

Molecular Cloning and Characterization of the *Helicobacter pylori* *fliD* Gene, an Essential Factor in Flagellar Structure and Motility

JANG SEONG KIM, JI HOON CHANG, SOO IL CHUNG, AND JUNG SUN YUM*

Mogam Biotechnology Research Institute, Koosung-myon, Yongin-city, Kyonggi-do 449-910, Korea

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***Helicobacter pylori* colonizes the human stomach and can cause gastroduodenal disease. Flagellar motility is regarded as a major factor in the colonizing ability of *H. pylori*. The functional roles of flagellar structural proteins other than FlaA, FlaB, and FlgE are not well understood. The *fliD* operon of *H. pylori* consists of *flaG*, *fliD*, and *fliS* genes, in the order stated, under the control of a σ^{28} -dependent promoter. In an effort to elucidate the function of the FliD protein, a hook-associated protein 2 homologue, in flagellar morphogenesis and motility, the *fliD* gene (2,058 bp) was cloned and isogenic mutants were constructed by disruption of the *fliD* gene with a kanamycin resistance cassette and electroporation-mediated allelic-exchange mutagenesis. In the *fliD* mutant, morphologically abnormal flagellar appendages in which very little filament elongation was apparent were observed. The *fliD* mutant strain was completely nonmotile, indicating that these abnormal flagella were functionally defective. Furthermore, the isogenic *fliD* mutant of *H. pylori* SS1, a mouse-adapted strain, was not able to colonize the gastric mucosae of host mice. These results suggest that *H. pylori* FliD is an essential element in the assembly of the functional flagella that are required for colonization of the gastric mucosa.**

Helicobacter pylori is a gram-negative, microaerophilic bacterium which colonizes the gastric antrum of the human stomach. Since Marshall and Warren first isolated and cultured this bacterium from biopsy specimens (25), extensive studies of its biology have been carried out and its complete genome sequence has been published (37). Epidemiological studies have consistently demonstrated that *H. pylori* is a causative agent of active chronic gastritis and peptic ulcers and that *H. pylori* is a primary risk factor for the development of intestinal type gastric adenocarcinoma (6, 15). More recently, this organism was also associated with mucosa-associated lymphoid tissue (MALT) and with B-cell MALT lymphomas (31). However, the actual mechanism by which gastroduodenal diseases develop in response to *H. pylori* infection remains unknown.

The putative pathogenic factors of *H. pylori* are categorized as colonization, persistence, and disease-inducing factors. Colonization in the host is a prerequisite of bacterial infection and subsequent pathogenesis. Motility by unipolar flagella, urease production, and adhesion to the gastric epithelial cells are all required for *H. pylori* colonization. A bacterial flagellum consists of three distinct parts connected in series: a basal body in the membrane, a short curved rod called the hook, and a long helical filament. The filament and the hook are each formed by regular assembly of a single species of protein subunit. Proteins called hook-associated proteins (HAPs) are required for joining the filament to the hook and for capping the distal tip of the filament. HAP1 and HAP3 are thought to function as structural adapters between the hook and the filament (13). HAP2 is thought to function as a capping structure at the distal end of the filament, which enables flagellin monomers to assemble into filaments (13, 14).

H. pylori is a motile, flagellated organism that probably penetrates the mucus layer by means of spiralling movements

associated with its unique shape. The motility of *H. pylori* is provided by two to six polar, sheathed flagella, the filaments of which consist of two flagellin types, encoded by the genes *flaA* and *flaB* (35). The majority of the filament is composed of FlaA, while FlaB is found only in the base (18). The flagellar filament is linked to the basal body by means of the hook, which is a polymer of the FlgE hook protein (30). In vitro experiments with isogenic *H. pylori* strains with mutated *flaA* and *flaB* flagellin genes have shown that both flagellins are required for full motility (16). Colonization experiments with spontaneous nonmotile mutants, as well as with isogenic *flaA*, *flaB*, and *flaA flaB* mutants in the gnotobiotic piglet model of *H. pylori* infection, have demonstrated that the establishment of persistent infection requires full motility and the presence of both flagellins (4).

Full motility is an essential virulence factor of *H. pylori* and a potential target for therapy and vaccine development.

We describe here the molecular characterization of the *H. pylori* *fliD* gene, which encodes a 76-kDa HAP-2 homologue, and the effects of a mutation in the *fliD* gene on the assembly of flagellar filaments and on motility. Infection of mice with a *fliD* mutant of *H. pylori* SS1, a mouse-adapted strain, demonstrated that the FliD protein is necessary for colonization. The *H. pylori* *fliD* gene is interesting in that it is a structural gene which appears to play a role in genetic regulation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *H. pylori* KCTC0217BP (Korean Collection for Type Cultures) was isolated from a Korean patient suffering from duodenal ulceration. *H. pylori* SS1 (Sydney strain) was kindly provided by A. Lee (University of New South Wales, Sydney, Australia). *H. pylori* strains were grown on selective blood agar plates containing 22 g of Columbia blood agar base (Difco)/liter, 20 g of tryptic soy agar (Difco)/liter, 7% (vol/vol) sheep blood, 10 μ g of vancomycin (Sigma)/ml, 300 U of colistin (Sigma)/ml, and 2.5 μ g of amphotericin B (Sigma)/ml. Plates were incubated at 37°C for 48 to 72 h under 10% CO₂.

Expression of recombinant FliD protein. The *fliD* gene was amplified by PCR with the oligonucleotide primers *fliD*/NNdeI and *fliD*/CBamHI (Table 1) according to standard PCR protocols. The amplified DNA fragment of 2,076 bp was digested with restriction enzymes *Nde*I and *Bam*HI and then ligated into *Nde*I/*Bam*HI-digested pET-15b (Novagen). The resulting plasmid was trans-

* Corresponding author. Mailing address: Mogam Biotechnology Research Institute, 341 Pojung-ri, Koosung-myon, Yongin-city, Kyonggi-do 449-910, Korea. Phone: 82-331-262-3851. Fax: 82-331-262-6622. E-mail: jsyum@kgcc.co.kr.

TABLE 1. Oligonucleotides used for PCR amplification

<i>H. pylori</i> gene	Primer	Sequence ^a	Position	Strand
<i>fliD</i>	fliD/NNdeI	<u>ggaattcc</u> atATGGCAATAGGTTTCATT	853–869	+
	fliD/CBamHI	c <u>gggatcc</u> TTAATTCTTTTGGCCGC	2893–2910	–
	fliDseq#2	GATATTTTGTAGCCAAGTGG	1216–1234	+
	fliDseq#4	TTCTTATAGCGTGCATACGG	2457–2476	+
	fliDseq#5	TCTAAAGCCTGCTGTATCGC	1621–1640	–
<i>ureA</i> ^b	UA5	TTGATGCTCCACTACGCTGG	2689–2708	+
	UA3	GGGGTATGCACGGTTACGAG	2935–2954	–
	UALP	CACCCCAAAAGAGTTAGATA	2667–2686	+
	UARP	ATGTCTAAGCGTTACCGAA	3142–3161	–

^a Lowercase letters indicate sequences not homologous to the *H. pylori* sequence. Underlined nucleotides represent the *NdeI* (fliD/NNdeI) and *BamHI* (fliD/CBamHI) restriction sites used to clone the PCR product.

^b The nucleotide sequence accession number is X57132 (21).

formed into *Escherichia coli* BL21(DE3). The recombinant FliD protein was expressed and purified by using the pET His tag system according to the manufacturer's instructions.

Antibody preparation. Polyclonal antibody against the outer membrane proteins (OMP) of *H. pylori* was produced in a rabbit. The animal was immunized with 140 µg of Sarkosyl-insoluble OMP prepared from *H. pylori* KCTC0217BP and boosted twice after 1 and 4 weeks, and blood was collected 10 days after the last injection. This anti-OMP antibody preparation was employed for genomic DNA library screening and Western blot analysis. Polyclonal antibody against *H. pylori* FliD was produced in a similar manner with 200 µg of purified recombinant FliD protein.

Electroporation of *H. pylori*. *H. pylori* KCTC0217BP cells were harvested from blood agar plates, washed in a 15% glycerol–9% sucrose solution, and suspended in a final volume of 50 µl of glycerol-sucrose solution at 4°C (10¹⁰ bacteria per ml). Supercoiled plasmids (700 ng) containing the targeted gene that had been disrupted with a kanamycin resistance cassette were added to the cells. After a 1-min incubation on ice, the cells and DNA were transferred to a prechilled electroporation cuvette (0.2-cm electrode gap) and placed in a Gene Pulser apparatus (Bio-Rad). Pulses were applied with the electronic variables set to 25 F, 1.25 kV, and 200 Ω, giving a time constant of 4.6 ms. After electroporation, bacteria were grown on nonselective plates for 48 h to allow expression of antibiotic resistance and then transferred onto plates containing kanamycin (20 µg/ml) and grown for up to 6 days.

RNA isolation and Northern blot analysis. Total RNA was extracted with Trizol reagent (Gibco BRL) from *H. pylori* KCTC0217BP and the *fliD* mutant of this strain grown for 48 h. Ten micrograms of RNA was resolved on 1% denaturing formaldehyde-agarose gels and transferred to a Hybond-N membrane (Amersham) by capillary blotting. The PCR product, amplified by primers flaA-5 (5'-GGAATTCATATGGCTTTTCAGGTCAA-3') and flaA-3 (5'-GCTCTAGA CTAAGTTAAAGCCTTAAG-3'), with genomic DNA of *H. pylori* KCTC0217BP as a template, was radiolabelled with the Megaprime DNA-labelling system (Amersham) and used as a probe. Hybridization was performed in ExpressHyb hybridization solution (Clontech) at 68°C for 18 h. The filter was washed at room temperature with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.05% sodium dodecyl sulfate (SDS) for 45 min and at 50°C for 30 min with 0.1× SSC–0.1% SDS. After autoradiography, RNA expression was quantified by densitometric analysis with a ScanJet 4c scanner (Hewlett-Packard) with SigmaGel, version 1.0, software.

Electron microscopy. Bacteria were harvested from blood agar plates and gently resuspended in phosphate-buffered saline. For negative staining, a Formvar carbon-coated grid was floated on a drop of the sample suspension for 5 min. Excess sample was withdrawn by touching the edge of the grid to a cut of Whatman no. 1 filter paper. The grids were then floated onto a drop of 1% phosphotungstate (pH 6.8) for 1 min. The grids were examined with a JEOL JEM-1200EX or JEM-1010 transmission electron microscope.

Motility testing. Motility was initially judged by viewing the movement of bacteria grown in liquid medium with an Olympus BX50 phase-contrast microscope. We also performed stab agar and single-colony motility tests to evaluate bacterial motility. Motility plates containing brucella broth (Difco) and 0.4% Bacto Agar were supplemented with 10% horse serum and antibiotics as described above. For the stab agar test, plates were inoculated by placing small slices of blood agar plates, densely grown with the strain to be tested, on the surface of the motility plates with the lawn side of the slice facing upward. Plates were incubated at 37°C for 4 days under microaerophilic conditions, and motility was assessed by examining the swarming halo. To examine single-colony motility, bacterial cells were harvested in brucella broth and diluted to about 10¹ to 10² cells/ml with motility medium. This bacterial suspension was poured onto the plates and incubated for up to 5 days. Single-colony morphology was examined with a phase-contrast microscope.

Animal experiments. Specific pathogen-free, 6-week-old, female, C57BL/6 mice (Charles River) were inoculated with the bacterial suspension. The animals were dosed three times in a 2-day period with 0.4 ml of bacterial suspension (approximately 10⁸ cells) by using a blunt-ended needle. Mice were sacrificed 10 days after the last inoculation. The stomach was excised, cut along the lesser curvature, and rinsed in saline to remove the contents. Half of the stomach was placed in 0.5 ml of brain heart infusion broth (Difco) and disrupted with a pellet pestle (Kontes Scientific Glassware), and the suspension was plated onto selective blood agar plates. The level of colonization was determined numerically by counting the viable bacteria thus recovered. Bacterial counts were expressed as CFU per gram of gastric tissue. The other half of the stomach was treated as previously described (9) and used for PCR amplification of the *H. pylori ureA* gene (21) to detect the presence of the bacteria. A nested PCR method was applied by using the *ureA*-specific oligonucleotide primers (UALP and UARP for primary PCR, UA5 and UA3 for nested PCR) listed in Table 1.

Nucleotide sequence accession number. The nucleotide sequences of the *fliD* operon have been deposited in GenBank under the accession no. U82981.

RESULTS

Library screening and identification of clones expressing a 76-kDa antigenic protein. A genomic expression library was constructed in Lambda ZapII (Stratagene) with *HaeIII*-digested genomic DNA of *H. pylori* KCTC0217BP and screened with anti-*H. pylori* OMP antibody. Approximately 1.5 × 10⁵ plaques were screened, and 11 positive plaques were selected. Each clone was transformed into a pBluescript SK(–) phagemid by in vivo excision (33). When the protein profile of each clone was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting, nine clones were found to express an *H. pylori*-specific protein with an estimated molecular mass of 76 kDa. A protein band of the same size was detected in *H. pylori* OMP as well as in whole-cell lysates with anti-*H. pylori* OMP antibody (data not shown). Plasmid DNA was isolated from each clone expressing the 76-kDa protein and digested with *EcoRI*; DNA inserts of 3.4 and 5 kb were observed, and a plasmid containing a 3.4-kb insert was selected for further study.

Nucleotide and amino acid sequence analysis of the *fliD* gene. A restriction map of the selected clone, which was designated pFLID, was constructed (Fig. 1). The entire 3,390-bp nucleotide sequence of the pFLID insert was determined. The sequenced DNA fragment contained three open reading frames (ORFs), which appeared to comprise a single operon. The products of these ORFs showed significant homology with the bacterial FlaG, FliD, and FliS proteins, as demonstrated by an amino acid sequence homology search with the BLAST network service at the National Center for Biotechnology Information (1). The organization of these ORFs is shown in Fig. 1. A σ²⁸-dependent promoter (5'-TtAA-N15-GCCGATAT-3' in the *H. pylori* –35/–10 region, consistent with the *E. coli*

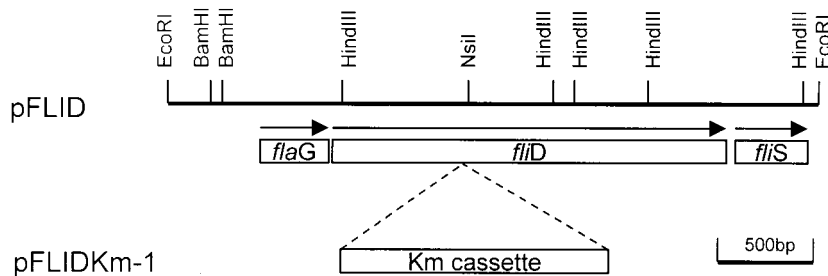


FIG. 1. Restriction maps of the recombinant plasmids pFLID and pFLIDKm-1. The locations of three ORFs (*flaG*, *fliD*, and *fliS* genes) are indicated by arrows. pFLIDKm-1 was constructed by inserting a kanamycin resistance cassette into the *NsiI* restriction site of pFLID. Only the inserts of the plasmids are depicted.

promoter sequence 5'-TAAA-N15-GCCGATAA-3' [nucleotides in the *H. pylori* sequence differing from those in the *E. coli* sequence are in lowercase]) was found upstream of the operon, and a Shine-Dalgarno ribosome binding site was found upstream of each ORF. Two transcription start points, nucleotide A at positions 417 and 419, were identified by primer extension experiments (data not shown). The *fliD* operon had the same gene organization in *H. pylori* KCTC0217BP and 26695, the complete genome sequences of which have been published previously (37). The amino acid sequences of FlaG and FliS from these two *H. pylori* strains showed significant homology, with 94% identity and 97% similarity for FlaG and identical sequences for FliS. However, FliD from these two strains showed size differences, having 685 amino acids in *H. pylori* KCTC0217BP and 674 amino acids in *H. pylori* 26695. The amino acid sequence divergence mainly occurred in the C-terminal region, while the first 654 residues from the N terminus showed 96% identity and 97% similarity. To rule out the possibility that this difference was due to misreading the *fliD* DNA sequences, we repeated the DNA sequencing and confirmed that the nucleotide sequences presented here are correct.

Based on the calculated molecular masses of the proteins encoded by each ORF, the 76-kDa protein which reacted with anti-*H. pylori* OMP antibody appeared to be encoded by the second ORF. To confirm this, the N-terminal amino acid sequence of the partially purified 76-kDa protein was determined. The amino acid sequence thus obtained, AIGSLSSLG LGSKVL, matched residues 2 to 16 of the deduced amino acid sequence encoded by the second ORF perfectly, confirming that this ORF, designated the *fliD* gene, encodes a 76-kDa protein which is a HAP2 homologue of *H. pylori*. When the amino acid sequence of *H. pylori* FliD was aligned with HAP2 sequences from other bacteria, such as *Salmonella typhimurium* (12), *Pseudomonas aeruginosa* (2), *Xenorhabdus nematophilus* (7), *Bacillus subtilis* (3), and *Vibrio parahaemolyticus* (26), sequence similarities were predominantly apparent in the N- and C-terminal regions. The only observed modification in the N-terminal sequence of *H. pylori* FliD protein was the absence of the first methionine residue. The N-terminal amino acid sequence of FliD did not contain any conventional signal sequences for export, indicating that the FliD is not transported via the primary cellular pathway for protein export. *H. pylori* FliD, like the other axial components of the flagellum, contained no cysteine residues. The proline content of *H. pylori* FliD was 1.75 mol%; there were 12 proline residues in the entire sequence, and these residues were not evenly distributed throughout the sequence but rather seemed to be clustered. There were no proline residues in the last 109 amino acids from the C terminus. In this region, we observed a repeated motif of hydrophobic amino acids at intervals of seven resi-

dues, starting from isoleucine at position 613 to alanine at position 680. Such hydrophobic heptad repeats have also been reported in other bacterial HAP2 proteins (3, 12, 26) as structural elements for quaternary interactions in the flagellar axial structures. In contrast, the N-terminal region of FliD lacked such hydrophobic heptad repeats and contained proline residues.

Construction of an isogenic *fliD* mutant by allelic-exchange mutagenesis. Bacterial HAP2 plays a role in flagellar morphogenesis as a flagellar capping protein, facilitating polymerization of the flagellin monomer at the tips of filaments (14). In order to examine the function of FliD in *H. pylori*, an isogenic mutant of *H. pylori* KCTC0217BP which would result in the null expression of FliD was constructed by the following method. A 1.4-kb *SmaI* restriction fragment of pILL600 (5) containing a gene encoding resistance to kanamycin (*aph3'-III*) was cloned into the unique *NsiI* site situated in the middle of the *fliD* gene after treatment of linearized pFLID with mung bean nuclease. The resulting plasmid was called pFLIDKm-1 (Fig. 1). *H. pylori* KCTC0217BP was transformed with pFLIDKm-1 by electroporation, and two independent transformants were obtained. Mutant strains were defined as descendants of a single colony of kanamycin-resistant *H. pylori*, and the genotypes of the mutants were characterized by PCR (Fig. 2A) with oligonucleotide primers specific for sequences upstream and downstream of the site of disruption (*fliD*seq#2 and *fliD*seq#5; Table 1). In mutant strains, a 1.8-kb DNA

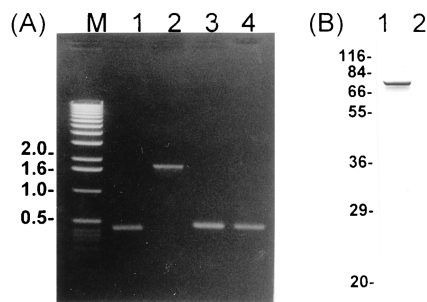


FIG. 2. Characterization of *H. pylori* KCTC0217BP wild type and isogenic *fliD* mutant by PCR (A) and Western blotting (B). (A) Lanes 1 and 2, DNA amplified by PCR with primers (*fliD*seq#2 and *fliD*seq#5) annealing up- and downstream of the kanamycin cassette insertion site in wild-type and mutant strains, respectively; lanes 3 and 4, DNA amplified by PCR with primers (*fliD*seq#4 and *fliD*/CBamHI) annealing downstream of the insertion site in wild-type and mutant strains, respectively; lane M, DNA size markers (in kilobases). (B) Western blotting of whole-cell lysates of *H. pylori* KCTC0217BP wild type (lane 1) and isogenic *fliD* mutant (lane 2). The blot was developed with anti-recombinant FliD antibody. Molecular mass markers (in kilodaltons) are shown on the left. Expression of the 76-kDa FliD protein was observed only in the wild-type strain.

fragment containing the 1.4-kb kanamycin cassette was amplified, whereas a 0.4-kb DNA fragment was amplified from the wild type. Specific integration was also verified by nucleotide sequencing the 1.8-kb PCR fragment. When PCR was performed with primers (*fliD*seq#4 and *fliD*/CBamHI; Table 1) annealing downstream of the disruption site, identical 0.5-kb DNA fragments were amplified from both wild-type and mutant strains (Fig. 2A). A double-crossover event had taken place in all the strains tested, leading to replacement of the intact allele by the allele disrupted with the kanamycin resistance cassette. The expression of the FliD protein in the isogenic mutant was analyzed by Western blotting with anti-*H. pylori* FliD antibody; these Western blots confirmed the disappearance of the 76-kDa FliD protein from the *fliD* mutant strain (Fig. 2B). All the mutant strains grew well and were not significantly affected in viability or growth characteristics.

Electron microscopy of *H. pylori fliD* mutant. Flagellar morphology was examined by transmission electron microscopy after negative staining (Fig. 3). In wild-type strains, typical sheathed *H. pylori* flagella were observed. Inside the sheaths, the filament was elongated to the end of the tip and typical terminal bulbs were often observed (Fig. 3A and B). In mutant strains, truncated sheathed flagellum-like appendages were observed, but the numbers and sizes of these structures were highly variable (Fig. 3C and D). In most cases, it was difficult to observe elongated filaments inside the appendages, which looked like extensions of empty sheaths, even under higher magnification. Occasionally, more-typical sheathed flagella were observed in the *fliD* mutant, but filaments were not fully extended even in these structures, and empty flagellar sheaths were visible at the end of the flagella (Fig. 3E and F). Terminal bulbs were not observed in any *fliD* mutant strains. These findings demonstrate the requirement for FliD in the morphogenesis of flagella and, more specifically, in the elongation of the flagellar filament.

Effect of *fliD* gene mutation on flagellin gene expression. To further characterize the biochemical differences in flagellar structure between wild-type and *fliD* mutant *H. pylori*, whole-cell lysates were analyzed by SDS-PAGE and Western blotting with an anti-Fla polyclonal antibody (32), which was raised against purified flagellar filaments. Only minor differences in protein profiles, at around 60 kDa, between wild-type and *fliD* mutant strains were observed (Fig. 4A). Western blotting demonstrated that FlaA and FlaB expression in the *fliD* mutant was reduced to 7 and 57% of wild-type levels, respectively, whereas hook protein (FlgE) expression levels in the wild type and *fliD* mutants were identical. *flaA* mutant strains possess a unipolar tuft of truncated sheathed flagella (16), similar to the flagellar morphology observed in the *fliD* mutant strains. Thus, the flagellar morphology of the *fliD* mutant strains may have been due to reduced expression of FlaA resulting from *fliD* gene disruption. Given that both the *flaA* (23) and *fliD* genes are under the control of a σ^{28} -dependent promoter, whereas the *flaB* and *flgE* genes are controlled by a σ^{54} -regulated promoter (30, 35), we thought that transcriptional regulation might be involved in the reduced expression of FlaA. To test this prediction, total RNA was analyzed by Northern blotting with an *flaA* probe (Fig. 4B). The transcription level of the *flaA* gene in the *fliD* mutant was reduced to 20% of the wild-type level. In contrast, transcription of the *ureA* gene, which is constitutively expressed and presumably not related to flagellar biogenesis, was not affected (data not shown). These results indicated that the loss of FliD expression resulted in down-regulation of *flaA* gene expression at the transcriptional level. However, since FlaA expression was reduced to a greater degree than *flaA* transcription, another control mechanism, such as secretion of

unpolymerized FlaA, appears to be involved in the regulation of FlaA levels.

Functional analysis by motility testing. Wild-type *H. pylori* and *fliD* mutants, growing exponentially in liquid media, were observed under the phase-contrast microscope. Motile bacteria were all members of wild-type strains, not of the isogenic mutant strain. Since colony morphology on motility plates is a reliable indicator of the motility phenotype, single-colony motility and stab agar motility tests were performed to compare the motilities of wild-type and mutant *H. pylori* strains (Fig. 5). In the single-colony test, motility was assessed with the phase-contrast microscope by comparing the swarming halos surrounding single colonies. The wild-type strain formed colonies with the large diffuse spreading halo typical of motile bacteria (Fig. 5A). The *fliD* mutant strain produced small and sharply delineated colonies, a morphology typical of nonmotile bacteria (Fig. 5B). Likewise, in the stab agar test, swarming halo formation was observed only with the wild-type strain and the mutant strain showed no apparent motility (Fig. 5C).

Functional analysis by infectivity testing. To determine whether the *fliD* mutant bacteria retained the ability to colonize the mucus layer of the stomach, an infection test using the *H. pylori*-mouse model was performed. *H. pylori* SS1, which is able to colonize mouse gastric mucosae and reach high infection levels (22), was used to assess the role of the *fliD* gene product in *H. pylori* colonization. We constructed an isogenic mutant of *fliD* in an *H. pylori* SS1 background by the method described above. Both wild-type and *fliD* mutant SS1 strains were administered orally to 6-week-old, female C57BL/6 mice. Ten days after oral inoculation, mice were sacrificed and one-half of each stomach was homogenized and plated on selective blood agar plates in order to culture colonizing *H. pylori*. We recovered 2.23×10^5 cells per gram of gastric tissue from the mice inoculated orally with wild-type *H. pylori* SS1 but none from those inoculated with the mutant (Table 2). The remaining half of the stomach tissue was used for PCR analysis in order to detect even very low levels of *H. pylori* in the gastric mucosa. The *ureA* gene was amplified only from the stomach tissues of mice inoculated with wild-type *H. pylori* SS1 (Table 2). These results strongly suggested that FliD is absolutely required for *H. pylori* to colonize and establish infection in the mouse model.

DISCUSSION

Many bacteria are propelled by the rotation of semirigid helical filaments called flagella. The flagellar filaments are joined via proteins called HAPs to a universal joint (the hook) which is connected to a motor (the basal body) embedded in the cytoplasmic membrane. HAPs are important in the formation of flagella, even though these proteins are only present in small amounts (13). HAP1 and HAP3 are involved in joining the filament to the hook. HAP2, also known as the distal capping protein, localizes to the tip of the flagellar filament, where it serves to plug the tip of the growing flagellum and promote polymerization of flagellin subunits (10, 13). Different HAP mutant phenotypes have been observed due to differences between sheathed and unsheathed flagella. *S. typhimurium* mutants with defects in genes encoding HAP1, HAP2, or HAP3 are immotile and secrete unpolymerized filament protein into the growth medium (11). When HAP2 protein was added exogenously to the HAP2 mutant, flagellin polymerization occurred and complete flagella were constructed (14). In contrast, *V. parahaemolyticus* mutants defective for these genes show different phenotypes (26). Mutants with defects in capping protein (HAP2) are motile but slow in semisolid motility

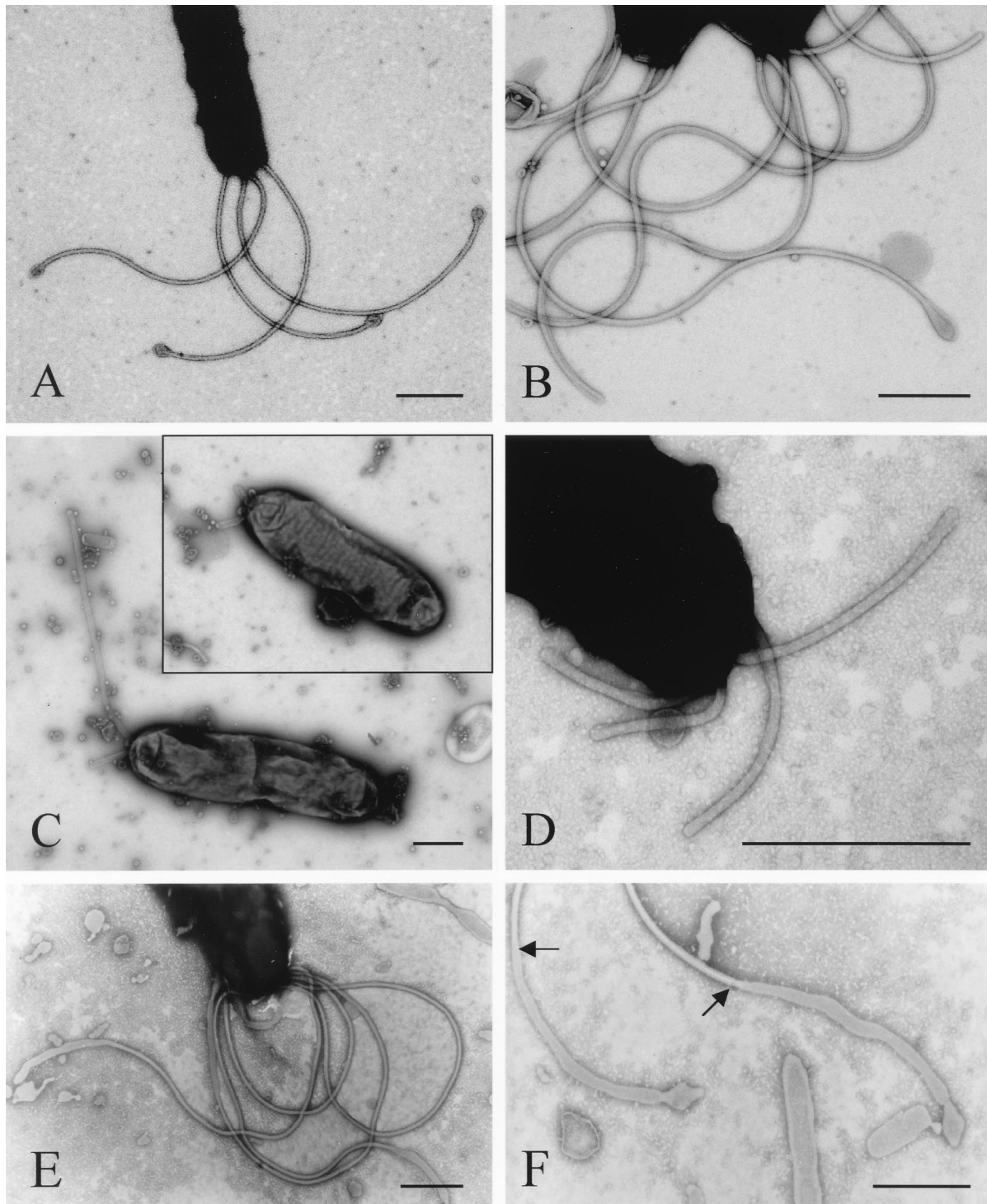


FIG. 3. Electron microscopy of *H. pylori* KCTC0217BP wild type and isogenic *fliD* mutant, negatively stained with potassium phosphotungstate (pH 6.8). In wild-type cells, full-length sheathed flagella are observed (A). Within the wild-type flagellar sheath, filaments are elongated to the tips of the flagella and terminal bulbs are observed (B). In the *fliD* mutant, truncated flagella, variable in number and size, are observed (C), and terminal bulbs are not apparent. Filament elongation is not observed in the *fliD* mutant even under higher magnification (compare panels B and D). Typical sheathed flagella with truncated filaments are occasionally seen in the *fliD* mutant strains (E). The end points of the truncated filaments are indicated by arrows (F). Bars, 500 nm.

plates, and mutants with defects in the joining proteins (HAP1 and HAP3) are immotile but do not secrete unpolymerized flagellin molecules. These mutants produced nonfunctional, severely truncated filaments that were not attached to the cell

body. It seems likely that the flagellar sheath is responsible for the differences in phenotype between *S. typhimurium* and *V. parahaemolyticus* HAP mutants. The sheath may trap secreted, unpolymerized flagellin and substitute for the capping protein.

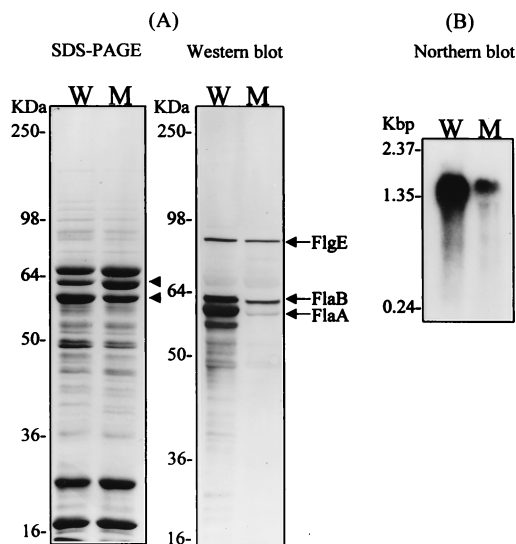


FIG. 4. Flagellin gene expression in wild-type and *fliD* mutant *H. pylori* strains. (A) Equal amounts of whole-cell lysates (20 μ g of total protein) from wild-type (lane W) and *fliD* mutant (lane M) *H. pylori* KCTC0217BP were separated by SDS-PAGE. Two protein bands of around 60 kDa, showing different levels of expression, are indicated by arrowheads. The same gel was also analyzed by Western blotting with polyclonal antibody anti-Fla. Note the significant reduction of FlaA in the *fliD* mutant. The positions of the hook (FlgE) and major (FlaA) and minor (FlaB) flagellin proteins are indicated on the right. (B) Northern blotting demonstrated that *flaA* RNA levels were also reduced in the *fliD* mutant.

In this study, we identified and characterized the *fliD* operon of the human pathogen *H. pylori*. This operon consists of *flaG*, *fliD*, and *fliS* gene homologues, in the order stated, downstream of a sequence which closely resembles the consensus for the so-called σ^{28} -dependent promoters. *fliD* operons containing *fliD*, *fliS*, and *fliT* genes have been found in other bacteria, including *E. coli*, *S. typhimurium*, and *B. subtilis* (3, 12, 17). The *fliD* genes of these bacteria encode the filament cap protein,

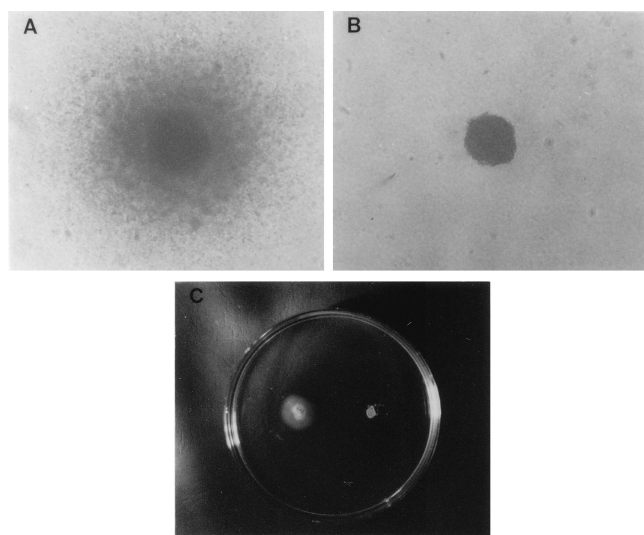


FIG. 5. Motility testing of *H. pylori* KCTC0217BP wild-type and isogenic *fliD* mutant strains. (A and B) Single-colony motilities of wild-type and *fliD* mutant strains, respectively. The wild-type strain formed diffuse colonies with large swarming halos, whereas the *fliD* mutant formed dense colonies. (C) Stab agar test of wild type (left) and *fliD* mutant (right).

TABLE 2. Colonization in mice by wild-type and *fliD* mutant *H. pylori* SS1

Inoculum ^a	No. of mice colonized/ total ^b		CFU/g of gastric tissue ^c \pm SD (10^5)
	By culture	By PCR	
<i>H. pylori</i> SS1	3/3	3/3	2.23 \pm 0.12
<i>H. pylori</i> SS1 <i>fliD</i> ::Km	0/3	0/3	0
Control	0/3	0/3	0

^a Each mouse (strain C57BL/6) received 10^8 CFU of *H. pylori* SS1 wild type or *fliD* mutant in 0.4 ml of brain heart infusion (BHI) broth, twice at 2-day intervals. Control mice received 0.4 ml of BHI broth alone.

^b *H. pylori* colonization in gastric tissue was assessed at 10 days postinoculation by culture and PCR.

^c Numbers of *H. pylori* CFU recovered following homogenization of gastric tissue samples. The results are geometric means for three mice per group.

also called HAP2, which facilitates the polymerization of endogenous flagellin at the tips of growing flagellar filaments. However, there are conflicting reports regarding the roles played by the *fliS* and *fliT* genes in flagellar formation (17). The *fliD* gene has been implicated as a chaperone involved in the export of flagellin, while *fliT* apparently has no effect on flagellar formation (38). In *P. aeruginosa*, the gene arrangement of the *fliD* operon is different; the *fliT* gene seems to be absent from this operon, and instead there is a duplication of the *fliS* gene (2). The gene arrangement of the *H. pylori fliD* operon also shows a distinctive feature in that the *flaG* gene homologue, instead of the *fliT* gene, is present. There appears to be no *fliT* gene homologue in the published *H. pylori* genome (37).

One of the interesting sequence features of flagellar axial proteins is a series of hydrophobic heptad repeats observed in their N- and C-terminal regions. These heptad repeats are characteristic of α -helical coiled coils and are believed to be important in quaternary interactions between respective protein subunits within the flagellar axial structures. Interestingly, hydrophobic heptad repeats were observed only in the C-terminal region in *H. pylori* FliD, as in other bacterial HAP2 homologues. These properties of HAP2 may reflect its special location. The filament cap occupies a unique location among all the axial substructures, in that its distal face is exposed to the environment (or probably to flagellar sheaths in sheathed flagella), rather than to quaternary interactions with other proteins. It has been suggested that the C-terminal region of HAP2 may be important in quaternary interactions with flagellin or with HAP3 at an earlier stage of the assembly process and that the N-terminal region is more free to diverge, perhaps because it is at the distal face of the subunit (12).

To elucidate the function of the *fliD* gene product of *H. pylori*, which possesses a unipolar bundle of sheathed flagella, we constructed isogenic mutants and compared their flagellar structures, motilities, and infectivities in a mouse model with those of the wild type.

In the *fliD* mutants, which were completely nonmotile, expression of the FliD protein was completely abolished and their flagella were sheathed but severely truncated. Interestingly, disruption of the *fliD* gene significantly decreased the expression of FlaA, the major flagellin subunit, partly by transcriptional down-regulation of *flaA*. This indicates that FliD, itself a flagellar structural component, plays a role in genetic regulation. In the process of flagellar assembly, filament elongation starts only after the hook-filament junction proteins (HAP1 and HAP3) and the filament-capping protein (FliD) are successively added to the full-length hook. Defects in the filament-capping protein may thus cause the failure of subse-

quent filament elongation. In this context, it is advantageous for the *H. pylori* *fliD* mutant strains to control the wasteful expression of FlaA by blocking gene transcription.

The regulation of motility and chemotaxis in *S. typhimurium* has been well studied (24). In *S. typhimurium*, flagellar operons are divided into three classes with respect to transcriptional hierarchy, and the genes required for flagellar biosynthesis are sequentially expressed according to a hierarchical pathway (19). Class 2 operons are positively regulated by class 1 genes, and class 3 operons are controlled by the FliA-FlgM regulatory system (29); FilA is a sigma factor (σ^{28}) specific for class 3, whereas FlgM is an anti-sigma factor which binds FliA to prevent its association with RNA polymerase core enzyme. The *fliD* operon of *S. typhimurium*, together with the genes for chemoreception and the flagellin gene, is a class 3 regulon, and the expression of these genes is under the control of a σ^{28} -dependent promoter. *fliD* mutations in *S. typhimurium*, in contrast to those in *H. pylori*, cause excessive export of the flagellum-specific anti-sigma factor, FlgM (39), resulting in overexpression of flagellar class 3 operons, including the flagellin gene, *fliC* (20). Therefore, the regulatory mechanism underlying flagellar biogenesis and control of motility in *H. pylori* appears to differ from that in *S. typhimurium*. Further evidence of such a difference in regulation is that no anti-sigma factor (FlgM) homologue has been found in the *H. pylori* genome (37). Instead, a transcriptional activator, FlgR, which functions both as an activator of σ^{54} -regulated genes such as basal body and hook genes and as a repressor of the σ^{28} -regulated *flaA* flagellin gene, has been identified (34). Given that the *fliD* gene is also under the control of a σ^{28} -dependent promoter, transcription of the *fliD* gene may be regulated by FlgR, but no experimental evidence is available to confirm this suggestion. Little is known about the flagellar regulon in *H. pylori*. To understand our present observations in the full context of the flagellar regulon will therefore require more information.

A remarkable structural feature of *H. pylori* flagella is the flagellar sheath. The effects of flagellin expression on flagellar morphology have been extensively studied (16). Empty flagellar sheaths are occasionally seen on *H. pylori* *flaA* and *flaA flaB* mutants, in which flagellar filaments are severely truncated or absent. Thus, generation of the flagellar sheath appears to be an active process independent of filament production. The *fliD* mutant also showed truncated sheathed flagella similar to those of *H. pylori* *flaA* mutants. However, the motilities of the *fliD* and the *flaA* mutant strains are different. The *fliD* mutant strains are nonmotile, while the *flaA* mutant strains are slightly motile. This result indicates that FliD expression is essential for the formation and function of *H. pylori* flagella.

Links between flagella and virulence have been observed previously. Colonization experiments with nonmotile mutants in the gnotobiotic piglet model of *H. pylori* infection have demonstrated that full motility is an essential virulence factor of *H. pylori* and a possible target for novel therapeutic substances. In other bacterial species, loss of the ability to produce flagella generally results in a less virulent organism. Nonflagellated isolates of *Campylobacter jejuni* (8), *Bordetella avium* (28), *Bacillus thuringiensis* (40), and *Clostridium chauvoei* (36) were all found to be less invasive or less virulent than the parental strains in their respective in vitro and in vivo models of pathogenesis.

Recently, FliD has been implicated as a virulence factor of *Proteus mirabilis*. A *fliD* mutant of *P. mirabilis* was shown to be defective in colonization of the urinary tract and attenuated in virulence in a mouse model of ascending urinary tract infection (27). FliD of *P. aeruginosa* participates in mucin-specific adhesion, which is the initial event in colonization by this organism

of the airways of cystic fibrosis patients (2). In the present study, FliD of *H. pylori* was shown to be essential for colonization as a result of its essential role in flagellar construction and establishment of motility. Construction of the flagellum is a complex process in which many genes are known to be involved. Although some of these genes have been characterized in *H. pylori*, little is known about the basic mechanisms and factors involved in flagellar synthesis in this organism. Recently, the sequence of the entire genome of *H. pylori* was published (37). Characterization of further *H. pylori* genes corresponding to known flagellar biosynthesis genes by reverse genetic approaches, as shown in this study, will provide more insights into the complex flagellar apparatus and the motility which these structures confer on bacteria. This information may provide practical ways to prevent or treat *H. pylori* infection in the gastric mucosa.

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