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Genetic and Molecular Characterization of the Polar Flagellum of *Vibrio parahaemolyticus*†

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Vibrio parahaemolyticus **possesses two alternate flagellar systems adapted for movement under different circumstances. A single polar flagellum propels the bacterium in liquid (swimming), while multiple lateral flagella move the bacterium over surfaces (swarming). Energy to rotate the polar flagellum is derived from the sodium membrane potential, whereas lateral flagella are powered by the proton motive force. Lateral flagella are arranged peritrichously, and the unsheathed filaments are polymerized from a single flagellin. The polar flagellum is synthesized constitutively, but lateral flagella are produced only under conditions in which the polar flagellum is not functional, e.g., on surfaces. This work initiates characterization of the sheathed, polar flagellum. Four genes encoding flagellins were cloned and found to map in two loci. These genes, as well as three genes encoding proteins resembling HAPs (hook-associated proteins), were sequenced. A potential consensus polar flagellar promoter was identified by using upstream sequences from seven polar genes. It resembled the enterobacterial σ^{28} consensus promoter. Three of the four flagellin genes were expressed in *Escherichia coli*, and expression was dependent on the product of the *fliA* gene encoding σ^{28}. The fourth flagellin gene may be different regulated. It was not expressed in *E. coli*, and inspection of upstream sequence revealed a potential σ^{54} consensus promoter. Mutants with single and multiple defects in flagellin genes were constructed in order to determine assembly rules for filament polymerization. HAP mutants displayed new phenotypes, which were different from those of *Salmonella typhimurium* and most probably were the result of the filament being sheathed.**

Many bacteria are propelled by rotation of semirigid helical filaments called flagella (32, 51). These propellers are joined via proteins called HAPs (hook-associated proteins) to a universal joint (the hook) which is connected to the motor (the basal body) embedded in the cytoplasmic membrane (25). There is much diversity in flagella (26, 58). They can be arranged on the cell body in a variety of configurations, including single polar, tufted polar (i.e., 2-6 polar), and many peritrichous (or lateral) configurations. Some flagella, the endoflagella of the spirochetes, are found in the periplasmic space (9). The composition of the flagellar filament can also vary: there are simple flagella such as those of *Escherichia coli* and *Salmonella typhimurium*, which are homopolymers composed of a single flagellin subunit, and complex flagella polymerized from multiple flagellins, such as the flagella of *Campylobacter* (16, 31), *Caulobacter* (11, 15, 42), and *Rhizobium* (7, 44, 57) species as well as many members of the domain *Archaea* (1, 14, 27). Moreover, flagellins may be modified, e.g., phosphorylated (*Pseudomonas aeruginosa* [29]) or glycosylated (*Halobacterium halobium* [14]), and the filament may be sheathed (*Bdellovibrio bacteriovorus* 109J, *Helicobacter pylori*, and many *Vibrio* spp.) (13, 52, 55).

The flagella of *Vibrio parahaemolyticus* are of particular interest because this organism possesses two distinct types of flagella that propel the bacterium under different circumstances (37, 48). A single polar flagellum propels the bacterium in liquid environments. This flagellar filament is sheathed by an extension of the cell outer membrane (3, 52). Energy for rotation of the polar flagellum is derived from the sodium transmembrane potential (5). Under conditions in which the polar system (Fla) is not suitable for movement, such as on surfaces or in highly viscous environments, synthesis of the second flagellar system (Laf) is induced. Many unsheathed, peritrichously arranged lateral flagella (controlled by the Laf system) rotate to move the bacterium over surfaces. This movement is called swarming. Lateral flagellar rotation is driven by the proton motive force (5). Genetic studies have demonstrated that the two flagellar systems are distinct, having no shared structural components (37).

Under certain circumstances, the bacterium simultaneously assembles these two distinct types of flagellar appendages. The polar flagellum is synthesized when *V. parahaemolyticus* is grown in liquid or on a surface, while the lateral flagella are synthesized only in response to growth on a surface. The signal that initiates induction of the Laf system is coupled to performance of the polar flagellum (35). So, in addition to its propulsive role in swimming in liquid environments, the polar flagellum is thought to also play a role as the tactile sensor, or dynamometer, informing the bacterium of contact with surfaces. Physical conditions that inhibit rotation of the polar organelle, such as a surface or a viscous environment or even agglutination with antibody directed against the sheathed flagellum, lead to induction of the alternative motility system. To learn more about the signal transduction mechanism that conveys physical information about the environment to the genes controlling the Laf system, it is important to characterize the structure of tactile sensor, namely, the polar flagellum. Moreover, molecular characterization of the components of both the polar and lateral flagella may provide important clues to the signals that direct export and morphogenesis of the flagellar organelle. Flagellar proteins do not possess signal sequences, and the flagellum-specific export pathway has not been elucidated (19). This organism, which assembles two types of fla-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source, parent, and/or reference
V. parahaemolyticus strains		
BB22	Wild type	6
LM899	*flaE1*::Kan^r	BB22; 35
LM915	Δ*flaAB2*	BB22; 35
LM989	Δ*flaEFCD2*::Kan^r Δ*flaAB2*	LM915; 35
LM1005	*flaA1375*::Tn*phoA*	BB22; 35
LM1006	*flaB1374*::Tn*phoA*	BB22; 35
LM1023	Δ*flaAB3*::Kan^r *lafX313*::*lux*	LM1017; this work
LM4056	*flaD1508*::Tn*phoA*	BB22; this work
LM4106	*flaC1555*::Tn*5*tac1	BB22; this work
LM4109	*flaD1545*::Tn*5*tac1	BB22; this work
LM4110	Δ*flaAB2 flaD1545*::Tn*5*tac1	LM915; this work
LM4174	*flaC1580*::Tn*5*tac1	BB22; this work
LM4176	*flaF1595*::Tn*5*tac1	BB22; this work
LM4181	Δ*flaAB2 flaC1555*::Tn*5*tac1	LM915; this work
LM4183	Δ*flaAB2 flaC1580*::Tn*5*tac1	LM915; this work
LM4218	*flaF1607*::Tn*5*tac1	BB22; this work
LM4221	Tn*5*tac1 insertion 1589	BB22; this work
LM4230	*flaE1615*::Tn*5*tac1	BB22; this work
LM4231	*flaE1608*::Tn*5*tac1	BB22; this work
LM4260	*flaH1705*::Tn*phoA*	BB22; this work
LM4261	*flaH1706*::Tn*phoA*	BB22; this work
LM4271	*flaB1374*::Tn*phoA* Δ*flaCD1708*::Cam^r	LM1006; this work
LM4272	*flaB1374*::Tn*phoA* Δ*flaD1710*::Cam^r	LM1006; this work
LM4273	*flaB1374*::Tn*phoA* Δ*flaC1709*::Cam^r	LM1006; this work
LM4275	*flaA1375*::Tn*phoA* Δ*flaD1710*::Cam^r	LM1005; this work
LM4276	*flaA1375*::Tn*phoA* Δ*flaC1709*::Cam^r	LM1005; this work
LM4277	Δ*flaCD1708*::Cam^r	BB22; this work
LM4278	Δ*flaD1710*::Cam^r	BB22; this work
LM4279	Δ*flaC1709*::Cam^r	BB22; this work
LM4280	Δ*flaCD1708*::Cam^r *lafX313*::*lux*	LM1017; this work
LM4281	Δ*flaAB2* Δ*flaCD1708*::Cam^r	LM915; this work
LM4283	*flaA1379*::Tn*phoA*	BB22; this work
LM4308	*flaB1373*::Tn*phoA*	BB22; this work
LM4309	*flaA1371*::Tn*phoA*	BB22; this work
LM4311	*flaB1377*::Tn*phoA*	BB22; this work
LM4329	Δ*flaD1710*::Cam^r *lafX313*::*lux*	LM1017; this work
LM4330	Δ*flaC1709*::Cam^r *lafX313*::*lux*	LM1017; this work
LM4331	Δ*flaAB2* Δ*flaD1710*::Cam^r	LM915; this work
LM4332	Δ*flaAB2* Δ*flaC1709*::Cam^r	LM915; this work
LM4342	*flaA1371*::Tn*phoA* Δ*flaCD1708*::Cam^r *lafX313*::*lux*	LM4280; this work
LM4344	*flaB1374*::Tn*phoA* Δ*flaCD1708*::Cam^r *lafX313*::*lux*	LM4280; this work
LM4345	*flaH1705*::Tn*phoA lafX313*::*lux*	LM1017; this work
E. coli strains		
CC118	*araD139* Δ(*ara leu*)*7697* Δ*lacX74* Δ*phoA20 galE galK thi rpsE rpoB argE*(Am) *recA1*	C. Manoil; 33
YK410	*araD139* Δ*lacU169 strA thi pyrC46 nalA thyA his*	M. Macnab
YK4181	YK410 *fliA*	M. Macnab
Plasmids		
pLAFRII	Tet^r	12
pRK415	Tet^r	28
pLM1105	Fla$^+$ immunoreactive pLAFRII recombinant cosmid containing *flaAB* locus	*V. parahaemolyticus* bank; 38
pLM1107	Fla$^+$ immunoreactive pLAFRII recombinant cosmid containing *flaAB* locus	*V. parahaemolyticus* bank; 38
pLM1327	2.75-kb *Hin*dIII subclone containing *flaAB*	pLM1107 and pACYC177
pLM1349	Fla$^+$ immunoreactive pLAFRII recombinant cosmid containing *flaCD* locus	*V. parahaemolyticus* bank; 38
pLM1350	Fla$^+$ immunoreactive pLAFRII recombinant cosmid containing *flaCD* locus	*V. parahaemolyticus* bank; 38
pLM1371	pLM1107 with *flaA1371*::Tn*phoA*	This work
pLM1374	pLM1107 with *flaB1374*::Tn*phoA*	This work
pLM1375	pLM1107 with *flaA1375*::Tn*phoA*	This work
pLM1386	12-kb *Eco*RI subclone containing *flaAB* locus	pLM1107 and PLAFRII; this work
pLM1417	Δ*flaAB2*	pLM1386; this work
pLM1489	Fla$^+$ immunoreactive clone; 9-kb *Xba*I fragment from *flaCD* locus	LM915 and pRK415; this work
pLM1545	pLM1489 with *flaD1545*::Tn*5*tac1	This work
pLM1569	3.1-kb *Pst*I subclone; *flaD*$^+$	pLM1489 and pRK415; this work
pLM1576	5.7-kb *Bam*HI subclone; *flaC*$^+$	pLM1545 and pRK415; this work
pLM1600	Tn*5*tac1 insertion 1600, Ptac aligned with *flaC*; *flaC*$^+$	pLM1576; this work
pLM1779	pLM1569 with Δ*flaC1709*::Cam^r	This work
pLM1780	pLM1569 with Δ*flaC1710*::Cam^r	This work
pLM1781	pLM1569 with Δ*flaCD1708*::Cam^r	This work

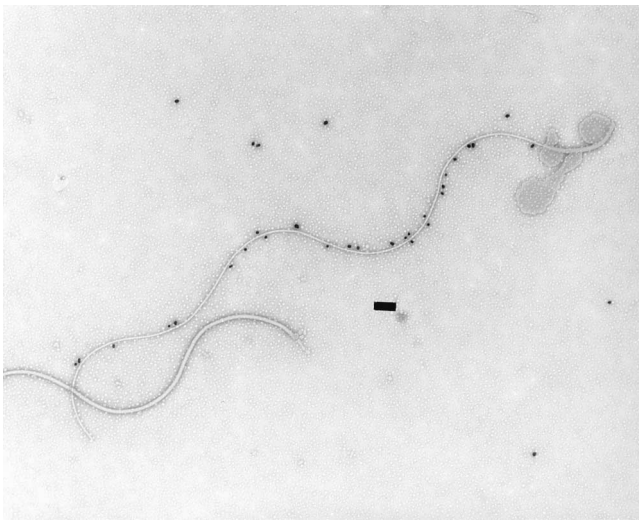

FIG. 1. Electron micrograph of purified sheathed and unsheathed polar flagella reacted with anti-polar flagellin antibody and 15-nm protein A-bound colloidal gold particles. Magnification is ×17,800; the bar indicates approximately 150 nm.

gella at the same time, may be ideal for dissecting flagellum-specific export signals.

The lateral flagellar filament has been previously characterized. It is composed of a single flagellin subunit with an apparent molecular size of 27 kDa on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (36). The gene encoding Laf flagellin has been cloned and sequenced: the deduced amino acid sequence resembles those of other flagellins, particularly at the amino and carboxyl termini (39). In contrast, composition of the polar filament is complex: there are multiple subunits in the flagellar polymer. Purified polar flagellar filament appears to be composed of two flagellin subunits on SDS-PAGE migrating at 44.5 and 45.5 kDa (35). This work presents evidence that there are four flagellin genes, *flaA*, *flaB*, *flaC*, and *flaD*, organized in two distinct loci. The genes were cloned, sequenced, and found to map with other flagellar genes, specifically those encoding HAPs important for joining the filament to the hook and capping the distal end of the filament. Mutant analysis revealed assembly rules for functional filament formation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains and plasmids used in this work are described in Table 1. For propagation of *V. parahaemolyticus* strains, the following media were used: 2216 (28 g per liter of marine broth 2216 [Difco Laboratories; broth was filtered after autoclaving to remove precipitate]), HI (25 g of heart infusion broth [Difco] and 15 g of NaCl per liter), and the minimal medium of Broach et al. (8) supplemented with 0.4% galactose, 20 mM NH_4Cl, and 2% NaCl (final concentration). Solidified medium was prepared by using 2% Bacto Agar (Difco). Motility medium was supplemented with 0.335% agar. *Vibrio* strains were grown at 30°C. LB, 2xYT, and NZCYM media for propagation of *E. coli* strains and phage lambda were prepared as described by Sambrook et al. (45). Ampicillin, chloramphenicol, gentamicin, kanamycin, and tetracycline were used at final concentrations of 75, 10, 125, 50, and 10 μg/ml, respectively.

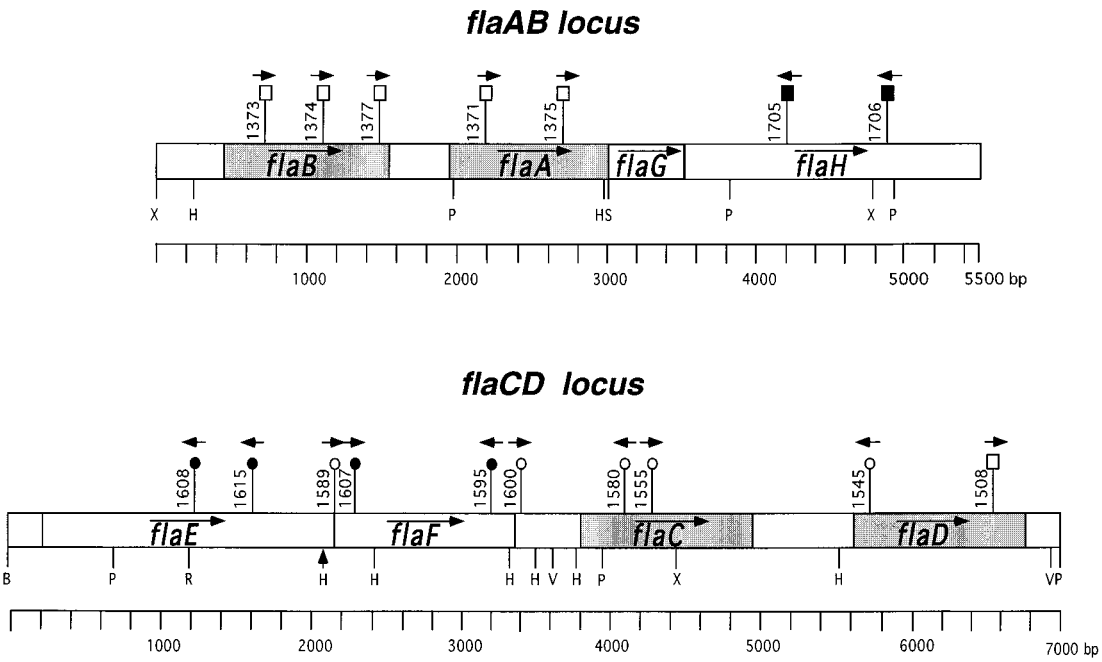

FIG. 2. Physical map derived from the nucleotide sequences of the two loci encoding the polar flagellin genes. The four flagellin genes (*flaA*, *flaB*, *flaC*, and *flaD*) are shaded. The genes *flaE*, *flaF*, and *flaH* encode proteins that resemble HAPs, and the function of *flaG* is not known. The positions of transposon insertions used for gene replacement mutagenesis in *V. parahaemolyticus* are shown on the restriction map. Circles indicate transposon Tn*5*tac1, and squares indicate transposon Tn*phoA*. Filled circles or squares indicate transposons that confer a motility defect when introduced into the chromosome. All of the transposons in the *flaCD* locus were mapped by priming and sequencing from the ends of each transposon insertion; insertions 1374 and 1375 in the *flaAB* locus were also mapped in this manner, and the remainder of the insertions in the *flaAB* locus were mapped by restriction analysis. Arrows indicate the direction of transcription of the indicated gene, *phoA*, or P_{tac} on Tn*5*tac1. The arrowhead marking the *Hin*dIII site at bp 2087 shows the point of insertion of the Kan^r cassette in strain LM899. The deletion (Δ*flaAB2*) in strain LM915 removed the 2.75-kb *Hin*dIII fragment encoding *flaA* and *flaB* and an adjacent 1.25-kb *Hin*dIII fragment mapping 5′ of *flaB*, and Δ*flaAB3*::Kan^r removed the 2.75-kb *Hin*dIII fragment and inserted the Kan^r cassette. Deletions in the *flaCD* locus remove the *Sac*I-*Avr*II fragment at bp 4255 to 6428 (Δ*flaCD1708*), the *Sac*I-*Spe*I fragment at bp 4255 to 4285 (Δ*flaC1709*), and the *Avr*II-*Sna*BI fragment at bp 6428 to 6528 (Δ*flaD1710*). Selected restriction sites are shown (H, *Hin*dIII; P, *Pst*I; R, *Eco*RI, S, *Sal*I; V, *Eco*RV; X, *Xba*I).

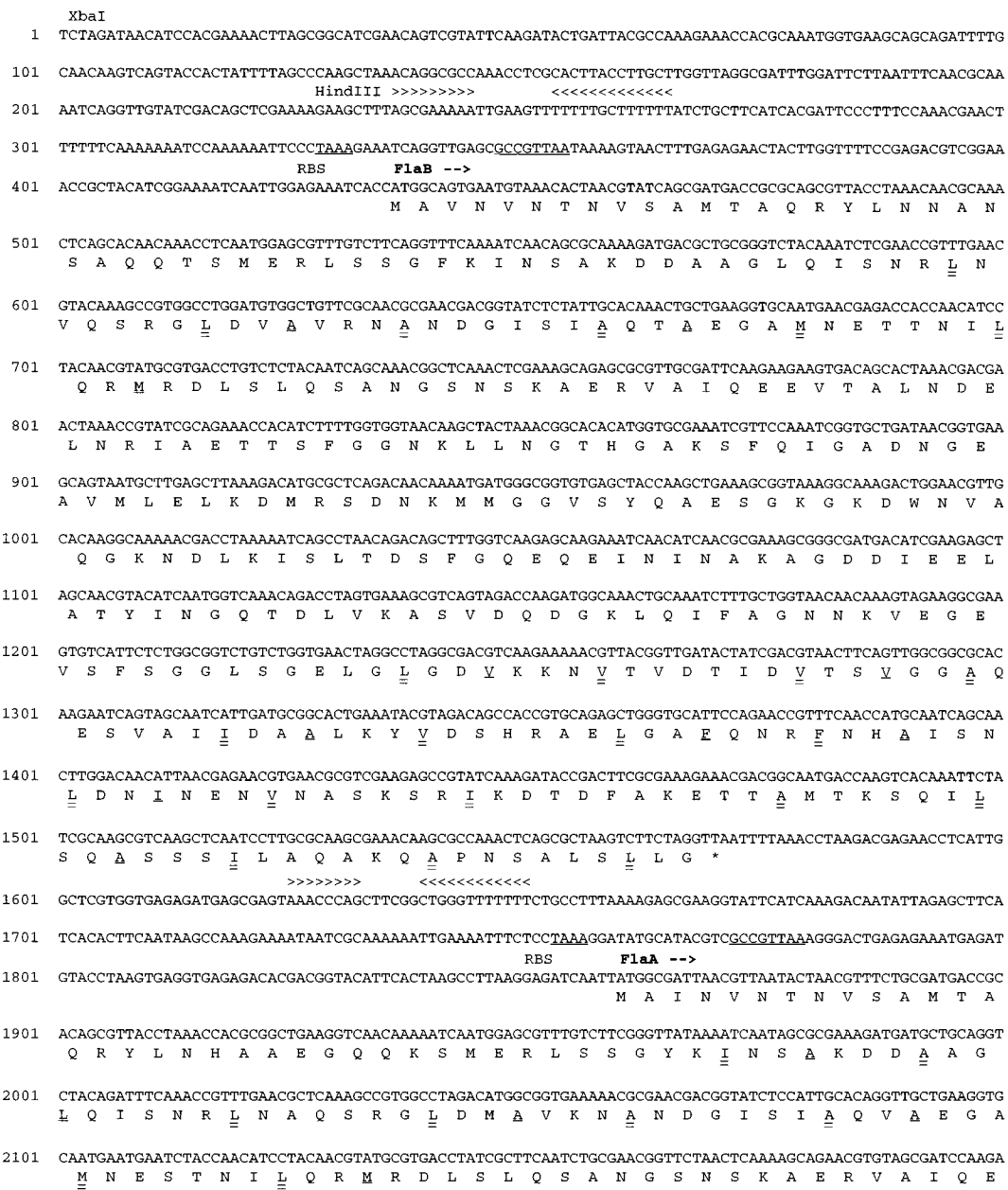

FIG. 3. DNA nucleotide sequence and deduced amino acid sequence of the *flaAB* locus. Selected restriction sites are shown to facilitate orientation to the physical map. The features of potential σ^{28} promoters are underlined. Sites resembling the *E. coli* ribosome binding site (RBS) and a structure that could serve as a potential rho-independent transcriptional terminator (chevrons) are indicated. Hydrophobic amino acids, which occur in a heptad repeat at the N and C termini of each deduced polypeptide and which may participate in the formation of α-helical coiled coils, are indicated by underlining: residues at positions a and d in an a-through-g heptad are double and single underlined, respectively.

Genetic techniques. Specific mutations were created on clones in *E. coli* and then transferred to *V. parahaemolyticus* by allelic replacement. Clones in *E. coli* were mutagenized with λ Tn*phoA* (from C. Manoil) (17) or λTn*5*tac1 (from D. Berg) (10). This procedure as well as the procedures for conjugation and gene replacement in *V. parahaemolyticus* has been described elsewhere (50). Mutations were also constructed in vitro. Transformations, ligations, and other general procedures were adapted from the methods of Sambrook et al. (45). Bacterial alkaline phosphatase was from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Deletions in the *flaCD* locus were introduced into plasmid pLM1569 with the concomitant introduction of a cassette encoding chloramphenicol resistance (from H. Schweizer) (47). Specifically, for Δ*flaCD1708*, pLM1569 was digested with *Sac*I-*Avr*II and ligated with the *Sac*I-*Xba*I fragment from pUC18CMR containing the chloramphenicol cassette; for Δ*flaC1709*, a *Sac*I-*Spe*I digest was ligated with *Sac*I-*Xba*I from pUC18CMR; and for Δ*flaD1710*, an *Avr*II-*Sna*BI digest was ligated with *Sma*I-*Xba*I from pUC18CMR. The deletions *flaAB2* and *flaAB3*::Kan^r were created by ligating a partial *Hin*dIII digest of pLM1386 in the absence or presence of a *Hin*dIII fragment encoding Kan^r isolated from Tn*phoA*. The mutations *flaE1*::Kan^r and Δ*flaEFCD2*::Kan^r were created by ligating a partial, phosphatased digest of a clone encoding genes in the *flaEFCD* locus in the presence of the Kan^r fragment. These mutations were originally called *flaC1*::Kan^r and Δ*flaCD2*::Kan^r, respectively (35). All strain constructions were confirmed by Southern blot analysis of restricted genomic DNA (34, 38) on 0.45-μm-pore-size Magna Charge nylon membranes (Micron Separations Inc., Westborough, Mass.).

Immunological techniques. Antibody production elicited in New Zealand White rabbits has been described elsewhere (35). Antibody 129 was prepared against polar flagellins, and antibody 127 was prepared against lateral flagellin. For immune electron microscopy, polyclonal antiserum 129 was purified to obtain an immunoglobulin G fraction by passing the antiserum through DEAE-

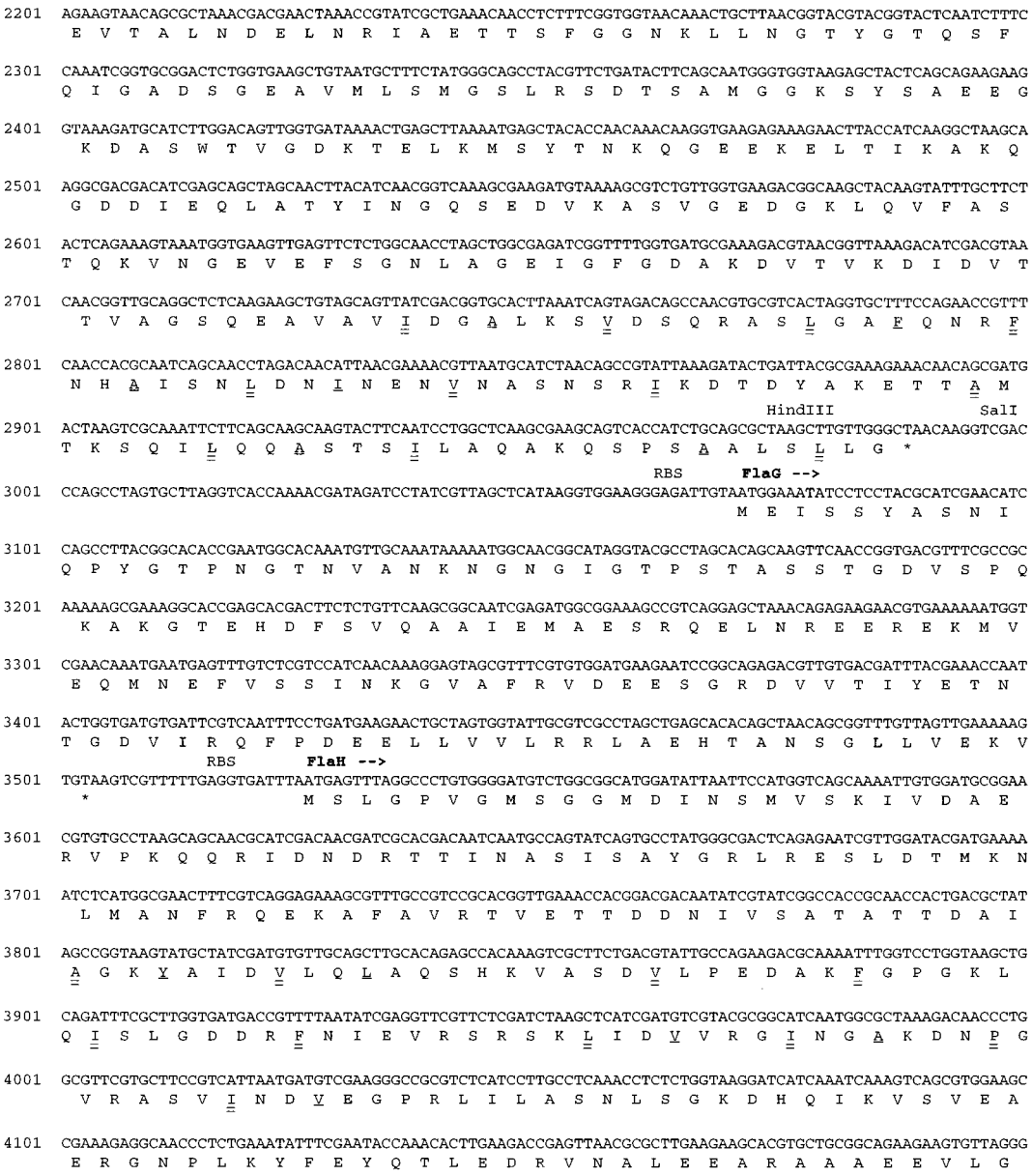

FIG. 3—*Continued.*

cellulose (Whatman International Ltd., Maidstone England) equilibrated with 10 mM potassium phosphate buffer (pH 7.8). The methods for preparing colony filter blots, immunodetection of cosmid clones producing antigen, and Western blot (immunoblot) analysis have been described elsewhere (38).

Electron microscopy. Electron microscopy was performed with a Philips 300 microscope and 400-mesh copper grids with 0.4% Formvar and light carbon coating. Flagella were purified as described previously (35). A drop of purified flagella was gently mixed with a 1/10 volume of 12.5% glutaraldehyde–10% paraformaldehyde in 0.15 M sodium cacodylate buffer (pH 7.4). Grids were floated on the fixed suspensions for 10 min, washed four times with phosphate-buffered saline (PBS; 20 mM $NaPO_4$ [pH 7.3], 100 mM NaCl), floated on antibody 129 (immunoglobulin G fraction diluted 1:25 in PBS) for 20 min, washed four times with PBS, floated on protein A-gold for 20 min, washed four times with distilled water, and negatively stained with 0.5% phosphotungstic acid. The protein A-conjugated colloidal gold particles were prepared by the method of Slot and Geuze (53) with the aid of S. J. Singer's laboratory. Protein A was from Pharmacia (Piscataway, N.J.).

DNA sequencing analysis. The *flaBAGH* locus was sequenced by subcloning the 4.7-kb *Xba*I fragment from pLM1107 in both orientations into M13 vector mp18 (40). The subclone and deletions of the subclone made by using the Cyclone I Biosystem (International Biotechnologies, Inc.) were used as sequencing templates. The 3′ end of the *flaH* gene was obtained by double-stranded sequencing of pLM1107, using oligonucleotide primers. Sequencing of the *flaEFCD* locus made use of a collection of transposon insertions mapping in this locus. Clones, each containing a transposon Tn*5*tac1 insertion, were primed with oligonucleotides directed against the unique ends of Tn*5*tac1. Alternatively, for insertions of transposon Tn*phoA*, fragments containing each end of Tn*phoA* and adjacent bacterial DNA were subcloned into M13 mp18 or mp19 (49). The oligonucleotides used as sequencing primers were TACC (matches left end of Tn*5*tac1; 5′ GATAAGCTGTCAAACATG 3′), TACE (matches right end; 5′ CACACAGGAAACAGAATTCC 3′), and TNPHOA (5′ GTCCAGGACGC TACTTG 3′). DNA sequence was obtained for both strands, using the dideoxy-chain termination procedure of Sanger et al. (46) with the Sequenase 2.0 kit from United States Biochemical. The source of radioactivity was α-^{35}S-dATP (Amersham). Synthetic oligonucleotides were prepared by Genosys Biotechnologies, Inc. (The Woodlands, Tex.). Sequence assembly was performed with the Genetics Computer Group (GCG) software package. Searches for homology were performed at the National Center for Biotechnology Information with the BLAST network service (4).

Nucleotide sequence accession number. The nucleotide sequence for the

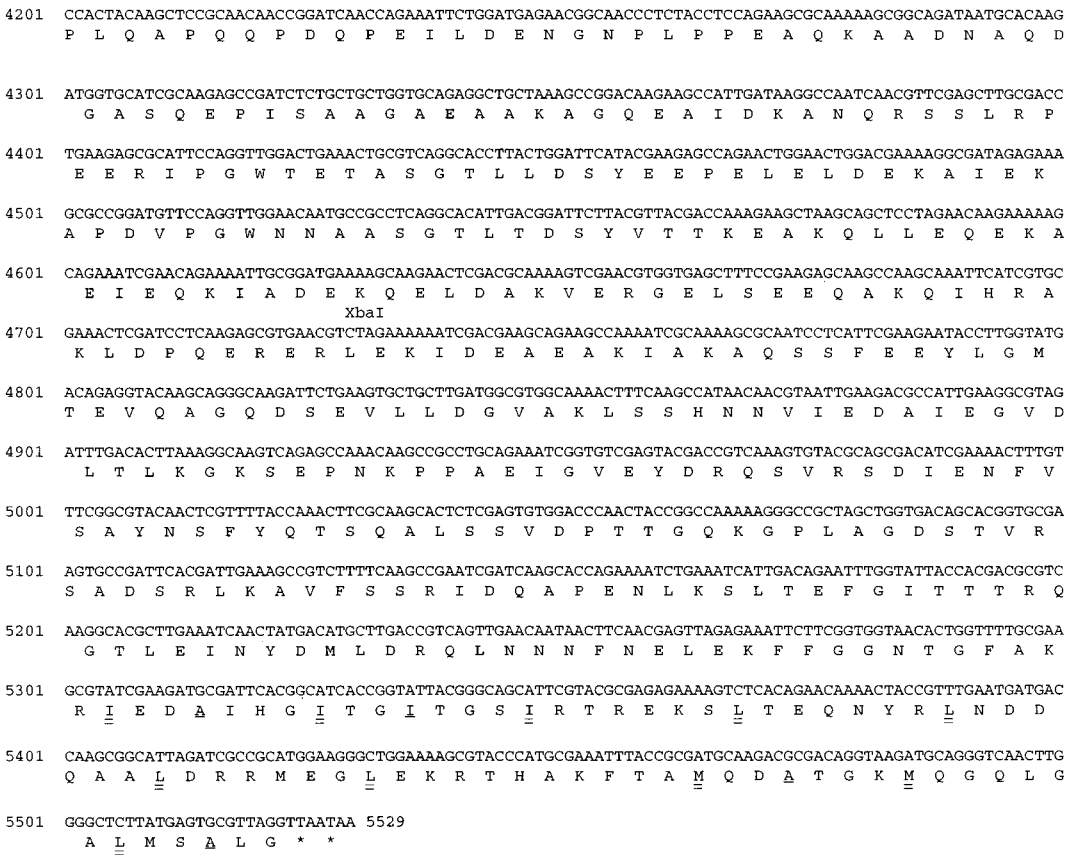

FIG. 3—*Continued.*

flaBAGH locus has been assigned GenBank accession number U12816, and that for the *flaEFCD* locus has been assigned GenBank accession number U12817.

RESULTS

Cloning of polar flagellin-encoding genes. Antibodies to polar flagellins were elicited in rabbits by using antigens excised from SDS-polyacrylamide gels. Purified flagella, prepared by shearing in a Virtis homogenizer and differential centrifugation to first remove the cell bodies and then pellet the organelles, contained proteins that migrated at 44.5 and 45.5 kDa on SDS-polyacrylamide gels stained with Coomassie blue (35). Figure 1 shows an electron micrograph of a sample of the purified flagellar preparation, which contained both sheathed and unsheathed flagellar filaments, reacted with the anti-polar flagellin serum and colloidal gold particles. The serum was specific for the polar flagellar filament and not the sheath.

This antiserum was used to screen a recombinant cosmid library for production of antigen. Two classes of cosmids were identified. On Western blots, one class, represented by pLM1105 and pLM1107, produced two proteins (FlaA and FlaB) that comigrated with the 45.5- and 44.5-kDa flagellins. The second class, represented by pLM1349 and pLM1350, produced what appeared to be a single cross-reactive protein that comigrated with the 45.5-kDa flagellin. Southern analysis showed the clones to cross-hybridize, and initially all of the clones were thought to be overlapping cosmids representing a single locus; however, restriction mapping and DNA sequencing revealed the classes to be representative of homologous but distinct, nonoverlapping regions of the chromosome. The second locus was also recovered directly from the chromosome of strain LM915, containing a deletion in the region encoding *flaA* and *flaB*, by using DNA homology to the deleted genes (probe pLM1327). This clone was named pLM1489, and it produced a single immunoreactive band (FlaD) that migrated at 45.5 kDa on Western blots.

Localization of polar flagellin genes with Tn*phoA*. Transposon Tn*phoA* was used to identify the location of the flagellin genes encoded by these large cosmids, which each contained more than 20 kb of recombinant DNA. Insertions in the structural genes were identified on chromogenic substrate as blue clones producing fusion proteins with alkaline phosphatase. Western analysis showed the clones to be missing flagellin protein and containing new proteins of larger molecular mass that cross-reacted with both anti-polar flagellin and anti-alkaline phosphatase serum. The points of insertion of the transposons were mapped by restriction analysis and are shown in Fig. 2.

DNA nucleotide sequence and predicted amino acid sequence. The DNA sequences corresponding to the regions shown in Fig. 2 are presented in Fig. 3 (*flaAB* locus) and Fig. 4 (*flaCD* locus) along with the deduced amino acid sequences. The sequence reveals four flagellin genes (shown by the shaded boxes) organized in tandem in two distinct loci. The genes encoding *flaA* and *flaB* are transcribed in the same direction but appear to be in different transcriptional units. The intergenic region spans 288 nucleotides and contains sequences that resemble an *E. coli* rho-independent transcriptional terminator. The genes encoding *flaC* and *flaD* are also transcribed in the same direction and are separated by 672 nucleotides. The deduced protein products of these four flagellins are highly

homologous, with FlaB and FlaD being almost identical and FlaA and FlaC being most dissimilar. A diagram of the relationships is presented in Fig. 5. The polar flagellins are similar to the lateral flagellin as well as to flagellins from other organisms, with most of this homology occurring at the N and C termini. Also, as noted in Fig. 3 and 4, each of the polar flagellins possesses hydrophobic heptad repeats within the N and C termini that are characteristic of α-helical coiled coils and believed to be important for flagellin polymerization (19, 23, 58).

In addition to the structural genes encoding flagellins, other genes were found closely linked. Immediately downstream of *flaA* is a small gene, *flaG*, encoding a potential protein of unknown function, and following *flaG* is *flaH*, which encodes a HAP2-like protein. The HAP2 protein of *S. typhimurium* (encoded by *fliD*) caps the distal tip of the filament, preventing excretion of unpolymerized flagellin (19, 24). The nucleotide sequence suggests that the *flaA*, *flaG*, and *flaH* genes constitute an operon, and in fact there are more flagellar genes in this potential transcriptional unit downstream of *flaH* (34a). Preceding *flaC* are two genes, *flaE* and *flaF*, encoding potential proteins that resemble HAP1 and HAP3, respectively (*S. typhimurium* genes *flgK* and *flgL*). These are the proteins responsible for joining the flagellar filament to the hook: HAP1 interacts with the hook and HAP3, while HAP3 interacts with HAP1 and flagellin (19). The two genes, *flaE* and *flaF*, are separated by only 16 nucleotides and may constitute an operon. A summary of the characteristics of the deduced proteins from the *flaAB* and *flaCD* loci is given in Table 2.

Like the flagellins, the *V. parahaemolyticus* homologs of the HAPs showed regions of hydrophobic heptad repeats at their N and C termini (Fig. 3 and 4). The extent of sequence similarity between the *Vibrio* gene products and the *Salmonella* HAPs is graphically presented in Fig. 6, which shows shared sequence similarities occurring predominately at the N and C termini. FlaH, like HAP2, is a little different from the other HAPs. The N terminus contains proline residues, and the hydrophobic repeats occur more internally to the N terminus than they do for the other proteins. Matrix plots and Bestfit analysis did not reveal striking similarity among the *Vibrio* HAPs, between *Vibrio* HAPs and noncognate *Salmonella* HAPs, or between FlaE (HAP1) or FlaH (HAP2) and the polar flagellins. However, FlaF (HAP3, which probably interacts with flagellin) showed some homology to the polar flagellins at its N and C termini. No outstanding sequence comparisons suggesting a conserved N-terminal motif that might be involved in export signalling were observed.

Flagellar promoter structure. Upstream of some of these *fla* genes and underlined in Fig. 3 and 4 are sequences that resemble the consensus derived for σ^{28}-dependent flagellar promoters of *E. coli* and *S. typhimurium*: TAAA n15 GCCG ATAA (18, 30). Finding these sequences is consistent with what is known about *Vibrio fla* expression in *E. coli*. Previously, it was shown that while expression of lateral genes required a unique, *Vibrio*-specific σ^{28}, production of polar flagellin in *E. coli* by pLM1489 (*flaC*$^+$ *flaD*$^+$) was dependent on *E. coli* σ^{28} (39). These observations have been extended with clones encoding *flaAB* (pLM1386) and *flaD* alone (pLM1569). Flagellins were not produced by these clones in the *E. coli* YK4181 (*fliA*), which is defective in the gene encoding the flagellar sigma factor, although they were produced in the congenic parent YK410 (data not shown). Five potential promoter sites were observed in the upstream sequence preceding *flaA*, *flaB*, *flaC*, *flaD*, and *flaE*. These sequences are aligned in Fig. 7 along with potential promoter sequences found upstream of two additional polar flagellar genes whose sequences are known (*motX* and *motY*).

The most divergent upstream sequences are those preceding the *flaE* and *flaC* genes. FlaC flagellin was not synthesized in *E. coli*. Single transposon hits in *flaD* on plasmid pLM1489 containing both *flaC* and *flaD* genes resulted in loss of production of flagellin (Fig. 8A, lane 8). No blue Tn*phoA* fusions with the *flaC* gene were isolated, while blue fusions were isolated with the other three flagellin-encoding genes. The region encoding *flaC* was subcloned in the absence of *flaD* (pLM1576), and the production of flagellin was not detectable (lane 9). However, the gene is not a nonfunctional gene, for when transposon Tn*5*tac1 carrying an outwardly directed isopropylthiogalactopyranoside (IPTG)-inducible promoter was introduced upstream of the coding region (pLM1600), IPTG-induced production of flagellin was observed (lane 10). Interestingly, close inspection of the upstream regions for both *flaE* and *flaC* revealed potential σ^{54}-like promoters (18, 43) (TGGCAC n5 TTGC; double underlined in Fig. 4).

Mutant analysis. Using two varieties of transposon Tn*5*, Tn*5*tac1 and Tn*phoA*, a large number of transposon insertions were isolated in the *flaAB* and *flaCD* loci. The positions of insertion for some of the transposons are presented in Fig. 2. These are the transposons that were used to construct mutants in *V. parahaemolyticus* by gene replacement. The effect of a representative set of insertions, as well as some deletions constructed in vitro, on flagellin production by clones in *E. coli* is presented in the immunoblot in Fig. 8A, and the flagellar profiles of some of the *V. parahaemolyticus* mutant strains derived from these mutated clones are shown in Fig. 8B.

The motility of the *V. parahaemolyticus* mutant strains was assessed in semisolid motility plates prepared with minimal medium and by examination using the light microscope of cultures growing exponentially in HI broth. Lateral flagella were not significantly induced under these cultural conditions and hence did not contribute to motility. The motility phenotypes of mutants with single defects in *fla* genes are presented in Table 3. Mutations in any one of the four flagellin genes failed to significantly affect swimming motility; swimming speed was slightly less than that of the wild type. Mutants with defects in the *flaE* or *flaF* were completely defective in swimming. Mutants with defects in *flaH* were swimming impaired; ring size on motility plates was smaller than that of the wild type.

Swimming of the wild type and a few mutant strains is shown in a sample motility plate in Fig. 9. In some cases, the mutants were constructed in a wild-type background (BB22; Fig. 9, strain 1) as well as a background defective for the lateral system (LM1017 [Laf$^-$], strain 2). Introduction of a deletion removing both genes from either locus failed to dramatically affect swimming motility in a Laf$^+$ or Laf$^-$ background (strains 3 to 5); ring size on motility plates was slightly less than that of the wild type. Introduction of double deletions removing both loci rendered the wild-type strain immotile (strain 6). For comparison, HAP-defective strains with lesions in *flaE* and *flaH* are shown (strains 7 and 8, respectively).

Thus, it appears that loss of two of the four flagellin genes was dispensable for flagellar function. Other combinations of defects were constructed (Table 4). Loss of function of one flagellin gene from each locus rather than a deletion of two genes from the same locus yielded mutant strains whose motility was slightly more impaired than those of strains with a deletion of either locus. Mutants with the combination of *flaC* Δ*flaAB* or *flaD* Δ*flaAB* defects were immotile. Strains with *flaB* and Δ*flaCD* defects were slow but motile; ring size was less

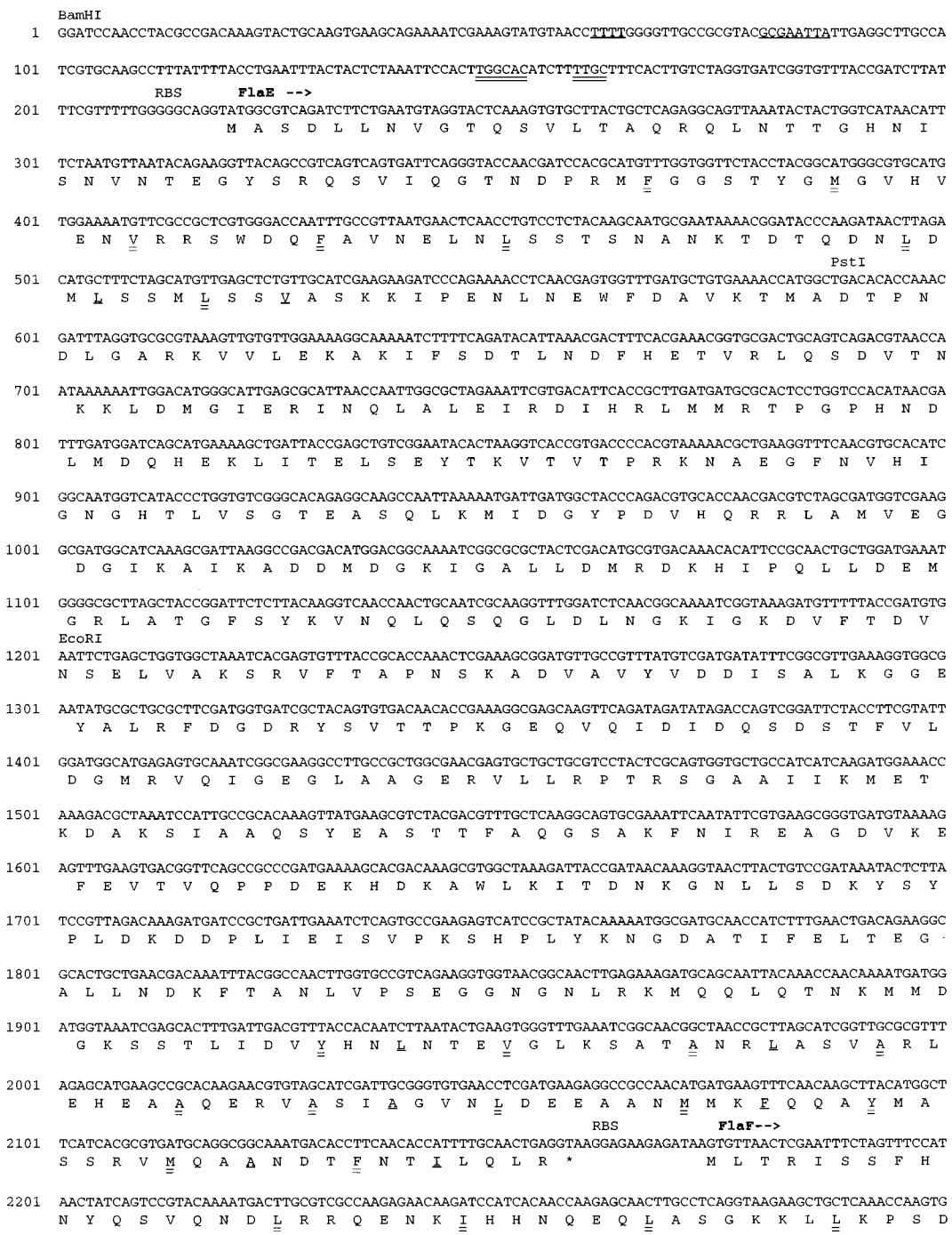

FIG. 4. DNA nucleotide sequence and deduced amino acid sequence of the *flaCD* locus. Selected restriction sites are shown to facilitate orientation to the physical map. The features of potential promoters are underlined, singly for σ^{28} and doubly for σ^{54}. Sites resembling the *E. coli* ribosome binding site (RBS) and structures that could serve as potential rho-independent transcriptional terminators (chevrons) are indicated. Hydrophobic amino acids, which occur in a heptad repeat at the N and C termini of each deduced polypeptide and which may participate in the formation of α-helical coiled coils, are indicated by underlining; residues at positions a and d in an a-through-g heptad are doubly and singly underlined, respectively.

than that produced by the wild type and approximately equivalent to the size for double-mutant strains which had single lesions in each locus. It has not yet been possible to construct a strain with *flaA*::Tn*phoA* and Δ*flaCD* lesions. To summarize, a strain with only the *flaA* gene intact was motile, while strains with only *flaC* or *flaD* were immotile. The ability of a strain with only the *flaB* gene intact to productively polymerize a flagellar filament remains to be determined.

FlaB migrated with an apparent mobility of 44.5 kDa on SDS-PAGE; mutants with defects in *flaB* failed to synthesize the 44.5-kDa protein (Fig. 8B, lanes 3 and 4). FlaA, FlaC, and FlaD migrated at approximately 45.5 kDa with mobilities too

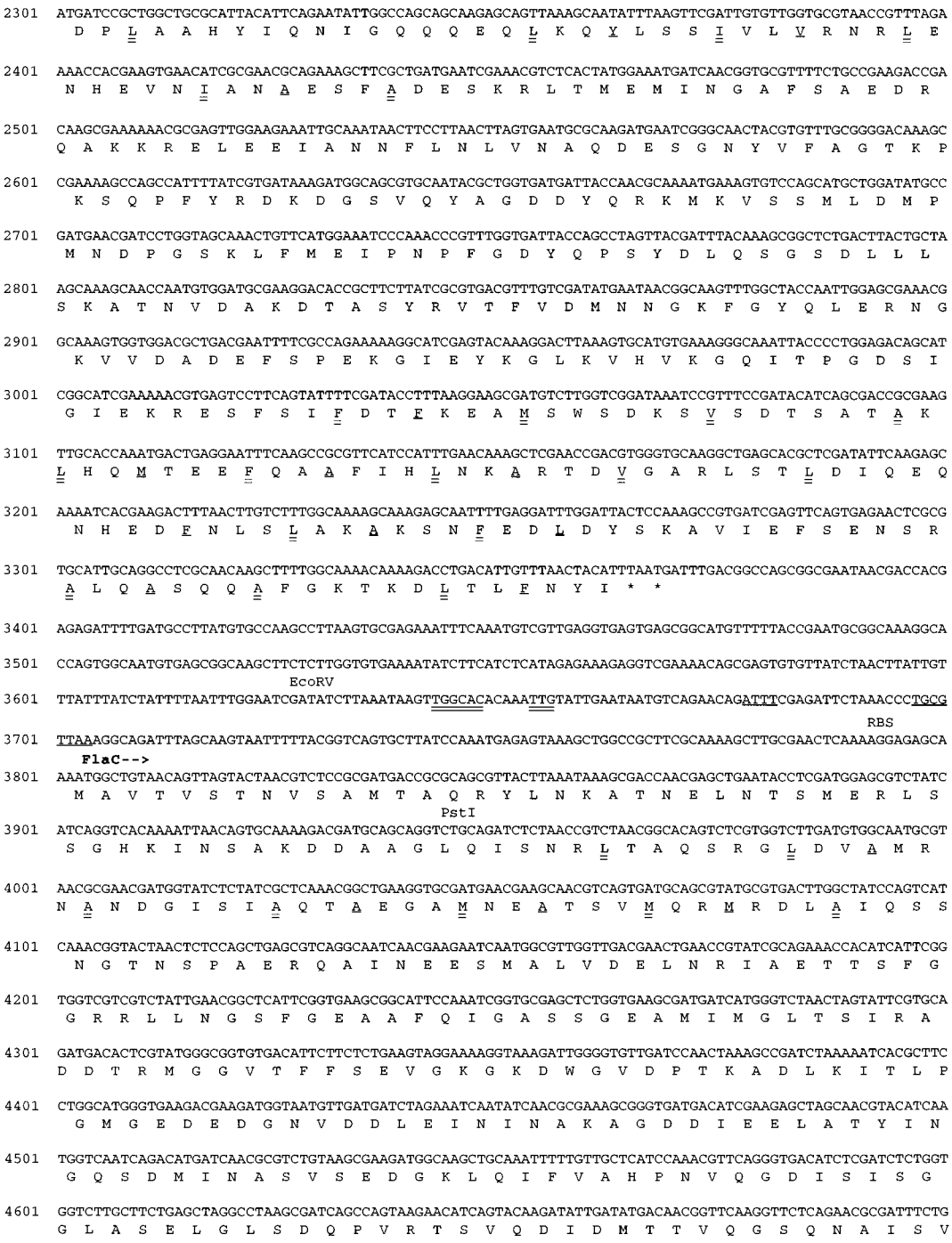

FIG. 4—*Continued.*

similar to be distinguished in the wild type; however, each individual flagellin was observed in mutants having only *flaA*, *flaC*, or *flaD* intact (Fig. 8B, lanes 8 to 10). Therefore, all four flagellin genes can be expressed. FlaC migrated with a slightly higher apparent molecular mass than FlaA and FlaD, and less FlaC than FlaA or FlaD was produced. Strain LM4281 (Δ*flaAB* Δ*flaCD*) completely failed to synthesize immunoreactive flagellin (lane 11).

Analysis of the HAP1 and HAP3 phenotypes. Initially the phenotypes of mutants with defects in *flaE* and *flaF* suggested that the defects caused production of nonfunctional filaments. Although these *V. parahaemolyticus* mutants were completely defective in swimming (Fig. 9, strain 7), they synthesized an apparent filament. A sedimentable fraction containing the flagellins was isolated after a 20,000 rpm (40,000 × *g*) spin in the standard flagellar preparation. However, the filament appeared fragile. For example, when LM899 (*flaE1*) was briefly vortexed after overnight growth and then centrifuged for 5 min in a microcentrifuge, immunoblots detected polar antigen in the growth supernatant, not attached to the cell body (Fig. 10,

```
4701  TACTGGACTCTGCACTGAAATACGTGGATTCACAACGTGCGGATCTTGGTGCGAAACAAAACCGTCTGAGCCACAGCATCAACAACTTGGCAAATATCCA
        L  D  S  A  L  K  Y  V  D  S  Q  R  A  D  L  G  A  K  Q  N  R  L  S  H  S  I  N  N  L  A  N  I  Q

4801  AGAAAACGTTGACGCGTCGAACAGCCGAATTAAAGATACGGACTTTGCGAAAGAAACAACGCAAATGACCAAAGCACAAATTTTGCAACAAGCAGGTACT
        E  N  V  D  A  S  N  S  R  I  K  D  T  D  F  A  K  E  T  T  Q  M  T  K  A  Q  I  L  Q  Q  A  G  T

4901  TCGATTCTTGCTCAAGCGAAACAGTTGCCAAACTCTGCAATGTCACTATTGCAGTAGTTTATACCAATTACAGTGAATAAGAGTTCAGAAATAGCGTAGA
      S  I  L  A  Q  A  K  Q  L  P  N  S  A  M  S  L  L  Q  *

5001  TTCAATGCTTGAGAACGAGGTAAAGTTTTTCGATAAGTAGTCATTCTACAGTCAAAAACTTTAACATAATTATCGAGCATTTTAGCAAGCTAGGGTGAGC

5101  AGTTATTTACTACGATTGGTATCCTCTAGCCTGTATTCAATACATTCAGACGTGGATGGGAATGCGGGCTTTTGGCAAGCAACTTTATAATAGAATGAAT

5201  GGCTCTCTCATCTCTCACCTGCCAATCATTTTCAATGTAGAGTTGCACCCGGTATGCCGGAAATGTGTTCATTTCTCTTGATCTCCACATAGGCTCTGGC

5301  GACCAACCTAGCCCCAGTTCTCTCAAAGGAAAAGGGGCTTTTTCTTTTCTGCATTTTCCTTCTTTTATTTTCTCTATTCATTTCCTTATCTCTTATTTTT

5401  GATGTTTTTCTGTACAATTTAGCCCTCTCGGCTCAATTTGTAGAAACTTGAGCACTTTTTCTGACTTTTCTTCAATTTTTTTAGCCAAATCTCGCAATGT

5501  TATTTTTTTAATTTTTTTTCTAAAGCTTCTGAATTTGGTGTCGTTAATAGAAGTAACTTTGAGAGAACTACTTGGTTTTCCGAGACGTCGGAAACCGCTA
                        RBS          FlaD-->
5601  CATCGGAAAATCAATTGGAGAAATCACCATGGCAGTGAATGTAAACACTAACGTATCAGCGATGACCGCTCAGCGTTACCTAAACAACGCAAACTCAGCA
                                  M  A  V  N  V  N  T  N  V  S  A  M  T  A  Q  R  Y  L  N  N  A  N  S  A

5701  CAACAAACCTCAATGGAGCGTTTGTCTTCAGGTTTCAAAATCAACAGCGCAAAAGATGACGCTGCGGGTCTACAAATCTCGAACCGTTTGAACGTACAAA
      Q  Q  T  S  M  E  R  L  S  S  G  F  K  I  N  S  A  K  D  D  A  A  G  L  Q  I  S  N  R  L  N  V  Q  S

5801  GCCGTGGCCTGGATGTGGCTGTTCGCAACGCGAACGACGGTATCTCTATTGCACAAACTGCTGAAGGTGCGATGAACGAGACCACCAACATCCTACAACG
        R  G  L  D  V  A  V  R  N  A  N  D  G  I  S  I  A  Q  T  A  E  G  A  M  N  E  T  T  N  I  L  Q  R

5901  TATGCGTGACCTGTCTCTACAATCAGCAAACGGCTCAAACTCGAAAGCAGAGCGCGTTGCGATTCAAGAAGAAGTGACAGCACTAAACGACGAACTAAAC
      M  R  D  L  S  L  Q  S  A  N  G  S  N  S  K  A  E  R  V  A  I  Q  E  E  V  T  A  L  N  D  E  L  N

6001  CGTATCGCAGAAACCACATCTTTTGGTGGTAACAAGCTACTAAACGGCACACATGGTGCGAAATCGTTCCAAATCGGTGCTGATAACGGTGAAGCAGTAA
      R  I  A  E  T  T  S  F  G  G  N  K  L  L  N  G  T  H  G  A  K  S  F  Q  I  G  A  D  N  G  E  A  V  M

6101  TGCTTGAGCTTAAAGACATGCGCTCAGACAACAAAATGATGGGCGGTGTGAGCTACCAAGCTGAAAGCGGTAAAGGCAAAGACTGGAACGTTGCACAAGG
        L  E  L  K  D  M  R  S  D  N  K  M  M  G  G  V  S  Y  Q  A  E  S  G  K  G  K  D  W  N  V  A  Q  G

6201  CAAAAACGACCTAAAAATCAGCCTAACTGACAGCTTTGGTCAAGAGCAAGAAATCAACATCAACGCGAAAGCGGGCGATGACATCGAAGAGCTAGCAACG
      K  N  D  L  K  I  S  L  T  D  S  F  G  Q  E  Q  E  I  N  I  N  A  K  A  G  D  D  I  E  E  L  A  T

6301  TACATCAACGGTCAAACAGACCTAGTGAAAGCGTCAGTAGACCAAGATGGCAAACTGCAAATCTTTGCTGGTAACAACAAAGTAGAAGGCGAAGTGTCAT
      Y  I  N  G  Q  T  D  L  V  K  A  S  V  D  Q  D  G  K  L  Q  I  F  A  G  N  N  K  V  E  G  E  V  S  F

6401  TCTCTGGCGGTCTATCTGGTGAACTAGGCCTAGGCGACGACAAGAAAAACGTTACGGTTGATACTATCGACGTAACTTCGGTTGGCGGCGCACAAGAATC
        S  G  G  L  S  G  E  L  G  L  G  D  D  K  K  N  V  T  V  D  T  I  D  V  T  S  V  G  G  A  Q  E  S

6501  AGTCGCGATCATTGATGCGGCACTGAAATACGTAGACAGCCACCGTGCAGAGCTGGGTGCATTCCAGAACCGTTTCAACCATGCAATCAGCAACTTGGAC
        V  A  I  I  D  A  A  L  K  Y  V  D  S  H  R  A  E  L  G  A  F  Q  N  R  F  N  H  A  I  S  N  L  D

6601  AACATTAACGAAAACGTGAACGCGTCGAAGAGCCGTATCAAAGATACCGACTTCGCGAAAGAAACGACGGCAATGACCAAGTCACAAATTCTATCGCAAG
      N  I  N  E  N  V  N  A  S  K  S  R  I  K  D  T  D  F  A  K  E  T  T  A  M  T  K  S  Q  I  L  S  Q  A
                                                                                          >>>>>>>>>>
6701  CGTCAAGCTCAATCCTTGCGCAACGGAAACAAGCGCCAAACTCAGCGCTAAGTCTTCTAGGTTAATCTACCTAACACGACAAGATACAAAAGCCAGGCTC
        S  S  S  I  L  A  Q  R  K  Q  A  P  N  S  A  L  S  L  L  G  *
       <<<<<<<<<<<
6801  ATCCCTGGCTTTTTGCTTATCGGCTATACCGATTTAGATGCGTCCTAACAGGGTGTTCGACAAGGCATTCATATCGATTAGATGGTCTCTTTAAATACCA
                                                                                  EcoRV
6901  ATGTTGCCGCAATGATGACGCAGCGACATCTTAGCCAAGCAGCTGATCAAAACGTTGAGTCACAGAGAAACCTGTCTTCGGGATATCGGATTAACTCAGC
                  PstI
7001  AAGCGACGATGCTGCAG  7017
```

FIG. 4—*Continued.*

lanes 4 to 6). This does not occur for strain BB22 (lanes 1 to 3). The wild-type polar flagellum was firmly attached to the cell; most of the flagellin antigen was found in the pellet. In fact, extremely vigorous vortexing or shearing with a Virtis homogenizer was required to detach the wild-type polar flagellum. Originally, these mutants were mistakenly considered flagellin gene defects resulting in a fragile, nonfunctional filament (35) (*flaC1* is now *flaE1*); however, examination of the DNA sequence revealed that the *flaE* and *flaF* defects mapped in two open reading frames encoding proteins resembling HAP1 and HAP3 of *S. typhimurium*. These proteins join the flagellar filament to the hook (21). For *S. typhimurium*, HAP mutants are filamentless and secrete unpolymerized flagellin into the

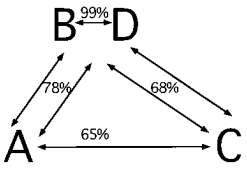

FIG. 5. Relatedness of the polar flagellins. Comparison of the deduced amino acid sequences of the four flagellins by GCG Bestfit analysis produced the following relationships: FlaB and FlaD are 99% similar and 99% identical, FlaA and FlaB are 86% similar and 78% identical, FlaA and FlaD are 86% similar and 78% identical, FlaC and FlaB are 78% similar and 68% identical, FlaC and FlaD are 78% similar and 68% identical, and FlaA and FlaC are 77% similar and 65% identical. For comparison, lateral flagellin is 65% similar and 49% identical with FlaD, and flagellin A of *P. aeruginosa* is 66% similar and 45% identical with FlaD. Percent identities are shown in the diagram, and the lengths of the arrows are representative of the distances of the relationships (i.e., the shorter the arrow, the higher the homology).

TABLE 2. Structural genes of the polar flagellum

Gene	Predicted size of gene product (Da)	Gene product
flaA	39,824	Flagellin
flaB	40,133	Flagellin
flaC	40,786	Flagellin
flaD	40,234	Flagellin
flaE	71,168	HAP1
flaF	44,928	HAP3
flaH	72,717	HAP2
flaG	15,752	Unknown

```
                flaE  Tttt n15 GCgaaTtA
                flaC  attt n15 tgCGTTAA
                flaD  TAAA n15 GtCGTTAA
                flaA  TAAA n15 GCCGTTAA
                flaB  TAAA n15 GCCGTTAA
                motX  TAAA n15 GCCGaTAA
                motY  TAAt n15 GCgcTTgA
VPA FLA CONSENSUS  TAAA n15 GCCGTTAA
σ28 CONSENSUS      TAAA n15 GCCGATAA
```

FIG. 7. DNA sequence alignment of the upstream regions of polar flagellar operons. Capital letters indicate identical or highly similar amino acids; lowercase letters indicate little or no similarity. The consensus *V. parahaemolyticus* polar flagellar (VPA Fla) promoter structure was derived from a minimum plurality of four matches. The *E. coli* and *S. typhimurium* σ^{28} consensus promoter is presented for comparison (30). GenBank accession numbers for *motX* and *motY* sequences are U09005 and U06949, respectively.

growth medium (20). Electron micrographs of the purified flagellar preparations from strain LM899 revealed small, oblong structures of approximately 25 by 75 nm in length that probably were short segments of polymerized flagellin encased in membrane blebs.

DISCUSSION

The polar flagellum of *V. parahaemolyticus* is a complex filament polymerized from multiple subunits and sheathed by an extension of the cell outer membrane. Four genes, organized in tandem in two loci, encode the flagellins. Single mutations introduced into any one of the four flagellin genes failed to affect flagellum function. Double mutations introduced by deletion of either locus also had little significant effect on swimming motility. Introduction of lesions in two flagellin genes in opposing loci (i.e., *flaA flaC*, *flaA flaD*, *flaB flaC*, or *flaB flaD*) yielded more partially defective phenotypes, i.e., strains that swam slower than Δ*flaAB* or Δ*flaCD* strains. Perhaps each flagellin works best with its contiguous partner or the ratio of flagellin type is important for filament formation. Although swimming in motility plates of a strain with a deletion in the *flaAB* locus was almost equivalent to swimming of the wild type, introduction of an additional defect (*flaC* or *flaD* into the Δ*flaAB* strain) yielded an immotile phenotype. These mutant strains with triple defects in flagellin genes produced polar flagellin antigen. Therefore, production of FlaD or FlaC alone was not sufficient to form a functional filament. Significantly less flagellin was synthesized in the Δ*flaAB flaD* ($FlaC^+$) strain than in Δ*flaAB flaC* ($FlaD^+$) or *flaB* Δ*flaCD* ($FlaA^+$) strains, and so it is possible that the amount of FlaC is limiting for filament polymerization. In contrast, FlaA seemed to be able to polymerize to form a functional homopolymer, for *flaB* Δ*flaCD* strains were motile, albeit slower than the wild type.

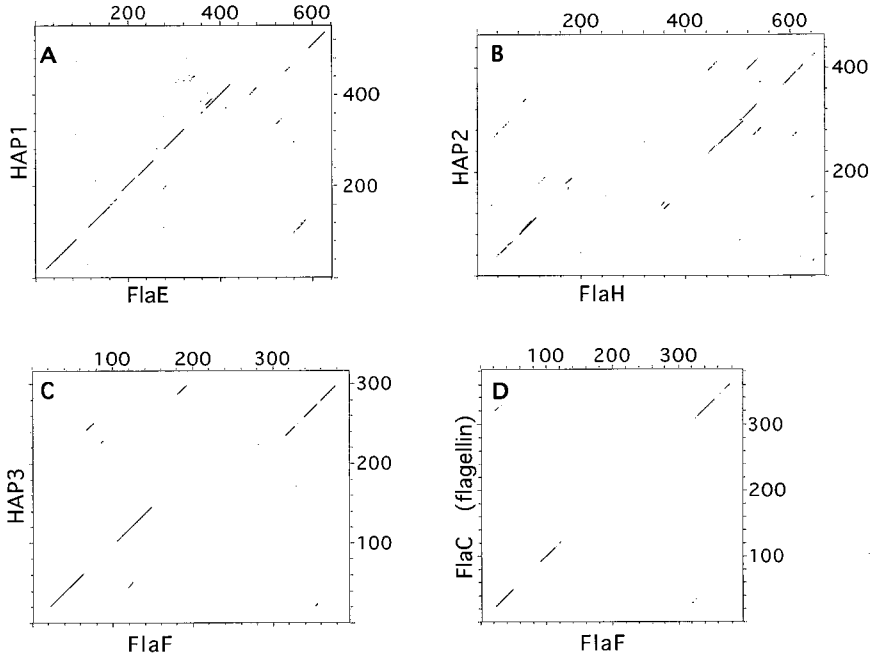

FIG. 6. Comparisons of the deduced amino acid sequences of *V. parahaemolyticus* polar flagellar HAPs with the sequences of *S. typhimurium* HAPs and with a polar flagellin sequence (FlaC). The sequences were compared by using the programs Compare and Dotplot from the GCG package with a window of 40 residues and stringency of 20. HAP1, HAP2, and HAP3 are the products of the *S. typhimurium flgK*, *fliD*, and *flgL* genes, respectively (19), and FlaC is the deduced sequence of a *V. parahaemolyticus* polar flagellin gene. GCG Bestfit analysis produced the following relationships for each panel: A, 50% similar and 27% identical; B, 50% similar and 27% identical; C, 44% similar and 25% identical; and D, 45% similar and 19% identical.

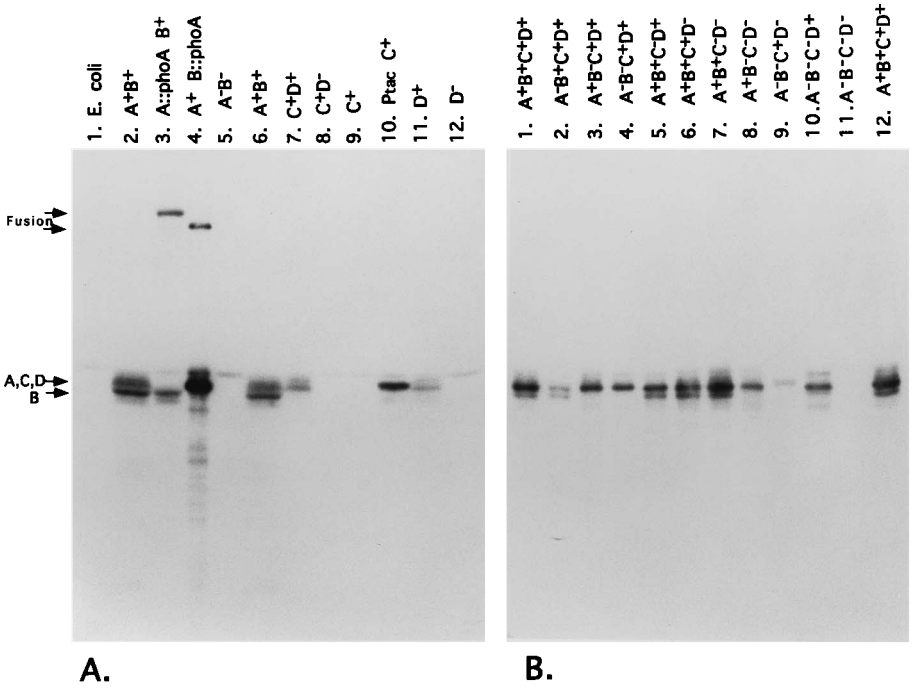

FIG. 8. Western immunoblot analysis of flagellin production in *E. coli* and *V. parahaemolyticus*. (A) Flagellin profiles of clones encoding flagellin genes with and without defects introduced by transposon insertion or deletion in *E. coli* CC118. Lanes: 1, no plasmid; 2, pLM1386 (*flaA*$^+$ *flaB*$^+$); 3, pLM1375 (*flaA*::Tn*phoA flaB*$^+$); 4, pLM1374 (*flaA*$^+$ *flaB*::Tn*phoA*); 5, pLM1417 (Δ*flaAB*); 6, pLM1386 (*flaA*$^+$ *flaB*$^+$); 7, pLM1489 (*flaC*$^+$ *flaD*$^+$); 8, pLM1545 (*flaC*$^+$ *flaD*); 9, pLM1576 (*flaC*$^+$); 10, pLM1600 (P_{tac} *flaC*$^+$; grown with 2 mM IPTG); 11, pLM1569 (*flaD*$^+$); 12, pLM1780 (Δ*flaD*). (B) Flagellin profiles of wild-type and mutant *V. parahaemolyticus* strains. Lanes: 1, BB22 (wild type); 2, LM1005 (*flaA*::Tn*phoA*); 3, LM1006 (*flaB*::Tn*phoA*); 4, LM915 (Δ*flaAB*); 5, LM4279 (Δ*flaC*); 6, LM4278 (Δ*flaD*); 7, LM4280 (Δ*flaCD*); 8, LM4271 (Δ*flaCD flaB*); 9, LM4331 (Δ*flaAB* Δ*flaD*); 10, LM4332 (Δ*flaAB* Δ*flaC*); 11, LM4281 (Δ*flaAB* Δ*flaCD*); 12, BB22 (wild type). The sample LM1005 in lane 2 was underloaded by approximately 50%. The resolving gels were 10% acrylamide. Immunoblots were reacted with polyclonal antiserum directed against subunits of the polar filament and subsequently with iodinated protein A. The antiserum (and preimmune serum) cross-reacted with an *E. coli* protein that migrated at an apparent molecular size slightly larger than those of the *Vibrio* polar flagellins.

The ability of FlaB to productively polymerize a homopolymer is not yet known, for the appropriate strain could not be constructed. The reason for this is being investigated. Only the strain with defects in all four flagellin genes failed to produce polar flagellin antigen, and this strain was immotile. More work remains to be done with these mutants. Although ring size on motility plates is a reliable indicator of motility phenotype (i.e., functionality of the flagellum), actual differences in swimming speed between strains should be assessed by measuring swimming speeds in the light microscope. Also, it will be of much interest to examine flagellar structure in the electron microscope.

On one-dimensional SDS-polyacrylamide gels, FlaA, FlaC, and FlaD migrated too closely to be resolved, while FlaB migrated with an apparent size slightly smaller than sizes of the others. Strains with *flaB* defects failed to synthesize the 44.5-kDa antigen. Mutants with lesions causing loss of two of the three 45.5-kDa flagellins (FlaA, FlaC, and FlaD) still synthesized a 45.5-kDa flagellin. Although these results demonstrate that all four genes can be expressed, the actual composition of the subunits in the filament of wild-type strain is not known. In the mutant strains, and with subclones in *E. coli*, FlaC migrates at a slightly higher apparent molecular mass than FlaA and FlaD. At the amino acid level, the flagellins are highly related to each other. It is curious that FlaB has such a different mobility, for the deduced amino acid sequences of FlaB and FlaD are different in only 2 of 379 amino acids. Two-dimensional gels of purified flagellar preparations from the wild type identified only three predominant spots (34a). Two flagellins may comigrate, or the fourth flagellin may be a minor compo-

TABLE 3. Relative swimming motility phenotypes of mutant strains with single transposon insertions

Allele number[a]	Relevant genotype	Swimming motility[b]
None	Wild type	+++
flaAB locus		
1371	*flaA*	++
1375	*flaA*	++
1373	*flaB*	++
1374	*flaB*	++
1377	*flaB*	++
1705[c]	*flaH*	+
1706	*flaH*	+
flaCD locus		
1608	*flaE*	−
1615[c]	*flaE*	−
1589	Intergenic insertion	+++
1607	*flaF*	−
1595[c]	*flaF*	−
1580[c]	*flaC*	++
1555[c]	*flaC*	++
1545[c]	*flaD*	++
1508[c]	*flaD*	++

[a] The transposon was either Tn*5*tac1 or Tn*phoA*. See Fig. 2 for transposon type, location, and orientation on the physical map. Transposon insertions were introduced into the wild-type strain by gene replacement.

[b] Examined in semisolid minimal motility plates and in the light microscope, using cultures growing exponentially in HI broth. In minimal medium, there is little induction of the lateral flagellar gene system and hence negligible contribution from swarming to motility.

[c] Transposon introduced into strains LM1017 (Laf$^-$) and BB22.

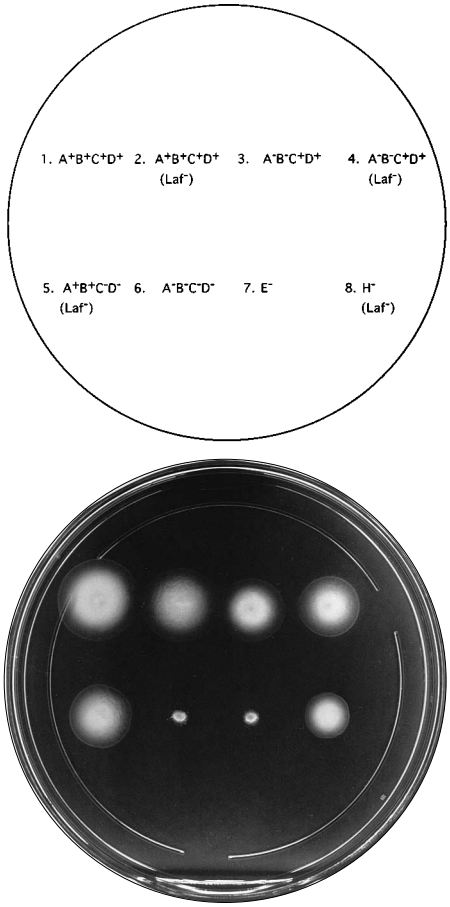

FIG. 9. Swimming in semisolid motility medium. Strains were inoculated from single colonies and grown overnight in HI broth, and 2 μl was inoculated into motility plates, which were then incubated overnight. Phenotypes are shown. Strains: 1, BB22; 2, LM1017; 3, LM915; 4, LM1023; 5, LM4280; 6, LM4281; 7, LM4218; 8, LM4345.

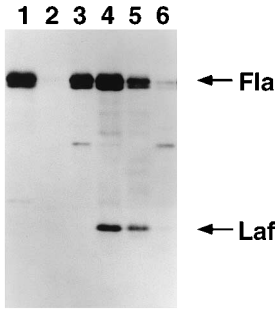

FIG. 10. HAP phenotype of *flaE1*. Western immunoblot analysis of wild-type and mutant strains. Overnight cultures grown in 2216 medium were diluted into sample buffer (complete) or spun in a microcentrifuge for 5 min to generate supernatant and pellet samples. Lanes: 1, BB22 (wild type) complete; 2, BB22 supernatant; 3, BB22 pellet; 4, LM899 (*flaE1*) complete; 5, LM899 supernatant; 6, LM899 pellet. The resolving gel was 12% acrylamide. The immunoblot was reacted with antisera specific for lateral (Laf) and polar (Fla) flagellin. LM899 produces lateral flagellin in liquid because the polar filament is not functional (37). Lateral flagella are fragile and can be sheared from the cell body by vortexing.

nent or under a different sort of regulation, e.g., environmental regulation analogous to that observed for *Campylobacter coli* (2, 31). Now that a set of various combinations of mutant alleles has been constructed, it should be possible to identify the spots on two-dimensional gels and dissect the composition of the wild-type polar flagellar filament.

The genes are arranged in two loci. Although there are two tandem flagellin genes in each location, the genes are probably not in transcriptional units. There is a potential rho-independent terminator structure in the intergenic region between *flaA* and *flaB*. The *flaA*, *flaB*, and *flaD* genes each possess upstream sequences that resemble flagellar promoters dependent on σ^{28}-directed transcription. In *E. coli*, expression of these three genes was dependent on the flagellar sigma, the product of the *fliA* gene. The *flaC* gene may be regulated differently. It was not expressed in *E. coli*, although it could be expressed when transcription was initiated at an exogenous promoter, e.g., the IPTG-inducible P_{tac}. There is an upstream sequence that might serve as a σ^{28}-dependent promoter; however, it matches poorly sequences observed for five other polar genes. Perhaps the gene is not highly expressed, is at a different level in the hierarchy of flagellar expression, or is under a distinct kind of environmental regulation requiring additional transcriptional factors. Interestingly, there is upstream sequence that shows much similarity to the σ^{54}-dependent promoters. There is precedent for finding such a sequence. Other bacteria, specifically *Caulobacter crescentus* (41), have flagellar genes directed by σ^{54} cascade of regulation, and both *Campylobacter* and *Helicobacter* species (2, 16, 54) have one flagellin gene transcribed from a σ^{28} promoter and a second flagellin gene transcribed from a σ^{54} promoter.

Adjacent to the flagellin genes are genes encoding three proteins that resemble the HAPs of *S. typhimurium*. HAP1 and HAP3 are important for joining the filament to the hook. HAP2 is also known as the distal capping protein (21, 24). HAP2 localizes to the tip of the flagellar filament, serves to plug the tip of the growing flagellum, and promotes polymerization of flagellin subunits, which are transported through a central channel in the filament to the distal tip. Mutants with defects in genes encoding HAP1, HAP2, or HAP3 are immotile and excrete unpolymerized filament (20). The phenotypes of *V. parahaemolyticus* mutants defective for these genes are different. Mutants with defects in the joining proteins that adapt the filament to the hook are immotile, but unpolymer-

TABLE 4. Relative swimming motility of strains with combinations of defects in polar flagellin genes

Mutation in *flaAB* locus	Motility with given mutation in *flaCD* locus[a]			
	None	Δ*flaC1709*::Cam^r	Δ*flaD1710*::Cam^r	Δ*flaCD1708*::Cam^r
None	++++	+++	+++	+++
flaA1375::Tn*phoA*	+++	++	++	Not constructed
flaB1374::Tn*phoA*	+++	++	++	++
Δ*flaAB2*	+++	−	−	−

[a] Examined in semisolid minimal motility plates.

ized flagellin molecules are not secreted. These strains produced nonfunctional, severely truncated filaments which were not attached to the cell body. Presumably the sheath acts to retain flagellin monomers, and abortive filament fragments polymerize. The fragments cannot be connected to the hook and bleb off surrounded by membrane vesicles. Mutants with defects in the capping protein were motile, but slow, in semisolid motility plates. Again, it seems likely that the flagellar sheath is responsible for the phenotype. The sheath could trap secreted, unpolymerized flagellin and substitute, although perhaps with less efficiency, for the capping protein. The entire polar filament is surrounded by a sheath, which remains to be characterized. Although the sheath appears to be an extension of the cell's outer membrane, its composition, like that for *Bdellovibrio bacteriovorus* (56), appears distinct from that of the outer membrane (34a).

This work initiates characterization of the complex polar flagellum. Construction of permutations of mutations in the four structural genes encoding flagellins has established a set of mutants that will prove valuable for future characterization of filament structure and function, as well as aid investigation of regulation of flagellin gene expression. The nucleotide sequences of the genes encoding seven axial proteins of the polar flagellum should provide important information for analysis of flagellar export and assembly, especially in comparison with lateral sequence information. Pairwise comparisons indicated that the deduced amino acid sequence for *Vibrio* flagellins and HAPs generally conform at their N and C termini to sequences of cognate axial proteins of other organisms. No consensus N-terminal motif could be identified, which may support the suggestion of Homma and colleagues (19) that export signals might derive from higher-order structure rather than from primary sequence. The deduced protein sequences revealed regions of hydrophobic heptad repeats that may be important conformational domains for flagellar formation. HAP phenotypes, which differ from phenotypes in *S. typhimurium*, underscore differences between sheathed and unsheathed flagella and may have implications for regulation of polar flagellar gene expression in *V. parahaemolyticus*, specifically with respect to excretion of FlgM, the important regulator that couples gene expression to flagellar assembly (22).

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