not reveal any open reading frames with significant homology to known genes. The predicted *flaE*, *flaD*, and *flaB* gene products have significant homology to numerous bacterial flagellins; they show the greatest homology to the *flaE flaD* and *flaB* gene products from *V. anguillarum* (81, 86, and 88% identity, respectively). Located downstream of *flaB* is an open reading frame encoding a homolog of the *flaG* gene product of *V. anguillarum* and *V. parahaemolyticus* (highest homology to the *V. anguillarum* FlaG, 78% identity in the portion we sequenced); these gene products have no known function.

The *V. cholerae* flagellins are homologous to each other. The predicted gene products of the five flagellin genes are similar in amino acid length (376 to 379 amino acids) and have calculated molecular masses of 40.4 kDa (FlaA), 39.5 kDa (FlaB), 39.9 kDa (FlaC), 39.9 kDa (FlaD), and 41.0 kDa (FlaE); therefore, they are predicted to be very similar in molecular mass. The gene products are highly homologous to each other (Fig. 4; Table 2), ranging from 61 to 82% identity. The predicted amino acid sequences have the highest homology at their amino and carboxyl termini.

Because the flagellin genes code for a structural subunit of the flagellar filament, and because every bacterial flagellin studied thus far is located within the flagellum, we assumed that all five *V. cholerae* flagellin gene products are located within the flagellum. In fact, the highly homologous flagellin gene products FlaA, FlaB, FlaC, and FlaD from *V. anguillarum* were shown to be located within the flagellum (34). We were able to identify a single dominant species corresponding to an approximate molecular mass of 40 kDa in partially purified *V. cholerae* flagellar preparations separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). We assume that this band corresponds to the flagellin gene products, but a monoclonal antibody which cross-reacts with bacterial flagellins from the family *Enterobacteriaciae* (11) did not recognize the *V. cholerae* flagellins in a Western blot, and we were unable to obtain any separation between the five flagellins.

Only *flaA* is essential for motility. We constructed *V. cholerae* strains containing chromosomal mutations in the various flagellin genes to assess their function (Table 1). A single mutation in *flaA* (KKV90 [Fig. 5] and KKV12 [data not shown]) results in a non-motile phenotype as assessed by swarm size in soft agar. The motility of the *flaA* strain KKV90 is recovered by complementation with a plasmid containing the entire *flaA* gene (pKEK89 [Fig. 5]). Single insertion mutations in *flaB*

```
        1                                                  50                                                 100
FlaA:   MtiNVNTNVS AMTAQRYLTk ATgelntSME RLSSGnrINS AKDDAAGLQI SNRLtaQSRG LDVAmRNAND GISIAQTAEG AMNEsTsILQ RMRDLaLQSA
FlaC:   MAvNVNTNVS AMTAQRYLTs ATnaqQsSME RLSSGyKINS AKDDAAGLQI SNRLnvQSRG LgVAVRNAND GISmAQTAEG AMkETTNILQ RMRDLSLQSA
FlaE:   MAmtVNTNVS AlvAQRhLns AsemlnqSlE RLSSGnrINS AKDDAAGLQI SNRLetQmRG LgiAVRNAND GISImQTAEG AMqETTqlLQ RMRDLSLQSA
FlaD:   MAvNVNTNVa AMTAQRYLTg ATnaqQtSME RLSSGfKINS AKDDAAGLQI SNRLnvQSRG LDVAVRNAND GISIAQTAEG AMNETTNILQ RMRDLSLQSA
FlaB:   MAiNVNTNVS AMTAQRYLng AadgmQkSME RLSSGyKINS ArDDAAGLQI SNRLtsQSRG LDmAVkNAND GISIAQTAEG AMNETTNILQ RMRDLaLQSs
        *   *****  *  *** *   *      * * *****  *** * ******** ****  * ** *  *  **** ***  ***** ** * *  ** ***** ***

        101                                                150                                                200
FlaA:   NGtNSaSERq AlnEEsvALq DELNRIAETT SFGGrKLLNG sFgeaSFQIG ssSGEAiimg LtsvRaDdfr MGGqSfiAeq pKtKeWgVpp tArDLkfeft
FlaC:   NGSNSkadRV AIQEEitALN DELNRvAETT SFGGNKLLNG tFatKSFQIG ADnGEAVMLn ikdMRSDnal MGGktYqAAn GKDKnWgVeA GktDLTitlk
FlaE:   NGSNSaaERV AlQEEmaALN DELNRIAETT SFaGrKLLNG qFmkaSFQIG AsSGEAVqLs LrnMRSDsle MGGfSYvAAa laDKqWqVtk GkqqLnisyv
FlaD:   NGSNSkSERV AIQEEitALN DELNRIAETT SFGGNKLLNG tFstKSFQIG ADnGEAVMLt LkdMRSDnrm MGGtSYvAAe GKDKdWkVqA GAnDiTftlk
FlaB:   NGSNSsSERr AIQEEvsALN DELNRIAETT SFGGNKLLNG sFgsKSFQIG ADSGEAVMLs mgsMRSDtqa MGGkSYrAqe GKaadWrVgA -AtDLTlsyt
        ** **   *  *  **  **  ***** **** **   *****  *   *****    ***        * *    ***    *        * *

        201                                                250                                                300
FlaA:   kKdGEavvld IiAKdGDDIE ELATYINGQT DlfKASVdqe GKLQiFvaep nieGnfniSG GLAtELGlng GpgVktTVqd iDiTSVGGsQ nAVgIIDAAL
FlaC:   dKregdvtIs INAKEGDDIE ELATYINGQT DmiKASVdEe GKLQLFtdnn ridGaatFgG aLAGELGigA aqdV--TVDt lDVTtVGGAQ esVaIvDAAL
FlaE:   naqGEnenIq IqAKEGDDIE ELATYINGkT DkVsASVnEk GqLQLyiagk etsGtlsFSG sLAnELqmnl lgye--aVDn lDisSaGGAQ rAVsvIDAAL
FlaD:   didGndqtIt vNAKEGDDIE EvATYINGQT DmVKASVnEk GqLQvFagnn kvtGdvaFSG GLAGaLnmqA Gtae--TVDt iDVTSVGGAQ qsVavIDsAL
FlaB:   nKqGEarevt INAKqGDDlE ELATYINGQT edVKASVgEd GKLQLFassq kvnGdvtigG GLgGEiGfdA GrnV--TVad vnVstVaGsQ eAVslIDgAL
                  ** *** * * ****** *     ***    * **          *     *  *              *          * *   *   * **

        301                                                350                                    380
FlaA:   KYVDSqRAdL GAkQNRlsHs ISNLsNIqEN VeASKSRIKD TDFAKETTqL TKsQILQQAg TSiLAQAKQl PNsAiSLLq
FlaC:   KYVDSHRAEL GAFQNRFnHA InNLDNINEN VNASKSRIKD TDFAKETTAL TKaQILsQAS sSVLAQAKQa PNsALaLLG
FlaE:   KYVDgHRsEL GAmQNRFqHA ISNLDNvhEN laASnSRIKD aDyAKETTqm iKqQILQQvS TSVLAQAKrq PkfvLfLLrn
FlaD:   KYVDSHRAEL GAFQNRFnHA ISNLDNINEN VNASKSRIKD TDFAKEiTAL TKsQILsQAS sSVLAQAKQa PNaALSLLG
FlaB:   KaVDSqRAsL GAFQNRFgHA ISNLDNvNEN VNASRSRIrD TDyArETTAm TKaQILQQAS TSVLAQAKQs PsaALSLLG
        * **  *  * ** ***  *  * ** *  **   ** *** *  * * * *    * *** *    * *****   *     **
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FIG. 4. Alignment of the deduced amino acid sequences of the five *V. cholerae* flagellin proteins. The sequences were aligned by Pileup (GCG Inc.). Amino acids which are identical in at least three of the flagellin genes are capitalized; amino acids which are identical in all five flagellin genes are denoted by an asterisk. The sequences used to design degenerate oligonucleotides for PCR are underlined (FLAX12; see Materials and Methods).

(KKV22), *flaC* (KKV171), *flaD* (KKV21), or *flaE* (KKV91) had no noticeable effect on the motility of *V. cholerae* in soft agar (data not shown).

We also constructed strains containing multiple mutations in the *flaBCDE* genes and assessed their motility phenotype in a similar manner. Strains containing mutations in *flaED* (KKV8), *flaDB* (KKV23), *flaEDB* (KKV34), and *flaCEDB* (KKV174) exhibited no obvious motility defect (Fig. 5 and data not shown).

A *flaA* strain lacks a flagellum. We used electron microscopy to directly observe cells of the various flagellin mutant strains of *V. cholerae*. Unlike the wild-type strain O395, which has a single polar flagellum, the *flaA* strain KKV90 lacks a flagellum (Fig. 6A to C). Some *flaA* cells could be seen with a small appendage at one pole (Fig. 6B and C); this may correspond to the flagellar hook. Complementation of the *flaA* strain with the entire *flaA* gene on a plasmid (pKEK89) results in flagellated bacteria resembling the wild-type strain (Fig. 6D).

Single mutations in *flaB*, *flaC*, *flaD*, or *flaE* (KKV22, KKV17, KKV21, and KKV91, respectively) did not noticeably affect the flagella in strains containing these mutations (data not shown). Strains containing *flaDB* or *flaEDB* mutations (KKV23 and KKV34) exhibited a mixed population of flagellated and nonflagellated bacteria, with noticeable numbers of bacteria having apparently shortened or sheared flagella (data not shown). The *flaCEDB* strain KKV174 had some bacteria with apparently full-length flagella, but the appearance of large numbers of nonflagellated bacteria and bacteria with shortened flagella indicates that the flagella of this strain may be

TABLE 2. Percent identity between flagellin amino acid sequences

Protein	% Identity to:				
	FlaA	FlaB	FlaC	FlaD	FlaE
FlaA	100	66	65	66	61
FlaB		100	70	71	61
FlaC			100	82	64
FlaD				100	66
FlaE					100

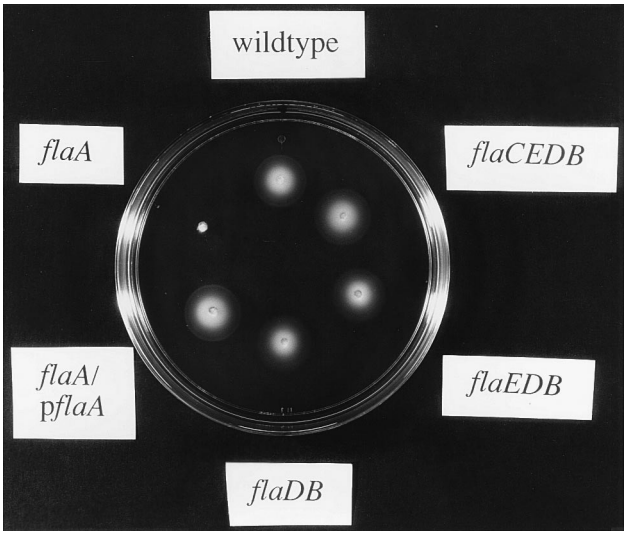

FIG. 5. Motility phenotypes of *V. cholerae* flagellin mutants. The bacteria were inoculated into motility agar (see Materials and Methods) at 37°C; motility is visualized by the swarm diameter. The strains shown (see Table 1) are O395 (wild type), KKV90 (*flaA*), KKV90 with pKEK89 (*flaA*/p*flaA*), KKV23 (*flaDB*), KKV34 (*flaEDB*), and KKV174 (*flaCEDB*).

particularly fragile and are sheared during growth or preparation of samples for microscopy (Fig. 6E).

All five flagellin genes are transcribed during logarithmic growth in *V. cholerae*. To determine if all five flagellin genes are expressed in *V. cholerae*, we constructed promoter-*lacZ* fusions of the flagellin promoters and measured the transcription of the plasmid-borne fusions in several background strains (Table 3). All five flagellin promoters are transcribed at relatively high levels in the wild-type strain CG842 (O395 Δ*lacZ*). These high levels of transcription are maintained in the *flaA* strain KKV90, as well as in the *toxR* strain KKV62.

***flaA* has a σ^{54}-dependent promoter, and the *flaE*, *flaD*, and *flaB* promoters are σ^{28} dependent.** To determine the regulatory characteristics of the various *V. cholerae* flagellin promoters, we measured transcription from the same *fla* promoter-*lacZ* fusions integrated into the chromosome of *S. typhimurium*, thus taking advantage of the extensive repertoire of genetic mutations in transcription components available in this organism (Table 4). The *flaE*, *flaD*, and *flaB* promoters were transcribed at relatively high levels in a wild-type *S. typhimurium* strain, and these high levels of transcription were dependent upon an intact *fliA* gene, which encodes σ^{28}, but were independent of an intact *ntrA* gene, which encodes σ^{54}. There is some residual transcription from the *flaD* promoter even in the absence of σ^{28}, indicative of a second promoter which is independent of σ^{28}. The *flaC* promoter was transcribed at low but significant levels in the wild-type strain, and this level of transcription remained essentially unaffected in *fliA* and *ntrA* strains. Promoter elements resembling the consensus σ^{28} binding site could be found in the *flaB*, *flaC*, *flaD*, and *flaE* promoters (Fig. 7).

The *flaA* promoter was not transcribed in the wild-type *S. typhimurium* strain or in the *fliA* and *ntrA* strains. σ^{54}-dependent promoters require a transcriptional activator protein in addition to RNA polymerase containing σ^{54} (28), and these activator proteins are generally bound to enhancer elements located within the promoter region to increase their local concentration with respect to RNA polymerase. However, DNA binding is not essential to transcriptional activation, and these activator proteins can activate σ^{54}-dependent transcription from solution when present in high enough concentrations (20, 21, 44, 45). We have identified a σ^{54}-dependent transcriptional activator from *V. cholerae*, FlrA, which will be characterized elsewhere (25). When overexpressed from the arabinose-inducible promoter P_{BAD}, this activator can activate transcription of the best-characterized σ^{54}-dependent promoter, *glnA*p, from *S. typhimurium* (Table 4), and this increased level of activation is dependent upon an intact *ntrA* (σ^{54}) gene. Overexpressed FlrA also activated the *flaA* promoter (an approximately 80-fold increase in transcription), and this high level of expression was dependent on an intact *ntrA* gene but independent of an intact *fliA* gene, consistent with *flaA* having a σ^{54}-dependent promoter. FlrA had no significant effect on the transcription of any of the other *fla* promoters. A promoter element resembling the consensus σ^{54} binding site could be found in the *flaA* promoter (Fig. 7).

DISCUSSION

In the present study, we identified and characterized five flagellin genes in the human pathogen *V. cholerae*. Many flagellated bacterial species contain just one or two flagellin genes, which code for the structural subunit of the flagellar filament, so the presence of five separate genes in *V. cholerae* is puzzling, especially since the five predicted gene products have significant homology to each other (61 to 82% identity). In this respect, *V. cholerae* is similar to other *Vibrio* spp., notably the human pathogen *V. parahaemolyticus* (four polar flagellin genes [33]) and the fish pathogen *V. anguillarum* (five polar flagellin genes [34]), which has an identical arrangement of flagellin genes with the highest homology to those from *V. cholerae*.

Phenotypes of *V. cholerae* flagellin mutants revealed that the FlaA protein is essential for motility and that *flaA* strains are nonflagellated. Expression of the other four flagellins in a *flaA* strain remains high, indicating that although highly homologous, they cannot substitute for some essential function of the FlaA protein in assembling a flagellum. Substitution of function is also not easily obtained by mutation, because no revertant motile mutants arise in a *flaA* strain (data not shown). In contrast, a *flaBCDE* strain was motile and cells with flagella could be visualized, although the flagella of this strain appeared to be particularly fragile and easily broken. These results are consistent with the FlaA protein being required to form a flagellar core or scaffold into which the other flagellins are inserted to provide structural integrity. Interestingly, *flaA* mutants of *V. anguillarum* are flagellated but exhibit decreased motility (42); apparently these proteins, although highly homologous, do not have identical functions in the two bacteria.

We have shown that in *S. typhimurium*, the *V. cholerae flaA* gene is transcribed by RNA polymerase complexed with the alternate sigma factor σ^{54} (σ^{54} holoenzyme) while the *flaE*, *flaD*, and *flaB* genes are transcribed by RNA polymerase containing the flagellar sigma factor σ^{28}. Neither σ^{54} nor σ^{28} regulates the expression of *flaC*, and so it remains unclear how this flagellin is regulated. We believe that the same differential regulation occurs in *V. cholerae*. We have created a mutation in the gene encoding σ^{54}, *rpoN* (25). The *V. cholerae rpoN* mutant is nonflagellated, and the *S. typhimurium ntrA* (σ^{54}) gene can complement this mutant for motility; likewise, the *V. cholerae rpoN* gene complements a *S. typhimurium ntrA* mutant for glutamine prototrophy. Thus, σ^{54} has maintained functional homology between these two organisms and must recognize the same promoter elements. We predict that σ^{28} has likewise maintained functional homology and activates the *flaE*, *flaD*, and *flaB* promoters in *V. cholerae*, although a σ^{28} homolog has yet to be identified. Interestingly, transcription of all five flagellin genes in *V. cholerae* is dependent on the presence of σ^{54}, implicating a heirarchy where σ^{54}-holoenzyme influences σ^{28}-dependent transcription (25).

The ability to differentially regulate the flagellins within the flagellum may enable *V. cholerae* to produce flagella which are particularly suited for motility within a given environment (high-viscosity mucus, low or high osmolarity or pH, etc.) by changing helical pitch, thickness, or other flagellar parameters. We considered the possibility that the multiple flagellins were present to provide antigenic variation to the flagellum. The bacterial flagellum is a large target with repeating subunits, so that it generally induces a strong immune response, and it is well known that many pathogens use antigenic variation as a means of evading host immune defenses. For example, flagellar antigenic switching in *S. typhimurium* is a well-defined means of antigenic variation in which only one flagellin gene is exclusively expressed at a given time while the other remains silent (52). This particular antigenic variation is accomplished by genetic rearrangement. Selective expression of individual members of a "pool" of nonessential flagellins would be a suitable means of antigenic variation. However, in *V. cholerae*, such a mechanism is apparently not occurring, because the five flagellin promoters are expressed simultaneously. It must be noted, however, that our data addresses flagellin expression in vitro only; it remains to be determined if flagellin expression

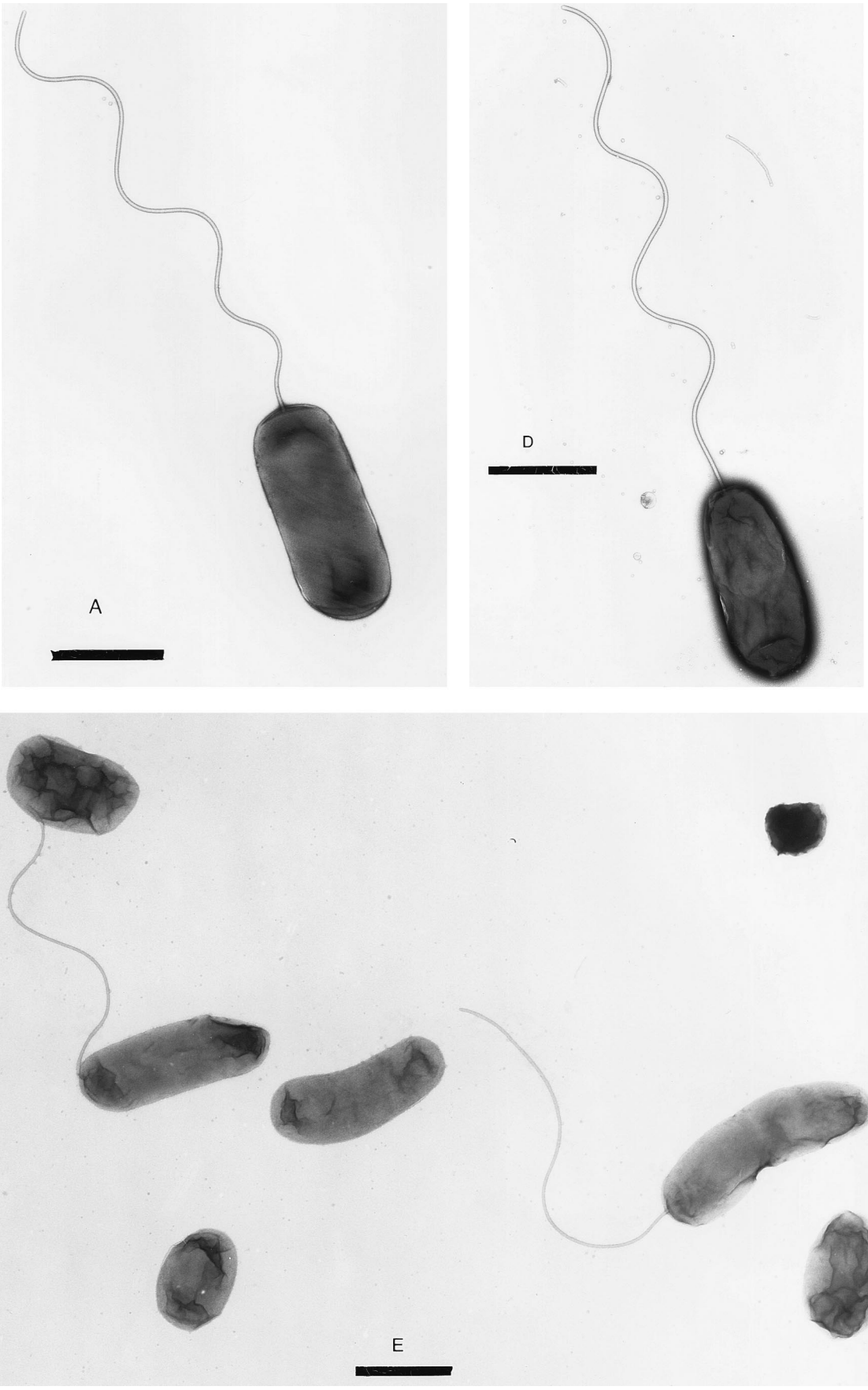

FIG. 6

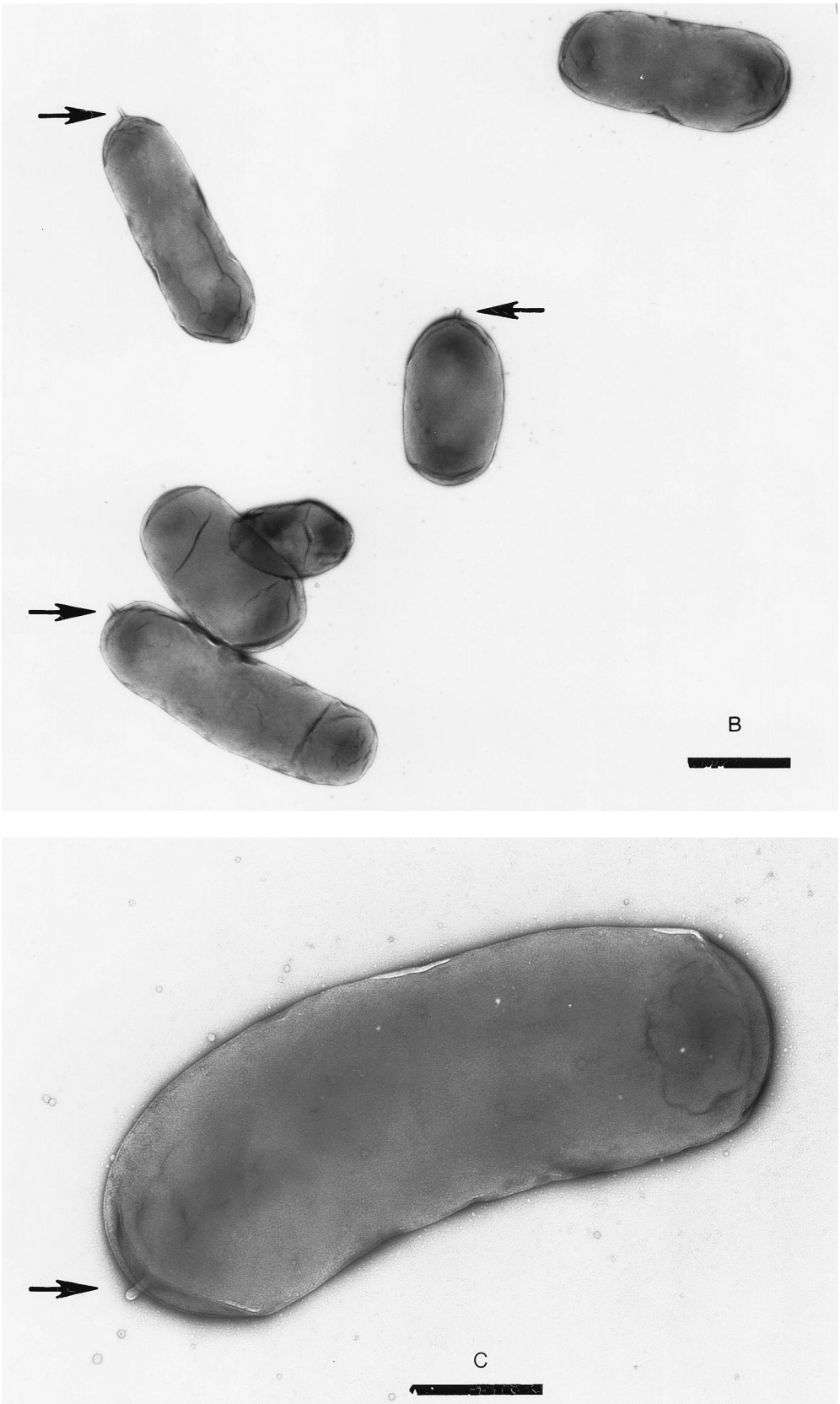

FIG. 6. Transmission electron microscopy of *V. cholerae* flagellin mutants (see Table 1). *V. cholerae* strains in logarithmic growth were resuspended in 0.15 M NaCl, spread onto carbon-coated grids and stained with 1% uranyl acetate. Bars, 1 μm (A, B, D, and E) and 500 nm (C). (A) O395, wild type. (B and C) KKV90, *flaA* (arrows indicate small appendages, possibly flagellar hooks. (D) KKV166, *flaA*/p*flaA* (KKV90 with pKEK89). (E) KKV174, *flaCEDB.*

TABLE 3. All five flagellin promoters are transcribed simultaneously in *V. cholerae*[a]

Genotype[b]	β-Galactosidase activity in strain with mutation:				
	*flaA*p-'*lacZ*	*flaC*p-'*lacZ*	*flaE*p-'*lacZ*	*flaD*p-'*lacZ*	*flaB*p-'*lacZ*
Wild type	9,641	11,047	3,417	14,639	16,298
Δ*flaA*::Cm^r	11,063	13,789	6,833	11,341	15,833
Δ*toxR*	9,436	15,441	5,101	13,846	13,407

[a] Assays were performed as described in Materials and Methods. Strains were grown in LB supplemented with 2 mM glutamine; cultures were assayed in triplicate at an optical density at 600 nm of ~0.2 to 0.4. Results are the average of three samples expressed in Miller units (38). Each strain harboring the vector pRS551 alone grown in this medium has 10 to 15 Miller units of activity, which can be considered background activity.

[b] The actual strains used (Table 1) were CG842 (wild type), KKV90 (Δ*flaA*::Cm^r), and KKV62 (Δ*toxR*), harboring plasmids pKEK80 (*flaA*p-'*lacZ*), pKEK76 (*flaC*p-'*lacZ*), pKEK81 (*flaE*p-'*lacZ*), pKEK77 (*flaD*p-'*lacZ*), and pKEK79 (*flaB*p-'*lacZ*).

within the host is significantly different. Also, the presence of the insertion element IS*1004*, which contains a transposase gene, downstream of the *flaC* gene provides a mechanism whereby chromosomal rearrangements could occur by illegitimate recombination within this locus; the presence of the left arm of IS*1004* within the O surface antigen locus *rfb* (4) suggests that such illegitimate recombination may be common in *V. cholerae.*

Another means by which pathogens evade immune response to flagellar antigens is by shutting off flagellar synthesis during colonization of the host. For example, in *Bordetella bronchiseptica*, the regulatory protein BvgA, which activates virulence gene expression, simultaneously represses flagellar gene synthesis, so that *B. bronchiseptica* is nonflagellated during infection (2). This may be an important means of immune system evasion, because *B. bronchiseptica* mutant strains that are flagellated during infection are more rapidly cleared (1). Alternatively, flagella are not needed during colonization, so that *B. bronchiseptica* may simply shut off synthesis for energetic reasons. Interestingly, the squid symbiont *V. fischeri*, which is closely related to *V. cholerae*, becomes aflagellate during colonization of the squid light organ (50). The likely reason for repressing flagellar synthesis in this case is to avoid unnecessary motility gene expression during a sessile phase of existence. In *V. cholerae*, genetic evidence suggests that motility phenotypes and the expression of some virulence genes are inversely related (14); i.e., some nonmotile mutants express higher levels of CT and TCP than does a wild-type strain under noninducing in vitro conditions, while *toxR* mutants, which express no CT or TCP, display a hyperswarmer phenotype. However, there is no direct evidence for repression of motility gene expression during infection. In this study, we were unable to detect any increase in *fla* gene transcription in a *toxR* mutant which might account for its hyperswarmer phenotype, and were also unable to detect any increase in CT and TCP expression by any of the *fla* mutants in vitro (data not shown).

Motility is important for full virulence of *V. cholerae* in the rabbit models of cholera (47), but various spontaneous nonmotile mutants show no defect for colonization in the infant mouse competition assay (14, 47). Consistent with these previous observations, the *flaA* mutant exhibited no defect for colonization of infant mice (data not shown). The attenuation of nonmotile mutants in rabbit animal models suggests that motility may be important for the organism to penetrate the mucus layer of the intestine and thus adhere to the apical surface of enterocytes. Perhaps the infant mouse has a sufficiently different (immature) mucus layer such that motility is not required to arrive at a permissive colonization site in this model.

Once the organisms colonize the intestinal surface, however, it might be advantageous to shut off flagellar synthesis to avoid immune system recognition and clearance, similar to *B. bronchiseptica*. Camilli and Mekalanos (6) identified a *V. cholerae* flagellin antisense transcript that was induced within the host during colonization. The reporter fusion they describe was inserted in a reverse orientation at a position corresponding to nucleotide 1677 of the *flaEDB* locus (Fig. 3) such that it would be measuring transcription originating in the *flaE-flaD* intergenic region or downstream within or past *flaD*. Notably, the antisense transcript would presumably regulate the expression of one of the σ^{28}-dependent flagellins, *flaE* and/or *flaD*; such a mechanism may be required to more rapidly shut off flagellin synthesis.

We have no additional evidence for flagellar gene repression during *V. cholerae* colonization, but the existence of differential

TABLE 4. The *flaA* promoter is transcribed by σ^{54}-holoenzyme and the *flaE*, *flaD*, and *flaB* promoters are transcribed by σ^{28}-holoenzyme in *S. typhimurium*[a]

Genotype[b]	β-Galactosidase activity in strain with mutation:					
	*flaA*p-'*lacZ*	*flaC*p-'*lacZ*	*flaE*p-'*lacZ*	*flaD*p-'*lacZ*	*flaB*p-'*lacZ*	*glnA*p-'*lacZ*[c]
Wild type	23	651	2,125	6,649	9,049	508
Wild type + FlrA[d]	1,808	493	1,239	5,165	5,133	2,047
ntrA	27	241	1,285	2,260	2,374	503
ntrA + FlrA[d]	33	564	1,372	5,931	6,014	288
fliA	28	206	21	173	40	
fliA + FlrA[d]	2,311	164	26	174	42	

[a] Assays were performed as described in Materials and Methods. Strains were grown in LB supplemented with 2 mM glutamine and 0.05% arabinose; cultures were assayed in triplicate at an optical density at 600 nm of ~0.2 to 0.4. Results are the average of three samples expressed in Miller units (38). Strain KK140 (*putPA*::'*lacZ*) grown in this medium has 8 Miller units of activity, which can be considered background activity.

[b] The actual strains used (Table 1) were KK164, KK173, KK167, KK156, and KK159 (wild type with *flaA*p-, *flaC*p-, *flaE*p-, *flaD*p-, and *flaB*p-'*lacZ* fusions, respectively), KK165, KK174, KK168, KK157, and KK160 (*ntrA209*::Tn*10* with *flaA*p-, *flaC*p-, *flaE*p-, *flaD*p-, and *flaB*p-'*lacZ* fusions, respectively), and KK166, KK175, KK169, KK158, and KK161 (*fliA5059*::Tn*10*dTc with *flaA*p-, *flaC*p-, *flaE*p-, *flaD*p-, and *flaB*p-'*lacZ* fusions, respectively).

[c] The genetic background in these reporter strains is Δ(*ntrB-C*); NtrC is the activator of σ^{54}-dependent transcription of the *glnA* promoter, so we wished to measure activation by the heterologous activator FlrA in the absence of NtrC. Residual transcription in a *ntrA* Δ(*ntrB-C*) strain originates from a second σ^{70}-dependent *glnA* promoter (32). The actual strains used (Table 1) were KK188 and KK189 [Δ(*ntrB-C*) and *ntrA* Δ(*ntrB-C*) with *glnA*p-'*lacZ*, respectively].

[d] These strains harbor plasmid pKEK94 (see Materials and Methods), which carries the gene encoding the σ^{54}-activator FlrA from *V. cholerae* (25) under the control of the arabinose-inducible promoter P_{BAD}. σ^{54} activators in high concentrations can activate σ^{54}-dependent transcription from solution (45); in the present study, this protein serves to identify any σ^{54}-dependent promoter.

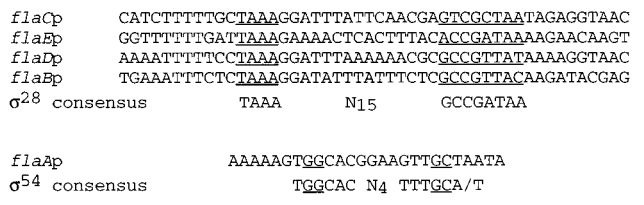

FIG. 7. Alignment of putative σ^{28} and σ^{54} promoter elements in *V. cholerae fla* promoters with σ^{28} and σ^{54} consensus promoter sequences. The consensus σ^{28} sequence for *E. coli* and *S. typhimurium* promoters is from reference 29, and the consensus σ^{54} sequence is from references 3 and 43.

regulation of the flagellin genes provides a potential mechanism for quickly shutting off flagellar synthesis. RNA polymerase containing σ^{54} (σ^{54} holoenzyme) can initiate transcription only in conjunction with an activating protein, which generally responds to environmental signals and activates transcription only under inducing conditions (28). In contrast, σ^{28} holoenzyme is active in the absence of any activating proteins but requires the export of the anti-sigma factor FlgM, which occurs through a correctly assembled hook–basal-body complex (22). Shutting off σ^{54}-dependent transcription only requires recognition of a change in environmental conditions, while shutting off σ^{28}-dependent transcription requires a buildup of anti-sigma factor within the cell, which presumably is a slower response mechanism. Because the FlaA protein is essential for the assembly of a flagellum, shutting off *flaA* synthesis through the absence of σ^{54}-dependent transcription would result in a nonmotile phenotype. We do not yet know the full extent of involvement of σ^{54} in flagellar gene synthesis in *V. cholerae*; σ^{54} may be required at multiple steps during flagellar synthesis, similar to the flagellar cascade of *Caulobacter crescentus* (5, 15). σ^{54} has also been shown to be required for flagellar synthesis in *V. anguillarum* (46), but it has not yet been determined whether it is directly involved in the transcription of flagellin genes, as it is in *V. cholerae.*

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