

Molecular Characterization of *fliD* Gene Encoding Flagellar Cap and Its Expression among *Clostridium difficile* Isolates from Different Serogroups

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The *fliD* gene encoding the flagellar cap protein (FliD) of *Clostridium difficile* was studied in 46 isolates belonging to serogroups A, B, C, D, F, G, H, I, K, X, and S3, including 30 flagellated strains and 16 nonflagellated strains. In all but three isolates, amplification by PCR and reverse transcription-PCR demonstrated that the *fliD* gene is present and transcribed in both flagellated and nonflagellated strains. PCR-restriction fragment length polymorphism (RFLP) analysis of amplified *fliD* gene products revealed interstrain homogeneity, with one of two major patterns (a and b) found in all but one of the strains, which had pattern c. A polyclonal monospecific antiserum raised to the recombinant FliD protein reacted in immunoblots with crude flagellar preparations from 28 of 30 flagellated strains but did not recognize FliD from nonflagellated strains. The *fliD* genes from five strains representative of the three different RFLP groups were sequenced, and sequencing revealed 100% identity between the strains with the same pattern and 88% identity among strains with different patterns. Our results show that even though FliD is a structure exposed to the outer environment, the flagellar cap protein is very well conserved, and this high degree of conservation suggests that it has a very specific function in attachment to cell or mucus receptors.

Clostridium difficile is an opportunistic human pathogen that causes nosocomial infections such as antibiotic-associated colitis (pseudomembranous colitis) and diarrhea. Its pathogenicity is mediated by two exotoxins, toxins A (308 kDa) and B (270 kDa), both of which damage the human colonic mucosa and are potent cytotoxic enzymes (4). Before these events take place, *C. difficile* must be implanted in the gut and colonize suitable epithelial cells which are protected by a layer of dense mucus. Confirmed and putative accessory virulence factors that could play a role in adhesion and in intestinal colonization have been identified, including the capsule (8), proteolytic enzymes (28, 30), and adhesins involved in the association with mucus and the cell (5, 13, 17, 35). During the colonization process, the bacterium penetrates the mucus layer and attaches to enterocytes; at these different stages flagella are suspected to play a role, but this has yet to be proven. In some bacterial species flagella have been implicated in adherence to mucus and cells and in colonization and virulence; these include *Pseudomonas aeruginosa* (2), *Vibrio cholerae* (29), *Vibrio anguillarum* (21), *Helicobacter pylori* (12), *Burkholderia pseudomallei* (6), *Campylobacter jejuni* (16), *Xenorhabdus nematophilus* (15), *Salmonella enterica* serovar Typhi (20), and *Proteus mirabilis* (22).

A bacterial flagellum consists of a basal body in the membrane, the hook, and a helicoidal filament. The major structural component of the filament, the flagellin FliC, is assem-

bled in subunits. Proteins called hook-associated proteins (HAP1, HAP2, and HAP3) are required to join the filament to the hook and to cap the distal tip of the filament. The *fliD* gene encodes HAP2, which functions as a capping structure at the distal end of the filament. It has been shown to have a function in mucin attachment by *P. aeruginosa* (1, 3), and *H. pylori* (18) and virulence in *P. mirabilis* (22).

We are interested in finding out whether flagella play a role in *C. difficile* intestinal attachment. Earlier studies from our laboratory have allowed characterization of the 39-kDa flagellin protein. The flagellin gene (*fliC*) was cloned and sequenced, and the recombinant protein was characterized (31). The diversity of the *fliC* gene among different isolates was studied, and it was found that the gene is present and expressed in both flagellated and nonflagellated strains (32).

In the study described here, in order to complete the findings concerning the proteins of the flagellar filament in *C. difficile*, we have characterized the *fliD* gene and its corresponding protein at the molecular level. We have investigated its presence and its variability among a series of *C. difficile* isolates from different serogroups and of various origins.

Forty-six *C. difficile* isolates belonging to 12 different serogroups (serogroups A1, A10, B, C, D, F, G, H, I, K, S3, and X) were selected at the Microbiology Unit of the Catholic University of Louvain, Brussels, Belgium, with care taken to choose strains isolated from several geographical locations (32). *Clostridium sordellii* (Institut Pasteur, Paris, France) was used as a negative control. All strains were grown under anaerobic conditions as described previously (32).

The primers used for amplification of the *fliD* genes from various *C. difficile* isolates were *fliD*-Nter (5'-ATGTCAAGTATAAGTCCAGTAAG-3') and *fliD*-Cter (5'-TTAATTACC

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TTGTGCTTGTG-3'), corresponding to the 5'- and 3'-end sequences of the *fliD* gene of strain *C. difficile* 630, respectively, the genome sequence of which is now available on the Internet (www.sanger.ac.uk). Amplification was performed as described previously (32). At the end of the amplification, 5 µl of each of the samples was digested with the restriction enzymes *AccI*, *DraI*, *EcoRI*, *HincII*, *HinfI*, *MboII*, and *XbaI* (Amersham-Pharmacia Biotechnology).

The PCR products of strain 79685, reference strains of serogroups A, B, and C, and strain EX482 were purified with the QIAquick PCR purification kit (Qiagen). The nucleotide sequences of both strands were analyzed with an ABI PRISM 310 genetic analyzer (Perkin-Elmer), as described previously (32). Protein sequence alignments were performed with the DNA Strider software and the CLUSTAL W program (33). Homologies with sequences stored in GenBank were searched for by using Fasta3 (European Bioinformatics Institute) or Blast (National Center for Biotechnology Information) software.

RNA was extracted from 10 ml of an 8-h *C. difficile* anaerobic culture as described previously (32). The reverse transcription (RT)-PCR was carried out with the SuperScript one-step RT-PCR system (Life Technologies). The RNA of *C. difficile* 79685 was used as a positive control, and the RNA of *C. sordellii* was used as a negative control. The cDNA synthesis step was performed at 50°C for 30 min, and a predenaturation step was performed at 94°C for 2 min. Thirty cycles of amplification were performed in a Thermocycler 2400 instrument (Perkin-Elmer). Each cycle consisted of three steps, as described previously (32). The amplified products were subjected to electrophoresis in a standard 1% (wt/vol) agarose gel.

For the cloning of the *C. difficile* 79685 *fliD* gene into an expression vector, two oligonucleotide primers, *fliD*-BamHI (5'-CCCCTGGGATCCATGTCAAGTATAAGTCCAGTAA G-3') and *fliD*-XhoI (5'-GGTCGACTCGAGTTAATTACCT TGTGCTTGTG-3'), which incorporated the *Bam*HI and *Xho*I restriction sites, respectively, were synthesized and used to amplify by PCR the full-length coding region of the *fliD* gene of strain 79685 (*Taq* polymerase [Promega] was used at 1 U/100 µl of the reaction mixture volume). The resulting 1,524-bp DNA product was digested with *Bam*HI and *Xho*I and cloned in-frame into the corresponding sites of pGEX-6P-1 (Amersham-Pharmacia Biotechnology). The nucleotide sequence of the junction between the vector and the insert was confirmed by sequencing analysis. The plasmid was transformed into *Escherichia coli* BL21. The expression and purification of the fusion protein were carried out as described previously (31). A polyclonal anti-FliD serum was raised against the purified recombinant FliD protein. The gel band corresponding to the purified protein was cut out, lyophilized, and injected subcutaneously into a rabbit. The polyclonal, monospecific antiserum was obtained by a previously described protocol (17) and was used at a 1:2,000 dilution in Western blots.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunoblotting were used to determine the presence of FliD proteins in clinical isolates. The proteins issued from the crude flagellar purification (9) were separated by SDS-PAGE (12% [wt/vol] acrylamide gel) as described by Laemmli (19). The gels were electrically transferred onto a

nitrocellulose membrane for immunoblotting, and proteins were detected with the rabbit polyclonal anti-FliD serum (1:2,000 dilution) as described previously for FliC (31).

PCR amplification with the specific N-terminal and C-terminal oligonucleotide primers derived from the *fliD* gene of *C. difficile* strain 630 was carried out to study the *C. difficile* isolates for the presence of *fliD*. A single 1,524-bp amplified product was generated from 43 of the *C. difficile* strains studied, including strain 630, whereas no product was obtained from strains EX560, CO109, and ATCC 43604 (Table 1). These strains did not show gene amplification, despite many attempts with various parameters, such as changing the annealing temperature, MgCl₂ concentration, or primers. For these strains the nonamplification of the *fliD* gene could result from either the absence of this gene or the presence of a genetically different gene which cannot be amplified with the primers used. The *fliD* gene was not amplified from the *C. sordellii* strain used as a negative control (data not shown). It is noteworthy that the *fliD* gene was present in both flagellated and nonflagellated strains.

In order to study the variability of the *fliD* gene among *C. difficile* isolates, the amplified *fliD* gene was digested with the *AccI*, *DraI*, *EcoRI*, *HincII*, *HinfI*, *MboII*, and *XbaI* restriction enzymes. The different restriction patterns obtained from the *C. difficile* strains are shown in Fig. 1. Three different restriction profiles were obtained with the *DraI* enzyme (designated a, b, and c), and two different profiles (designated a and b) were obtained with the *AccI*, *EcoRI*, *HincII*, *HinfI*, *MboII*, and *XbaI* enzymes. Clinical isolates could be subdivided into two major restriction fragment length polymorphism (RFLP) groups (group a or b), each of which was represented by the profile (profiles a and b, respectively) obtained with the different restriction enzymes (Table 1; Fig. 1). The *fliD* RFLP analysis of strain EX482 revealed a unique RFLP group (group c), defined by profile c obtained with *DraI*, profile a obtained with *HinfI* and profile b obtained with the *AccI*, *EcoRI*, *HincII*, *MboII*, and *XbaI* enzymes. RFLP group a (20 strains) comprises all strains that belong to serogroups C, F, I, and X; one strain each of serogroups A10 and K; and two strains of serogroup S3. The second major RFLP group (group b; 22 strains) encompasses all strains of serogroups D, G, and H; the majority of the strains of serogroups A and K; and three strains of serogroup S3.

Different methods have been developed for *C. difficile* typing, particularly serogrouping by slide agglutination (10, 11) and comparison by PAGE of cell protein migration patterns. Newer molecular biology-based techniques have been used to study the genetic diversity of the flagellar genes, and RFLP analysis has been carried out to study the *fliC* genotypic variabilities in *S. enterica* (7), *C. jejuni* (24, 25, 27), *P. aeruginosa* (23, 36–38), *H. pylori* (26), and *C. difficile* (32). This is the first instance in which typing has been performed with the *fliD* gene. Since the results showed a little variability of this gene among the different isolates with two main patterns (patterns a and b), this gene is not an excellent biomarker for the study of diversity. We can note, nevertheless, that the strains belonging to the same serogroup generally exhibit the same pattern by RFLP analysis. Strains of serogroups A, G, H, and K have RFLP pattern b; interestingly, Delmée et al. (9) showed that flagellated strains of serogroup H were agglutinated by anti-

TABLE 1. *C. difficile* isolates studied^a

Strain ^b	Serogroup	<i>fliD</i> gene present (PCR)	<i>fliD</i> transcribed (RT-PCR)	<i>fliD</i> translated into protein (immunoblotting)	Flagellar structure visible by EM ^c	RFLP group
ATCC 43594 ^d	A1	+	ND ^{d,e}	+	+	b
24573	A1	+	ND	+	+	b
EX482	A1	+	ND	+	+	c
SE810	A10	+	ND	+	+	a
TO005	A10	+	ND	+	+	b
55787	A10	+	ND	+	+	b
EX560	B	-	ND	-	-	ND
CO109	B	-	ND	-	+	ND
ATCC 43593	B	+	ND	+	+	b
ATCC 43596 ^d	C	+	+	-	-	a
54637	C	+	+	-	-	a
54828	C	+	+	-	-	a
51936	C	+	+	-	-	a
1075	C	+	+	-	-	a
BR058	D	+	ND	+	+	b
ATCC 43597	D	+	+	-	-	b
55944	D	+	+	-	-	b
ATCC 43598 ^d	F	+	ND	+	+	a
5168	F	+	ND	+	+	a
6058	F	+	ND	+	+	a
6100	F	+	ND	+	+	a
54126	G	+	ND	+	+	b
51187	G	+	ND	+	+	b
ATCC 43599 ^d	G	+	ND	+	+	b
SE956	G	+	+	-	-	b
ATCC 43600 ^d	H	+	+	-	-	b
50673	H	+	ND	+	+	b
53444	H	+	ND	+	+	b
ATCC 43601 ^d	I	+	+	-	-	a
54823	I	+	+	-	-	a
56026	I	+	ND	+	+	a
55684	I	+	+	-	-	a
52356	K	+	+	-	-	a
51659	K	+	ND	+	+	b
48515	K	+	ND	+	+	b
SE752	K	+	ND	+	+	b
ATCC 43602 ^d	K	+	ND	+	+	b
79685	S3	+	ND	+	+	a
57207	S3	+	+	-	-	a
37561	S3	+	ND	+	+	b
EX596	S3	+	ND	+	+	b
35962	S3	+	ND	+	+	b
36678	X	+	ND	+	+	a
12934	X	+	ND	+	+	a
20356	X	+	+	-	-	a
ATCC 43603 ^d	X	-	ND	-	+	ND

^a +, positive result; -, negative result.

^b The *C. difficile* strains studied are the same as those described in reference 32.

^c EM, electron microscopy. The electron microscopy results were presented elsewhere (32).

^d Serogroup reference strain.

^e ND, not determined.

sera raised against the flagellins of strains belonging to serogroups A, G, H, and K. The same results were observed with strains of serogroups C, F, I, and X with pattern a. It is interesting that there was a correlation between cross-agglutination of specific serogroups and RFLP profiles.

On the basis of the differences between the *fliD* genes obtained by RFLP analysis, we decided to sequence two strains with pattern a, two strains with pattern b, and one strain with one pattern c. Analysis of the DNA sequence of each strain revealed an open reading frame composed of 1,524 nucleotides corresponding to 507 amino acids. The *C. difficile* flagellar cap protein has a calculated molecular mass of 56 kDa, and thus, its mass does not differ from the estimated molecular mass of

56 kDa determined by SDS-PAGE (see Fig. 3a). The deduced amino acid sequences of the FliD proteins of strains 630 and 79685 and a reference strain of serogroup C were more than 99% identical but were different (88% identity) from those of the reference serogroup A and B strains and strain EX482, which were also more than 99% identical (data not shown). The deduced amino acid sequences of strain 79685 and the reference serogroup A strain were compared to known FliD protein sequences in GenBank. The FliD proteins of *E. coli* and *S. enterica* serovar Typhi, two genetically closed microorganisms, showed high degrees of identity (51%), whereas the degrees of identity between FliD of *C. difficile* and those of other bacterial genera ranged from 19 to 27%. The deduced

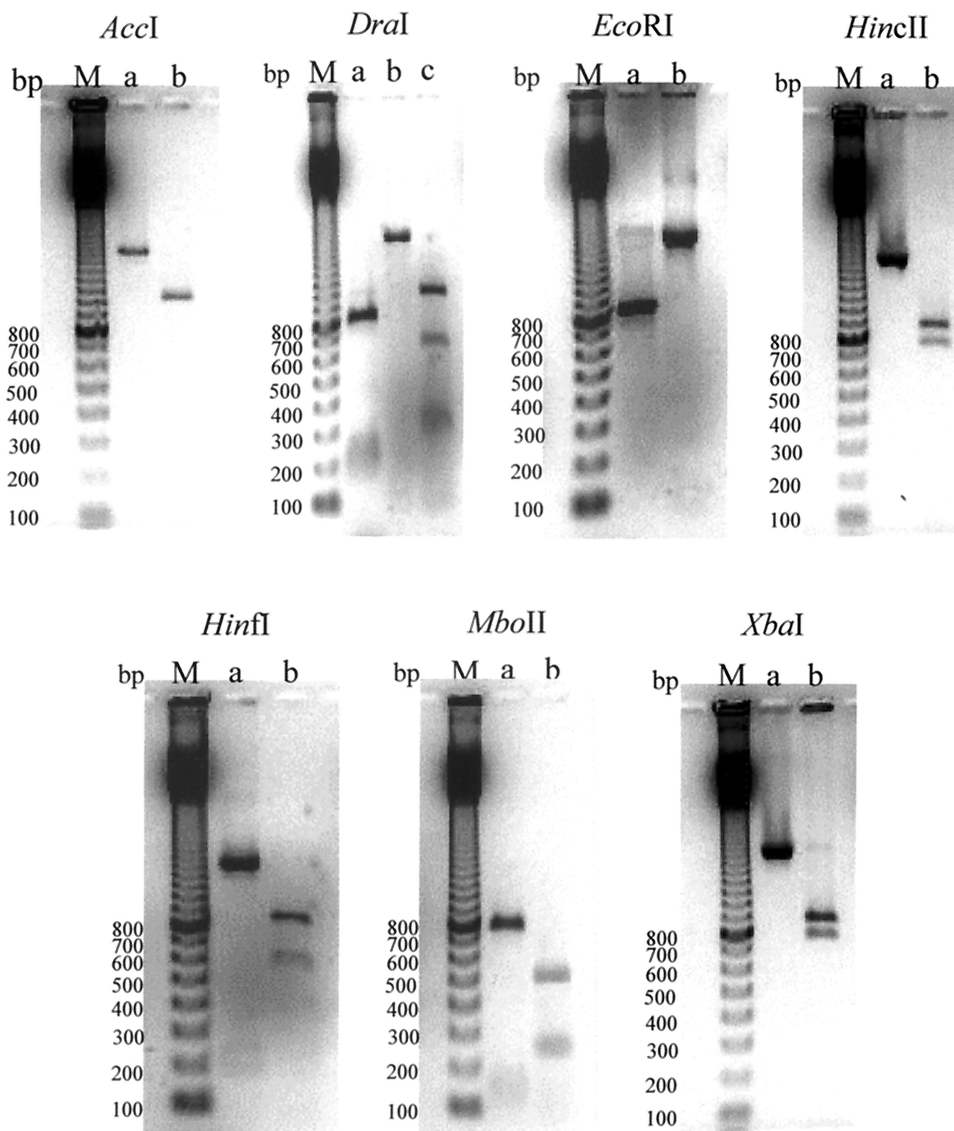


FIG. 1. RFLP patterns of PCR-amplified flagellar cap genes. The amplified *fliD* genes of *C. difficile* isolates were digested with *AccI*, *DraI*, *EcoRI*, *HincII*, *HinfI*, *MboII*, and *XbaI*. The different restriction profiles obtained with each enzyme were designated a, b, and c. Lanes M, 100-bp ladder (Amersham-Pharmacia Biotechnology); lanes a, profile a; lanes b, profile b; lanes c, profile c. The digested amplified products were subjected to electrophoresis in a 1.2% (wt/vol) agarose gel. The numbers next to the gels are in base pairs.

amino acid sequences show that the structure of this protein is extremely well conserved, with no variable domains present.

To gain insights into why certain strains are nonflagellated, we investigated the transcription of the *fliD* gene by detection of cap protein mRNA by RT-PCR in the nonflagellated strains. Nonflagellated strain EX560, the *fliD* gene of which was not amplified by PCR, was not studied. The results show that a single 1,524-bp product was obtained in all nonflagellated *C. difficile* strains (Fig. 2). Thus nonflagellation is not a result of the absence of transcription of the *fliD* gene.

Purification of the cap protein was carried out to produce a monospecific antiserum in order to investigate the translation of the *fliD* gene. The *fliD* gene of *C. difficile* strain 79685 was cloned into the *E. coli* expression vector pGEX-6P-1, and the expression was induced with isopropyl- β -D-thiogalactopyrano-

side. The recombinant FliD protein was purified by affinity chromatography on glutathione-Sepharose, and the fusion protein glutathione *S*-transferase (GST)-FliD was cleaved with Precision protease, as described previously (31). As shown in Fig. 3a, a major 56-kDa band free of contaminating GST was observed in the final eluate in SDS-polyacrylamide gels. Antibodies raised against the purified FliD protein recognized the purified 56-kDa protein and a protein with the same molecular mass in a crude flagellar preparation from strain 79685 (Fig. 3b). This result shows the specificity of the antiserum for FliD. In order to determine whether the *fliD* gene is translated in flagellated and nonflagellated strains, these antibodies were used to probe crude flagellar preparations of all *C. difficile* strains studied. The results showed that the antiserum recognized the 56-kDa FliD proteins of all flag-

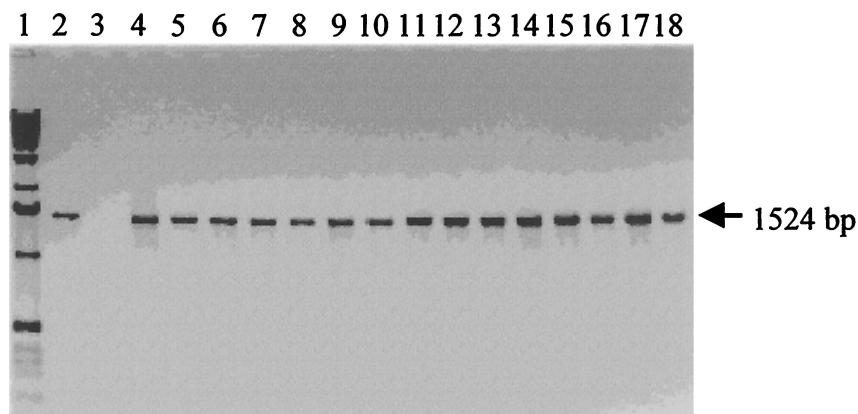


FIG. 2. RT-PCR products with specific *fliD* primers *fliD*-Nter and *fliD*-Cter and RNA isolated from nonflagellated *C. difficile* strains. Lanes: 1, 1-kb ladder (Amersham-Pharmacia Biotechnology); 2, strain 79685 (positive control); 3, RNA from *C. sordellii* (negative control); 4, strain ATCC 43596 (reference serogroup C strain); 5, strain 54637; 6, strain 54828; 7, strain 51936; 8, strain 1075; 9, strain ATCC 43597 (reference serogroup D strain); 10, strain 55944; 11, strain SE956; 12, strain ATCC 43600 (reference serogroup H strain); 13, strain ATCC 43601 (reference serogroup I strain); 14, strain 54823; 15, strain 55684; 16, strain 52356; 17, strain 57207; 18, strain 20356. To confirm the purity of the RNA preparation and the specificity of the target RNA, an RNA sample treated with RNase was submitted to an RT-PCR as described in the text. Furthermore, the absence of genomic DNA contamination in the RNA samples was verified by PCR with *fliD* gene-specific N-terminal and C-terminal primers. No amplified products were detected in these two control experiments.

ellated *C. difficile* strains with the exception of those of strains CO109 and ATCC 43604. In contrast, no 56-kDa protein immunoreacted with the antiserum in nonflagellated strains (Table 1). This result suggests that (i) the FliD of each flagellated strain contains cross-reacting epitopes due to the presence of FliD monomers and (ii) in nonflagellated strains the absence of translation of the *fliD* gene could explain the lack of flagellation. We have shown previously that in strains in which no flagellar structure is visible by electron microscopy, the nonflagellated strains possess a cryptic flagellin gene (*fliC*) (32). The present study demonstrates that they also have a cryptic cap protein gene. Cryptic genes have been characterized in non-

flagellated bacteria, and expression of surface flagella has been induced by modifying culture conditions *in vitro* in *S. enterica* serovar Pullorum (14) and in *Shigella flexneri* and *Shigella sonnei* (34). So far, little is known about the *in vivo* expression of flagella, and it can be hypothesized that flagellar switching on and off occurs through modification of microenvironmental factors *in vivo* during the host-pathogen interaction.

In conclusion, except for Arora et al. (1), who identified two distinct type of *fliD* genes among a group of *P. aeruginosa* strains, no study concerning the molecular variability of the *fliD* gene in other bacteria has been carried out; the protein has been studied only for its functionality. In our study, the analysis

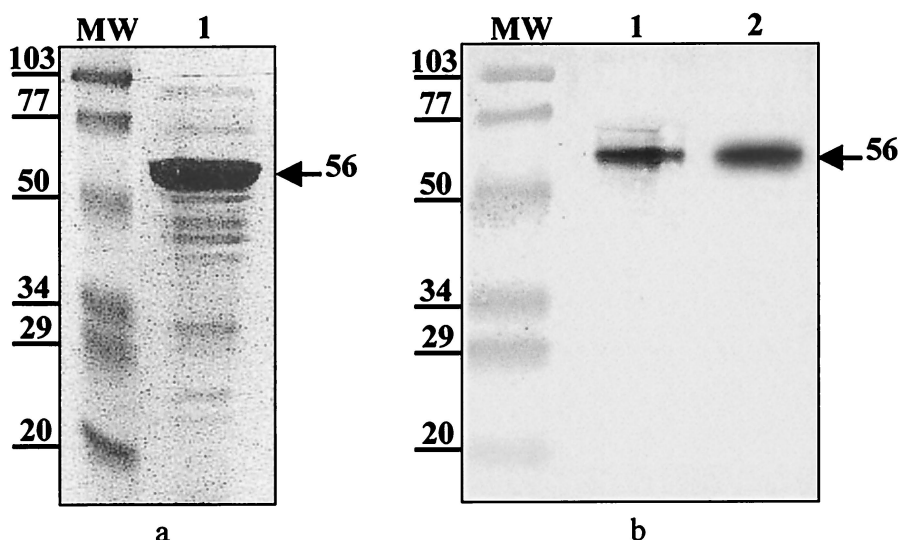


FIG. 3. (a). Purification of *C. difficile* 79685 FliD protein. The SDS-polyacrylamide gel shows low-molecular-mass standards of 103, 77, 50, 34, 29, and 20 kDa (Bio-Rad Laboratories) (lane mw) and FliD eluted from glutathione-Sepharose columns after digestion of GST-FliD with PreScission protease (lane 1). A major band is observed at 56 kDa (b). Immunoblotting of crude flagellar preparation of *C. difficile* strain 79685 reacted with a 1:2,000 dilution of polyclonal antiserum raised against purified FliD (lane 1). FliD was eluted from a glutathione-Sepharose column after digestion of GST-FliD with PreScission protease (lane 2). The arrow indicates the band corresponding to the 56-kDa flagellar cap protein.

of the sequences of FlhD proteins from different *C. difficile* strains showed scarce variability but revealed variable domains between different bacterial genera. This suggests that the FlhD protein could possess specific conserved domains, which could have a function in attachment to highly specific cell or mucus receptors. The flagellar cap protein could play a role in adherence by mediating initial binding of the flagellar tip to mucin during the first stage of pathogenesis. Microenvironmental factors and host interactions could induce the production of flagella and gut colonization by *C. difficile*. Important questions remain to be answered concerning the exact role of the flagellar proteins in colonization and their vaccine potential.

Nucleotide sequence accession numbers. The nucleotide sequences of the *flhD* loci of strains 79685, ATCC 43594, ATCC 43593, ATCC 43596, and EX482, corresponding to serogroup S3, reference strains as serogroups A, B, and C, and serogroup A1, respectively, were submitted to GenBank and were assigned accession numbers AF297024, AF297025, AF297026, AF297027, and AF297028, respectively.

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