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The *flgE* Gene of *Campylobacter coli* Is under the Control of the Alternative Sigma Factor σ^{54}

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The *flgE* gene encoding the flagellar hook protein of *Campylobacter coli* VC167-T1 was cloned by immunoscreening of a genomic library constructed in λZAP Express. The *flgE* DNA sequence was 2,553 bp in length and encoded a protein with a deduced molecular mass of 90,639 Da. The sequence had significant homology to the 5′ and 3′ sequences of the *flgE* genes of *Helicobacter pylori*, *Treponema phagedenis*, and *Salmonella typhimurium*. Primer extension analysis indicated that the VC167 *flgE* gene is controlled by a σ^{54} promoter. PCR analysis showed that the *flgE* gene size and the 5′ and 3′ DNA sequences were conserved among *C. coli* and *C. jejuni* strains. Southern hybridization analyses confirmed that there is considerable sequence identity among the hook genes of *C. coli* and *C. jejuni* but that there are also regions within the genes which differ. Mutants of *C. coli* defective in hook production were generated by allele replacement. These mutants were nonmotile and lacked flagellar filaments. Analyses of *flgE* mutants indicated that the carboxy terminus of FlgE is necessary for assembly of the hook structure but not for secretion of FlgE and that, unlike salmonellae, the lack of *flgE* expression does not result in repression of flagellin expression.

Flagella are the locomotory organelles of bacteria. The motility imparted to the bacteria by flagella appears to be an important virulence determinant for a number of species, including the thermophilic *Campylobacter* spp. Worldwide, *Campylobacter jejuni* and *C. coli* are among the most common causes of bacterial diarrhea in humans. Campylobacters colonize the gastrointestinal mucous layer, exhibiting motility within this viscous environment. Nonmotile mutants of *C. jejuni* and *C. coli* are unable to colonize the gastrointestinal tract and subsequently are unable to produce disease in animal models and human volunteers (4, 5, 36, 42).

The flagellar filament of *C. coli* VC167 has been extensively characterized. This strain possesses two tandemly oriented flagellin genes that express highly related species of flagellin protein (10, 13). The FlaA protein is the predominant flagellin species in the filament, although mutational analysis has shown that both FlaA and FlaB flagellins are required for maximum motility (10). The *flaA* gene is under the control of a σ^{28} promoter, while *flaB* is regulated by a σ^{54} promoter (11). The rotation of the flagellar filament is driven by a complex motor located within the cell envelope and mediated through an axial coupling structure, the flagellar hook (30). The 105- by 24-nm campylobacter hook displays a conical protrusion at the proximal end, a concave cavity at the distal end, and helically arranged subunits. The average apparent subunit molecular weight of the campylobacter hook protein measured by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) is 92,500. The FlgE protein of VC167 has been isolated and partially characterized biochemically and immunochemically (43).

In this study, we extended the characterization of the campylobacter flagellar hook. We report here on the sequence of the *flgE* gene and its conservation in campylobacters, as well as the role of the hook in motility, flagellar filament assembly, and the regulation of flagellin synthesis and secretion. In addition, we report on the regulation of the *flgE* gene and on structural requirements for FlgE secretion and hook assembly.

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MATERIALS AND METHODS

Bacterial strains, vectors, and growth conditions. The campylobacter strains used in this study and their Lior serotypes (LIO) are *C. coli* VC167-T1 (LIO8), *C. jejuni* VC156 (LIO8), *C. jejuni* VC159 (LIO8), *C. jejuni* VC87 (LIO1), *C. jejuni* VC83 (LIO4), *C. jejuni* VC88 (LIO5), *C. jejuni* 81-176 (LIO5), *C. jejuni* VC84 (LIO6), *C. jejuni* 81-116 (LIO6), *C. coli* VC95 (LIO7), *C. jejuni* VC91 (LIO11), *C. coli* VC92 (LIO12), *C. coli* VC97 (LIO20), and *C. fetus* VC78. *Campylobacter* spp. were grown on Mueller-Hinton (MH) agar (Difco) or motility agar (10) at 37°C in an atmosphere containing 5% (vol/vol) oxygen and 10% (vol/vol) CO_2. *Escherichia coli* XL1-MRF and XL1-OLR were purchased from Stratagene (La Jolla, Calif.) for propagation of λZAP Express, while *E. coli* DH5α, from New England Biolabs (Beverly, Mass.), was used as the host strain for plasmid cloning experiments (15). Agar was supplemented with 50 μg of kanamycin/ml (Sigma) or 100 μg of ampicillin/ml (Sigma) when required.

Antisera. Antiserum MEP1 was prepared by immunizing an adult New Zealand White rabbit subcutaneously and intramuscularly with SDS-denatured hook protein from *C. coli* VC167-T1 as previously described (43). Polyclonal anti-*C. coli* flagellar antiserum (SML2) was used as previously described (29).

DNA isolation and *flgE* cloning. DNA was isolated from campylobacters as described previously (3) and was partially digested with *Sau*3A. A genomic library of *C. coli* VC167-T1 was constructed in the lambda vector by standard methodology (44). Libraries were screened by plaque immunoblot with antiserum MEP1 against the hook protein of *C. coli* VC167-T1, and reacting plaques were processed as described elsewhere (44). Standard procedures were employed for experiments involving pUC18 plasmid (50) cloning in *E. coli* (44).

DNA sequencing. Sequence reactions were performed on double-stranded templates by using *Taq* polymerase (Perkin Elmer Cetus, Norwalk, Conn.), either dye-labeled terminators or primers, and a Perkin Elmer 480 thermal cycler for analysis with an ABI 373A automated sequencer (Applied Biosystems, Foster City, Calif.). Universal forward and reverse sequencing primers (Pharmacia Canada Inc., Baie d'Urte, Quebec, Canada) and custom primers synthesized on an Applied Biosystems ABI 391 DNA synthesizer were used. Sequence data was collated, assembled, and analyzed with GeneWorks version 2.5 (Intelligenetics, El Camino, Calif.).

Primer extensions. The primer used for the *flgE* primer extension reaction was 5′-ACGCCAGACCAAAGTGATC-3′. This oligonucleotide binds to the mRNA strand 7 bp after the translational start. The primer was labeled with [γ-^{32}P]ATP (DuPont New England Nuclear, Wilmington, Del.) by using T4 polynucleotide kinase (Promega, Madison, Wis.). RNA was extracted from VC167 T1 cells grown on MH agar for 18 h with RNeasy kits from Qiagen (Chatsworth, Calif.). The purified RNA was used in primer extension reactions with a kit from Promega and the *flgE* primer. The primer extension product was run on a 6%

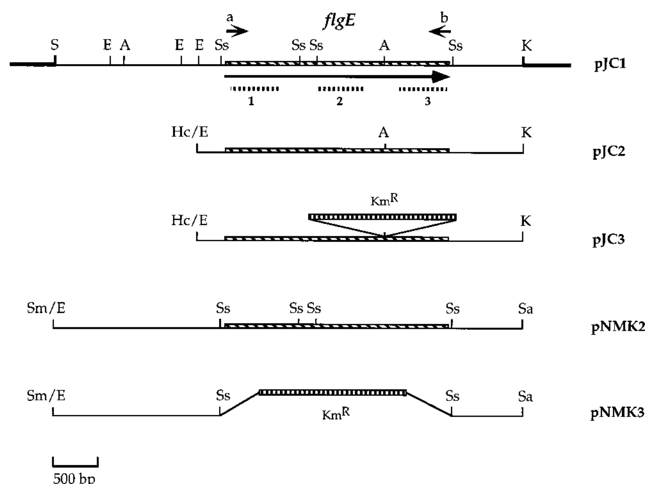

FIG. 1. Physical maps of cloned *C. coli* VC167-T1 chromosomal DNA containing the *flgE* gene (indicated by cross-hatching). The plasmid nomenclature is shown on the right, and the orientation of *flgE* is indicated. Small horizontal arrows represent DNA primers NK1021 (a) and NK1022 (b). The locations of the three PCR products used as probes in Southern blots are also indicated. Abbreviations; S, *Sac*I; E, *Eco*RV; A, *Afl*II; Ss, *Ssp*I; K, *Kpn*I; Hc, *Hin*cII; Sm, *Sma*I; Sa, *Sal*I; Km^R, kanamycin resistance cassette.

(wt/vol) acrylamide sequencing gel in parallel with a DNA sequencing ladder primed with the same ^{32}P-labeled oligonucleotide used in the primer extension. The sequencing ladder was generated with Sequenase version 2.0 (U.S. Biochemicals, Cleveland, Ohio) and pJC1 was used as the template.

PCR amplification of DNA. Oligonucleotides used for amplification of flagellin information were constructed on a Applied Biosystems ABI 391 DNA synthesizer. PCR amplification of chromosomal DNA was performed in a Perkin Elmer 480 thermal cycler essentially as described by Gustafson et al. (14). The samples were subjected to 35 cycles of 1 min of denaturation at 94°C, 1 min of primer annealing at 55°C, and 2 min of extension at 72°C. PCR-generated DNA products were detected by gel electrophoresis on 1.0% agarose. A PCR control containing water instead of DNA was routinely included with each reaction. The 18-bp primer NK1021 (5′-GATGAGATCACTTTGGTC-3′) is located on the plus strand, beginning 1 bp upstream of the translational start site of *flgE*. The 20-bp primer NK1022 (5′-GCTGGATTAGAGTTTGGAGC-3′) is located on the noncoding strand beginning 8 bp from the end of the open reading frame (ORF). Primers NK1032 (5′-TGCAGTAGCTTTAACCCATG-3′) and NK1015 (5′-CACCATAAAAAAGCCATCGC-3′) were used to generate probe 1 (563 bp; Fig. 1). Probe 2 (522 bp; Fig. 1) was generated by PCR amplification with primers NK1039 (5′-CCTTCACTGCCCCAACCGATA-3′) and NK1029 (5′-CAACCCCATATCTAGCATCTC-3′). Probe 3 (554 bp; Fig. 1) was generated with primers NK1041 (5′-CATCGTAGGAACAGCAAG-3′) and NK1022.

Southern hybridizations. Southern hybridization was performed at 68°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate) incorporating 10× Denhardt's solution, 0.25-mg/ml yeast tRNA, 1% (wt/vol) SDS, and 2 mM EDTA. Blots were washed twice in 2× SSC–0.5% (wt/vol) SDS at 68°C for 15 min and twice in 0.5× SSC–0.1% (wt/vol) SDS at room temperature for 15 min (high stringency).

Site-specific mutagenesis of *flgE*. The gene disruption and replacement methods used were essentially those described by Labigne-Roussel et al. (23). The natural transformation method used was that of Wang and Taylor (49). Selection of campylobacter transformants was done on MH agar supplemented with 50 μg of kanamycin/ml.

Fractionation of campylobacter cells. Cells were grown in 200-ml biphasic culture flasks until cultures were at mid-logarithmic phase. Cells were harvested by centrifugation at 10,000 × *g* for 15 min. The supernatant was removed, dialyzed, concentrated 100-fold by lyophilization, and then stored at −70°C. The cell pellet was resuspended in 3 ml of Tris-saline (10 mM Tris-Cl, 30 mM NaCl, pH 7.5), and the cells were lysed by sonication. The sonicate was separated into crude soluble and envelope fractions by centrifugation at 45,000 × *g* for 30 min. The soluble fraction was decanted and centrifuged at 100,000 × *g* for 30 min to remove any remaining envelope protein. The supernatant was concentrated 10-fold by lyophilization and stored at −70°C. The envelope pellet was resuspended in 0.5 ml of Tris-saline and stored at −70°C.

Electrophoresis. SDS-PAGE was performed by the method of Laemmli (24), with a minislab gel apparatus (Hoefer Scientific Instruments, San Francisco, Calif.). Protein solubilized in sample buffer was stacked in 4.5% (wt/vol) acrylamide (constant voltage of 100 V) and separated in 7.5 or 12% (wt/vol) acrylamide (constant voltage of 150 V). For immunological detection, separated protein was electrophoretically transferred to nitrocellulose paper in a Novablot apparatus (Pharmacia LKB, Bromma, Sweden) at 15 V for 40 min by using the methanol-Tris-glycine buffer system of Towbin et al. (47).

Immunoblotting. Following electrophoretic transfer, the reactive sites on the nitrocellulose paper were blocked with 2% (wt/vol) skim milk in 10 mM Tris HCl–0.9% NaCl (Tris-NaCl, pH 7.4) for 1 h. The nitrocellulose paper was then incubated with an appropriate dilution of antiserum in Tris-NaCl for 2 h, washed three times in Tris-NaCl, and incubated with goat anti-rabbit antibody conjugated to alkaline phosphatase (Caltag Laboratories, San Francisco, Calif.) diluted 1:3,000 in Tris-NaCl. After incubation for 1 h, the nitrocellulose was washed three times in Tris-NaCl and the immunoreactive bands were visualized by using the substrate 5-bromo-4-chloro-3-indoylphosphate (Sigma) and nitroblue tetrazolium (Sigma).

Electron microscopy. Formvar-coated grids were floated on 20-μl drops of bacterial cell suspensions. Excess sample was withdrawn by touching the edge of the grid to a cut edge of Whatman no. 1 filter paper. The grids were negatively stained by being floated on drops of 1% (wt/vol) ammonium molybdate (pH 7.0) for 5 min and examined in a Hitachi H7000 or a JEOL JEM-1200EX transmission electron microscope operated at an accelerating voltage of 75 kV under conventional bright-field illumination conditions. Images were recorded on sheet film (Ilford Technical Film) at a nominal magnification of ×50,000.

Nucleotide sequence accession number. The DNA sequence reported here has been deposited in the GenBank database under accession number AF004221.

RESULTS

Cloning of *C. coli* VC167-T1 *flgE*. To clone the *flgE* gene of *C. coli*, a genomic library of strain VC167-T1 in lambda expression vector λZAP Express was constructed as previously described (3). The library was screened for immunoreactivity with antiserum MEP1 (43) raised against purified, SDS-denatured *C. coli* VC167-T1 FlgE. A number of reacting clones were picked and purified, and the pBK-CMV-derivative plasmids were excised (the phagemid pBK-CMV is the vector excised from λZAP Express). One of these FlgE-expressing clones, pJC1, was selected for further study. The physical maps of the plasmids, pJC1 and several pUC and pBR322 subclones derived from it, are presented in Fig. 1.

Western immunoblot analysis indicated that recombinant FlgE was expressed in *E. coli* DH5α harboring pJC1 (data not shown). The apparent molecular mass of the recombinant protein produced in *E. coli* was 92,500 Da as estimated by SDS-PAGE (43), which is indistinguishable from that of the protein expressed in *C. coli* VC167-T1.

Sequencing of *C. coli* VC167-T1 *flgE*. The location of the region comprising the ORF encoding *C. coli* FlgE was initially determined by subcloning the 5.3-kb insert from pJC1 and sequencing various subclones with forward and reverse universal primers. The sequence of the entire gene was subsequently determined by using custom primers to sequence pJC1 directly. The position and orientation of the 2,553-bp ORF encoding FlgE are indicated in Fig. 1. The 851-amino-acid FlgE translation product, which lacks an amino-terminal signal sequence, was predicted to have an M_r of 90,639. The protein encoded by the *C. coli* VC167-T1 hook gene had an overall amino acid composition similar to those of other hook proteins despite its higher predicted molecular weight. Comparison of the deduced primary amino acid sequence of *C. coli* VC167-T1 FlgE with the FlgE sequences of *Helicobacter pylori*, *Salmonella typhimurium*, and *Treponema phagedenis* showed that the four proteins are clearly related. Alignment analysis of the four FlgE proteins for maximal matching by using the algorithm of Needleman and Wunsch (37) showed that the *C. coli* and *H. pylori* proteins share the highest level of identity at 40%, while the *T. phagedenis* and *S. typhimurium* proteins share 30 and 27% identity, respectively, with the *C. coli* protein. This compares to the 28% similarity shown between the *C. coli* VC167-T1 FlgE protein and the flagellins (FlaA and FlaB) from *C. coli* (10, 13, 27). The sequence similarity among the four hook proteins was especially obvious when the 50 residues

A

C. coli FlgE	1	MM-RSLWSG-VSG-LQAHQVAM--DV-EGNNI----SNVNTT-GFKY-SR----------ADF--GTMF--SQTVK--	50
H. pylori FlgE	1	.L-::::::-:N:-M::::I:L--:-I:S::::----A::::-::::-::----------:S:--VD:L--::-::L-	50
S. typhimurium FlgE	1	:---:-F:QA:::-:N:A--:TNL::I-:::::----A:SA:Y-:::--:-GT--------:S:--AD::AG:K-:GL-	50
T. pallidum FlgE	1	::-:::F::-:::-M:N::TR:--::I-:::V----A:::::-::::---:GR--------VN:--QD-LI-::Q--LS	50
C. coli FlaA	1	:GF:INTN--:AA-:N:K--:NS-:L---:SRALDQ:LSRLSS:LRIN:A----------::DAS:-:A--I------	50
C. coli FlaB	1	:GF:INTNI---:A:N::--:--------:------:V::ARELD:SL::LSSGLRINSA::DAS:-:A--I------	50

B

C. coli FlgE	801	EM---K-TA-ALE-----MSN---VDL-SRSLTELII--I--QRG-Y---QAN-SKT-I---STSDQM-LQT-LI-Q---LK--Q	851
H. pylori FlgE	668	SISGS:-----::-----S::---::-::::N::VV---:::-F---:::-::A-V---T::::I-:N:-:L-N---::--:	718
S. typhimurium FlgE	353	---G-:L:NG:::A-----::---:::-:K---::-VNM:VA::N-:---:SN-AQ:-:K---:Q::I-:N:-:V-N---:---R	403
T. pallidum FlgE	413	-----:-----:IAGTLE:::---:::-TDQF:DM:---:T-:::-F---::G-A::-:Q---:::T:-:E:V:--N---::--R	463
C. coli FlaA	523	NV---:-S:---:------:QIRD::FA:E:ANYSKAN:-LA:S:S:AMA:::-:-------:Q--:NV:R--:------:---:	573
C. coli FlaB	523	NV---:--:-:-:------:TIRD::FAA----:S---------------::F::YN:LAQ:G:YA:S-:R-NAV:QNV::LL:	573

FIG. 2. Alignment of deduced amino-terminal (A) and carboxy-terminal (B) 50-amino-acid sequences of *C. coli* FlgE, *H. pylori* FlgE, *S. typhimurium* FlgE, *T. pallidum* FlgE, *C. coli* FlaA, and *C. coli* FlaB. Hyphens indicate gaps introduced to facilitate sequence alignment, and colons indicate residues identical to those of *C. coli* FlgE. Residue numbers are indicated to the left and right of the amino acid sequences.

at the amino-terminal end and the 50 residues at the carboxy-terminal end of the proteins were compared. Alignment analysis showed that at the amino-terminal end, the *H. pylori*, *T. phagedenis*, and *S. typhimurium* FlgE proteins share 72, 58, and 47% identity, respectively, with *C. coli* FlgE, while at the carboxy-terminal they share 61, 56, and 50% identity (Fig. 2). A similar level of amino acid residue conservation was not seen between *C. coli* FlgE and FlaA and FlaB. FlgE shares only 25 and 27% identity with FlaA and FlaB, respectively, at the amino-terminal end and 34 and 32% identity, respectively, at the carboxy-terminal end (Fig. 2).

Phylogenetic tree analysis of the four hook proteins and the *C. coli* VC167 FlaA and FlaB proteins (Fig. 3) shows that the hook proteins evolved on a different branch from the flagellins. However, within the hook protein family, the campylobacter hook protein diverged before that of *H. pylori*, followed by those of *T. phagedenis* and *S. typhimurium.*

Analysis of the promoter region of campylobacter *flgE*. Primer extension analysis was performed to determine the transcriptional start point by using a primer which binds to the coding strand at a position starting 23 bases downstream of the translational start. The results, shown in Fig. 4A, indicate that the mRNA starts 12 bp downstream of a typical σ^{54} polymerase binding site (GG-N_{10}-GC) (39). Figure 4B compares the 5′ noncoding regions of the *flgE* gene to another σ^{54}-regulated flagellar gene of VC167, *flaB*. The two promoters are very similar in the characteristic GG-N_{10}-GC polymerase binding region and in the ribosome binding region but dissimilar elsewhere. The transcriptional start for *flgE* is 70 bp upstream of the translational start, and there is a short inverted repeat (AACGC-N_{20}-GCGTT) at the start of the *flgE* mRNA, suggesting the possibility of translational regulation. In contrast, the *flaB* mRNA starts only 27 bp upstream of the translational start and contains no secondary structures. σ^{54} promoters are also characterized by the presence of upstream enhancer sequences to which regulatory proteins bind and facilitate interaction with the holoenzyme in a manner more typical of eukaryotic polymerases than of prokaryotic polymerases (45). A sequence similar to the upstream enhancers found in front of several nitrogen-regulated genes, TGT-N_{10}-ACA (22), has been reported for *flaB* and is shown in Fig. 4B (TGT-N_7-ACA) (10). A similar sequence (TGT-N_{19}-ACA) is found 88 bp upstream of the transcriptional start point of *flgE*. In addition, both promoters have exceptionally high A+T content in the region between the enhancer and the polymerase binding site (74% A+T for the *flaB* promoter and 77% A+T for the *flgE* promoter in comparison to an overall genomic A+T content of approximately 65%). A high A+T content is thought to facilitate DNA bending and interaction of the polymerase and enhancer, either by binding of host integration factor (22) or by the inherent ability of phased AT-rich tracts of DNA to bend (7).

0.185
S. typhimurium FlgE
0.205
0.185
T. phagedenis FlgE
0.108
0.462
0.389
H. pylori FlgE
0.497
C. coli FlgE
0.018
C. coli FlaA
0.942
0.018
C. coli FlaB

FIG. 3. Unweighted pair group method with arithmetic mean (38) tree showing the evolutionary relatedness of *C. coli* VC167-T1 FlgE to FlgE proteins from *H. pylori*, *S. typhimurium*, and *T. phagedenis* and the FlaA and FlaB proteins from *C. coli* VC167-T1. The numbers are calculated evolutionary distances.

Conservation of campylobacter *flgE*. The primer pair NK1021 and NK1022 selectively amplified a 2,543-bp product from the *C. coli flgE* gene by PCR (data not shown). The same primer pair was used to amplify the *flgE* gene from 16 *Campylobacter* strains from nine Lior serogroups, and similar-size PCR products were obtained from all of the *C. coli* and *C. jejuni* strains tested (data not shown). No PCR product was obtained from *C. fetus* VC78 (data not shown), indicating that the 5′ and 3′ DNA sequences are not conserved in *C. fetus.*

Southern blot analysis of *Cla*I-digested chromosomal DNAs from selected *Campylobacter* strains was performed by using

A

```
        1      GATC        A
                           A
                           G
                           T
                           G           C
   *                       T* ->       A*   +1
                           A           T
                           T           A
                           T
                           T
                           G
```

B

```
TGTGATTTTAGTAATTTTTATTACACAAAAGCCCCAAAGCTTCATAAACTCCCAAAATAC  flgE
 ::   :  :  : : ::::        :         :         ::  :    : ::
AGTATATAAAACATTCTTTAGCGTTTACTGTAATTTATACAAATCCAAGCCTAGTAGTAA  flaB

                                                     -26
CTTTAAATTTTTTCTATAAATTTTTAAAAAAATTCATAAAAAAATTTCACAAGTTGGAACAGA flgE
   ::    ::::: :::    :    :::::  ::::       :::  :: :: ::::::::
TACTAGGCTTTTTTTATTTCTAAATAAAAT-TTCAATTTGAAT--CAAAACTTGGAACACT  flaB

              mRNA
  -12           *      ____                  ____
ACTTGCTGTAAACTTCACATAAACGCAAAAGTTTTTTAAAGCCAAAGCGTTAAATTTTTTA flgE
 :::::: ::: :::   :
TCTTGCTTTAATCTTTTCGAT---------------------------------------- flaB

                rbs
AAGCAATATTTTATAAAGGATTTAAGATG                                flgE
  ::::::::::   ::::::::::: :::
--GCAATATTTTGA-AAGGATTTAAAATG                                flaB
```

FIG. 4. Analysis of the *flgE* promoter. (A) Determination of the *flgE* transcriptional start site by primer extension. Lane 1 shows the product of the *flgE*-specific primer extended on VC167 T1 mRNA; the corresponding sequencing ladder generated with the same primer is also shown (GATC), and the 5′ end of the mRNA sequence is indicated on the right. (B) Comparison of two σ^{54} promoters from *C. coli* VC167. The regions 5′ to the coding regions of *flgE* and *flaB* are compared; colons indicate identical residues, and dashes indicate gaps introduced into the *flaB* sequence to maximize alignment. The ATG in bold letters indicates the translational start of each gene. The mRNA start sites (in bold letters) are indicated, and the putative RNA polymerase binding sites (−26 and −12) and the ribosome binding site (rbs) are indicated. An inverted repeat at the start of the *flgE* mRNA is overlined. Putative upstream enhancer sequences are underlined and italicized.

FIG. 5. Southern blot hybridizations of the complete *flgE* gene (A) and probes for the 5′ (B), central (C), and 3′ (D) regions of *Campylobacter* DNA. A 1.5-μg sample of chromosomal DNA, digested with *Cla*I, from each strain was loaded onto each lane. Lanes: 1, *C. coli* VC167-T1 (LIO8); 2, *C. jejuni* VC156 (LIO8); 3, *C. jejuni* VC159 (LIO8); 4, *C. jejuni* VC87 (LIO1); 5, *C. jejuni* VC83 (LIO4); 6, *C. jejuni* VC88 (LIO5); 7, *C. jejuni* 81-176 (LIO5); 8, *C. jejuni* VC84 (LIO6); 9, *C. jejuni* 81-116 (LIO6); 10, *C. coli* VC95 (LIO7); 11, *C. jejuni* VC91 (LIO11); 12, *C. coli* VC92 (LIO12); 13, *C. coli* VC97 (LIO20); 14, *C. fetus* VC78. Molecular size markers are indicated on the right.

the entire *flgE* gene, generated by PCR with oligonucleotides NK1021 and NK1022, as the probe. Although this probe hybridized to all of the *C. coli* and *C. jejuni* strains tested, there was considerable restriction fragment length polymorphism, as well as some variation in signal strength (Fig. 5A). For example, the probe hybridized weakly to *C. jejuni* VC91 (Fig. 5A, lane 11) and *C. coli* VC97 (Fig. 5B, lane 13). To examine conservation in different regions of the gene, probes for the 5′ end (probe 1; Fig. 1), the central region (probe 2), and the 3′ end (probe 3) of the *flgE* gene were generated by PCR (Fig. 1). The most conserved regions were those represented by probes 1 (Fig. 5B) and 3 (Fig. 5D); these probes hybridized strongly to all of the strains of *C. coli* and *C. jejuni* examined. Probe 2 also hybridized very strongly to most of the strains examined (Fig. 5C) but hybridized weakly to strain 81-176 (Fig. 5C, lane 7) and failed to hybridize at all to the two strains which hybridized weakly to the full-length *flgE* probe: VC91 (Fig. 5C, lane 11) and VC97 (Fig. 5C, lane 13). None of the probes hybridized to DNA from *C. fetus* VC78 (Fig. 5, lane 14).

Site-specific mutagenesis of FlgE. Two mutations in the *flgE* gene, an insertion and a deletion-replacement, were constructed by using a kanamycin resistance cassette from pILL600. To construct the first mutant, an *Sma*I-ended kanamycin resistance cassette was inserted into the *Afl*II site of pJC2 (Fig. 1). The resulting plasmid, pJC3, was transformed into *C. coli* VC167-T1, and kanamycin-resistant colonies were isolated. Mutants were confirmed to represent double crossovers by PCR with primers NK1021 and NK1022 and by hybridization with probes for *flgE* and the kanamycin resistance gene (data not shown). The results were consistent with allelic replacement of the wild-type *flgE* gene with the mutated allele, which was predicted to encode a truncated protein lacking the terminal 248 amino acids with an M_r of 64,460. Western immunoblot analysis using polyclonal antihook antibody MEP1 (43) showed that this hook mutant, *C. coli* VC167-T1*flgE*::Km^r,

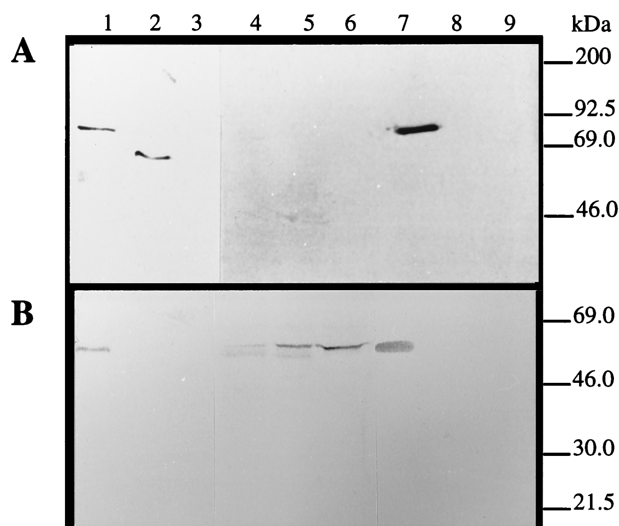

FIG. 6. SDS-PAGE analysis of the effect of insertional inactivation mutagenesis on FlgE and flagellin expression in *C. coli* VC167-T1. Cell fractions from the wild type and mutants were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with a 1:5,000 dilution of rabbit polyclonal anti-*C. coli* FlgE protein antiserum (A) and a 1:10,000 dilution of rabbit polyclonal anti-*C. coli* flagellin antiserum (B). Lanes: 1, *C. coli* VC167-T1 supernatant fraction; 2, *C. coli* VC167-T1*flgE*::Km^r supernatant fraction; 3, *C. coli* VC167-T1Δ*flgE* supernatant fraction; 4, *C. coli* VC167-T1 soluble fraction; 5, *C. coli* VC167-T1*flgE*::Km^r soluble fraction; 6, *C. coli* VC167-T1Δ*flgE* soluble fraction; 7, *C. coli* VC167-T1 envelope fraction; 8, *C. coli* VC167-T1*flgE*::Km^r envelope fraction; 9, *C. coli* VC167-T1Δ*flgE* envelope fraction. Molecular size standards are indicated on the right.

produced a truncated hook protein with a molecular mass of 64.5 kDa which was secreted into the culture supernatant (Fig. 6A, lane 2). This same blot shows that the hook protein was also detected in the supernatants from the parental strain (Fig. 6A, lane 1) and that the amount of hook protein found in the supernatant was approximately the same in both the wild type and the mutant. The wild-type hook protein was present at high levels in the envelope fraction of cells (Fig. 6A, lane 7), but the truncated hook protein was not detectable in the envelope fraction of the mutant (Fig. 6A, lane 8). No hook protein was visible in the soluble fractions of either the wild type or the insertion mutant (lanes 4 and 5, respectively). These data suggest that the carboxy-terminal region of FlgE is necessary for its assembly into a hook structure but not for its secretion.

The second mutant was constructed by cloning the 5.3-kb *Sal*I-*Sma*I fragment from pJC1 into the *Eco*RV-*Sal*I site of pNMK1, a derivative of pBR322 which lacks the *Ssp*I site, generating pNMK2. The *Sma*I-ended kanamycin resistance cassette from pILL600 was inserted between the two outermost *Ssp*I sites of pNMK2 indicated in Fig. 1, resulting in a deletion from 21 bp before the start codon to 22 bp after the stop codon of the *flgE* gene. The resulting plasmid, pNMK3, was transformed into *C. coli* VC167-T1. The resulting mutant, *C. coli* VC167-T1Δ*flgE*, was again confirmed to have resulted from a double crossover by PCR with primers NK1021 and NK1022 and by Southern hybridization analyses with probes for *flgE* and the kanamycin resistance cassette (results not shown). Immunoblot analysis of this mutant with MEP1 antiserum confirmed that the mutant did not produce hook protein in any cellular fraction (Fig. 6A, lanes 3, 6, and 9).

Mutants were nonmotile when examined by phase-contrast microscopy and motility agar and lacked the flagellar filament and hook when examined by electron microscopy (data not shown).

Effect of *flgE* mutation on flagellin production. In *S. typhimurium*, mutation of the hook protein resulted in FlgM-mediated repression of σ^{28}-controlled flagellin synthesis (17, 31). However when cell fractions of both hook mutants of *C. coli* VC167-T1 were examined by Western immunoblot analysis with polyclonal antiflagellin serum SML2, both mutants were shown to produce flagellin, which, compared to the wild type, appeared to accumulate in the soluble fraction (Fig. 6B, lane 5 and 6). The level of intracellular flagellin was similar in both hook mutants but more than that observed in wild-type cells (Fig. 6B, lane 4). No flagellin was detected either in the supernatant (Fig. 6B, lanes 2 and 3) or cofractionating with the envelope fraction (Fig. 6B, lanes 8 and 9) of either hook mutant. Predictably, flagellin was seen in the wild type in both the supernatant (Fig. 6B, lane 1) and at higher levels in the membrane fraction where it cofractionated (Fig. 6B, lane 7). Primer extension analyses using RNA isolated from VC167-T1Δ*flgE* indicated that both the *flaA* and *flaB* genes were expressed (data not shown).

DISCUSSION

The size of the campylobacter *flgE* gene appears to have remained conserved among various strains and is consistent with a gene encoding a 90,639-Da protein, the largest hook protein reported to date. It is significantly larger than the 42,000-Da hook proteins of *S. typhimurium* (1) and *E. coli* (8), the 55,000-Da hook protein of *T. phagedenis* (26), and the 70,000-Da FlgE protein of *C. crescentus* (25) and is also larger than the 78,000-Da and 87,500-Da hook proteins of *H. pylori* and *H. mustelae* (41). Despite this larger subunit size, however, the overall amino acid composition of the campylobacter FlgE protein is consistent with that of other hook proteins. Indeed, campylobacter FlgE is also a characteristic flagellar axial protein. For example, the absence of cysteine from the protein is consistent with the absence of this amino acid in the various axial proteins comprising the *Salmonella* flagellum (16). In addition, at both termini, the *C. coli* FlgE protein contains the heptad repeats of hydrophobic residues characteristic of the axial proteins of the flagellar system (16). Beginning at residue 10, *C. coli* FlgE also contains the SGL consensus typical of the axial components, and beginning at residue 23, the GNNISN sequence is clearly a variant of the extended ANNLAN consensus found among these flagellum components (16). Similarly, beginning at residue 818 in the carboxy-terminal end, *C. coli* FlgE displays the diverged variant SLTELI of the ELVNMI axial component consensus (16). The role of these various consensus sequences is unclear, but Homma et al. (16) have suggested that they may possibly be involved in subunit-subunit interaction or in recognition of the protein for secretion. The results obtained here with the truncated FlgE mutant *C. coli* VC167-T1*flgE*::Km^r show that the carboxy terminus of the protein is not required for secretion of the hook protein but is necessary for the polymerization of FlgE monomers and/or interaction with the rod of the basal body. This is consistent with other findings that the carboxy-terminal region of the salmonella hook protein is necessary for stabilization of the hook filament (48), while the amino-terminal region of the *Caulobacter* hook protein has been shown to be necessary for secretion (21).

As indicated by the strain-to-strain differences in signal strength in Southern hybridization analyses and by the presence of restriction fragment length polymorphisms, there appears to be sequence divergence among campylobacter *flgE*

genes. The data indicate that there is considerable variation in the central region of the gene and higher conservation in the 5′ and 3′ ends. The high conservation at the 3′ end is consistent with the mutational data described above, strengthening the notion that this region encodes an area of the hook protein monomer which plays a key role in the functional interaction with other hook monomers to form the hook structure. This arrangement of *flgE* sequence homology is typical of other flagellar components (16, 31) and is consistent with the observed antigenic diversity among campylobacter hook proteins (18, 28). Indeed, antigenic divergence of the campylobacter hook protein is more complex than the *Bacillus*, *Caulobacter*, and *Salmonella* hook proteins, perhaps because of the size of the central variable domain. Since the flagellar hook protrudes from the bacterial cell surface, it is well positioned to contribute to the antigenic diversity of this group of pathogenic bacteria. Previous studies in this laboratory (28) and by Jin and Penner (18) have shown that a 92,500-Da protein can contribute to the antigenic differences observed among *Campylobacter* strains. Moreover, a protein with an M_r of 92,000 has been shown be immunogenic in *Campylobacter* infection of humans (34).

C. coli was the first organism reported to have the genes encoding its flagellar filament proteins under the control of two alternative sigma factors. Both flagellin proteins are produced concomitantly, but *flaA*, which is controlled by a σ^{28} promoter, is expressed at much higher levels than σ^{54}-regulated *flaB* (10). The relative signal strength of the *flgE* promoter in *C. coli* is stronger than that of the *flaB* promoter as measured by primer extension analyses done in parallel (data not shown). We have shown here that FlgE is expressed at high levels from its σ^{54} promoter in *E. coli*. However, despite the fact that the *flaB* σ^{54} promoter has been shown to be active in *E. coli* when fused to chloramphenicol acetyltransferase (2), we have been unable to detect a FlaB product in *E. coli* by Western analysis (data not shown). These apparent differences in expression of FlaB and FlgE in *E. coli* may reflect either differences in recombinant protein stability or differences in promoter strength due to sequence differences outside of the polymerase binding site in these two σ^{54}-regulated genes. These differences include the positioning of the promoter relative to the translational start codon, the corresponding length of the untranslated mRNA leader, and/or the positioning and sequences of the putative upstream enhancer sequences. In addition, the *flgE* mRNA has a unique short inverted repeat which starts 4 bp from the message start point, which could affect translation. Previous studies have shown that the *flaB* σ^{54} promoter is subject to environmental and growth phase-dependent regulation (2). Given the differences between the noncoding regions of these two genes and the apparent differences in the level of expression from these two σ^{54} promoters, it will be interesting to determine the effects of environment and growth phase on *flgE* expression in a quantitative manner as has been done for *flaB* (2).

Only a few genes in the campylobacter flagellar regulon have been described. In addition to the hook gene described here, other reported genes include the two flagellin structural genes (13, 40), two genes involved in glycosylation of the flagellin subunits (12), a gene required for motor function (51), and a gene called *flhA* (32, 33), whose homologous products in *S. typhimurium* and *B. subtilis* are involved in either regulation of flagellin expression or flagellar biogenesis (6, 20). In *S. typhimurium* and *B. subtilis*, early flagellar gene expression is coupled to late flagellar gene expression by an anti-σ^{28} factor, FlgM (9, 35). Since FlgM is normally exported through the flagellar filament, any mutation affecting filament structure results in increased intracellular levels of FlgM and repression of the σ^{28}-controlled flagellin gene. However, unlike mutations affecting flagellar structure in the family *Enterobacteriaceae*, mutation of *flgE* in campylobacters and helicobacters, which also have two flagellin genes, one controlled by a σ^{28} promoter and one controlled by a σ^{54} promoter (19), results in intracellular accumulation of flagellin. Mutation of *flhA* in campylobacters and the corresponding mutation in *H. pylori* (called *flbA*) does result in lack of expression of flagellin (32, 46). However, in a *flbA* mutant of *H. pylori*, the *flgE* gene is expressed and a hook structure is assembled, suggesting another difference in the regulatory cascade.

In summary, motility plays an important role in the ability of *Campylobacter* spp. to colonize their intestinal niche and produce disease. The flagellar hook plays an essential role in this process, being required for flagellin secretion, flagellar filament formation, and motility. Regulation of expression of FlaA, FlaB, and FlgE appears to be complex, involving not only two different classes of promoters but also, in the case of the *flaB* and the *flgE* genes, two different σ^{54}-type promoters which appear to be differentially regulated.

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