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Phenotypic and Genotypic Diversity of the Flagellin Gene (*fliC*) among *Clostridium difficile* Isolates from Different Serogroups

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Phenotypic and genotypic diversity of the flagellin gene (*fliC*) of *Clostridium difficile* was studied in 47 isolates from various origins belonging to the serogroups A, B, C, D, F, G, H, I, K, X, and S3. Electron microscopy revealed 17 nonflagellated strains and 30 flagellated strains. PCR and reverse transcription-PCR demonstrated that the flagellin gene was present in all strains and that the *fliC* gene was expressed in both flagellated and nonflagellated strains. Southern blotting showed the presence of only one copy of the gene and three different hybridization patterns. DNA sequence analysis of *fliC* from the strains belonging to serogroups C, D, and X, representative of each profile, disclosed great variability in the central domain, whereas the N- and C-terminal domains were conserved. The variability of the flagellin gene *fliC* was further studied in the isolates by PCR-restriction fragment length polymorphism (RFLP) analysis. Nine different RFLP groups were identified (I to IX), among which three (I, VII, and VIII) corresponded to numerous serogroups whereas the six others (II, III, IV, V, VI, and IX) belonged to a single serogroup. Flagellin gene RFLP analysis could constitute an additional typing method employable in conjunction with other typing methods currently available.

Clostridium difficile is the major etiological agent of pseudomembranous colitis and antibiotic-associated diarrhea. In addition to the two major toxins, A and B, which represent the major virulence factors (7), a number of other putative accessory virulence factors have been described. These include adhesins mediating adherence to mucosa (15, 23, 48), fimbriae, and capsule and tissue-degradative enzymes (6). However, in some bacterial species, flagella may also be a virulence factor and play a role in colonization of the gastrointestinal tract. The flagellar structure plays a role in internalization of *Campylobacter jejuni* (18), *Salmonella enterica* serovar Typhi (26), and *Proteus mirabilis* (31) into cultured epithelial cells. Motility is an important factor in the virulence of *Vibrio cholerae* (39) and *Vibrio anguillarum* (30). Flagella are also involved in chemotaxis and have been implicated in mucus-cell adherence and colonization by *Pseudomonas aeruginosa* (1, 40), *Helicobacter pylori* (14), and *Burkholderia pseudomallei* (8). Since flagella are believed to constitute one of the virulence factors of various infectious bacteria, the flagellin gene could be considered a useful genetic marker for epidemiological and phylogenetic studies (20, 52).

One aspect of *C. difficile* that we have studied is its interaction with target cells (15, 23, 48). Adhesion to and colonization of target tissues by bacteria are frequently important first steps in establishing infection. It is likely that *C. difficile* is unable to colonize without attachment and will be quickly removed by nonspecific host defense mechanisms.

Our laboratory is interested in finding out whether flagella play a role in *C. difficile* intestinal colonization. Few studies concerning *C. difficile* flagella have been performed; Delmée et al. established that flagella were involved in cross-reactions of serogroups (11). In a previous study we characterized the 39-kDa flagellin protein (45). The flagellin gene was cloned and sequenced, and the recombinant protein was characterized.

The aim of this work was to study the phenotypic and genotypic variability of the flagellin gene (*fliC*) and its correlation with serogroups in *C. difficile* isolates from different origins. Strains were investigated by electron microscopy (EM). The presence of the *fliC* gene was verified by PCR amplification, and the expression of the flagellin gene was studied by reverse transcription (RT)-PCR. In order to investigate the flagellin gene structure, Southern analysis with serogroup reference strains and sequencing of *fliC* genes from three strains were performed. PCR amplification of flagellin genes combined with restriction fragment length polymorphism (RFLP) analysis were used in an attempt to study the variability among *C. difficile* isolates.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Forty-seven 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. The 10 reference strains for specific serogroups were A (ATCC 43594), B (ATCC 43593), C (ATCC 43596), D (ATCC 43597), F (ATCC 43598), G (ATCC 43599), H (ATCC 43600), I (ATCC 43601), K (ATCC 43602), and X (ATCC 43603). *Clostridium sordellii* (Institut Pasteur, Paris, France) was used as a negative control, and *C. difficile* 79-685 was used as a positive control for the flagellin gene (Table 1).

Clostridium strains were grown under anaerobic conditions on agar plates (Oxoid Ltd., Basingstoke, Hampshire, England) supplemented with 7% horse blood (BioMérieux, Marcy l'Etoile, France) or in TGY (tryptone glucose yeast infusion broth) (Difco Laboratories, Detroit, Mich.) for 48 h.

EM. The strains of *C. difficile* were grown overnight on agar plates supplemented with 7% horse blood as described above. A bacterial suspension was made in 100 μl of phosphate-buffered saline. Copper grids (Touzard et Matignon, Paris, France) were placed facedown on the cell suspension for 5 min and then negatively stained with a 2% phosphotungstic acid solution (pH 7.2). The grids were air dried and observed under a transmission electron microscope (EM 301; Philips).

DNA extraction and Southern blotting. DNA was extracted from 10 ml of overnight culture according to the protocol provided in the Puregene DNA gram-positive bacteria and yeast DNA extraction kit (Gentra Systems, Minneapolis, Minn.).

Southern blotting was carried out with 5 μg of DNA digested with 10 U of

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TABLE 1. *C. difficile* isolates studied[c]

Strain	Serogroup	Origin[a]	Toxin A in vitro	Toxin B in vitro	Flagella shown by EM	PCR	RT-PCR	RFLP group
ATCC 43594[b]	A1	Brussels 3, Belgium	+	+	+	+	ND[d]	VII
EX482	A1	Brussels 2, Belgium	+	+	+	+	ND	VII
24573	A1	Charleroi, Belgium	+	+	+	+	ND	VII
SE810	A10	Annecy, France	−	−	+	+	ND	I
TO005	A10	Toronto, Canada	+	+	+	+	ND	IV
55787	A10	Brussels 1, Belgium	−	−	+	+	ND	V
EX560	B	Mauscron, Belgium	−	−	−	+	+	I
CO086	B	Jouy en Josas, France	−	−	+	+	ND	III
CO109	B	Jouy en Josas, France	−	−	+	+	ND	III
ATCC 43593[b]	B	Brussels 1, Belgium	−	−	+	+	ND	VII
ATCC 43596[b]	C	Namur, Belgium	+	+	−	+	+	I
54637	C	Brussels 4, Belgium	+	+	−	+	+	I
54828	C	Brussels 1, Belgium	+	+	−	+	+	I
51936	C	Sambreville, Belgium	+	+	−	+	+	I
1075	C	Brussels 1, Belgium	−	−	−	+	+	I
BR058	D	Brussels 1, Belgium	−	−	+	+	ND	VIII
ATCC 43597[b]	D	Brussels 1, Belgium	−	−	−	+	+	VIII
55944	D	Brussels 1, Belgium	−	−	−	+	+	VIII
ATCC 43598[b]	F	Brussels 1, Belgium	−	+	+	+	ND	II
5168	F	Brussels 1, Belgium	−	+	+	+	ND	II
6058	F	Brussels 1, Belgium	−	+	−	+	+	II
6100	F	Brussels 1, Belgium	−	+	+	+	ND	II
54126	G	St. Ode, Belgium	+	+	+	+	ND	VII
51187	G	Brussels 1, Belgium	+	+	+	+	ND	VII
ATCC 43599[b]	G	Brussels 3, Belgium	+	+	+	+	ND	VII
SE956	G	Annecy, France	+	+	−	+	+	VII
ATCC 43600[b]	H	Brussels 3, Belgium	+	+	−	+	+	VII
50673	H	Tournai, Belgium	+	+	+	+	ND	VII
53444	H	Brussels 1, Belgium	−	−	+	+	ND	VIII
ATCC 43601[b]	I	Brussels 1, Belgium	−	−	−	+	+	I
54823	I	Brussels 1, Belgium	−	−	−	+	+	I
56026	I	Brussels 1, Belgium	−	−	+	+	ND	I
55684	I	Brussels 1, Belgium	−	−	−	+	+	I
52356	K	Brussels 1, Belgium	+	+	−	+	+	I
51659	K	Soignies, Belgium	+	+	+	+	ND	VII
48515	K	Brussels 1, Belgium	+	+	+	+	ND	VII
SE752	K	Annecy, France	+	+	+	+	ND	VII
ATCC 43602[b]	K	Brussels 1, Belgium	−	−	+	+	ND	VII
79685	S3	Strasbourg, France	+	+	+	+	ND	I
57207	S3	Brussels 1, Belgium	−	+	−	+	+	I
37561	S3	Charleroi, Belgium	+	+	+	+	ND	VII
EX596	S3	Brussels 3, Belgium	+	+	+	+	ND	VII
35962	S3	Brussels 1, Belgium	−	+	+	+	ND	VIII
36678	X	Brussels 1, Belgium	−	+	+	+	ND	I
12934	X	Verviers, Belgium	−	−	+	+	ND	VI
20356	X	Brussels 1, Belgium	−	+	−	+	+	VI
ATCC 43603[b]	X	Brussels 1, Belgium	−	−	+	+	ND	IX

[a] Brussels 1, Brussels 2, etc., represent different units or different hospitals in Brussels.
[b] Serogroup reference strain.
[c] +, positive result; −, negative result.
[d] ND, not determined.

*Hin*dIII for 3 h under the conditions recommended by the provider (Life Technologies, Cergy Pontoise, France). Products of digestion were separated by electrophoresis on a 0.8% (wt/vol) agarose gel. The fragments were transferred onto a positively charged nylon membrane (Roche, Mannheim, Germany) using a vacuum blotter (Appligene-Oncor, Illkirch, France). The amplified *fliC* gene of the *C. difficile* 79-685 strain (45) was used as a *C. difficile* flagellin gene-specific probe. The DNA probe was labeled and detected by using the ECL direct nucleic acid labeling and detection system (Amersham-Pharmacia Biotech, Les Ulis, France). Hybridization and washing of membranes were carried out at low stringency (0.5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] at 50°C).

PCR amplification and PCR-RFLP. For amplification of the *fliC* gene from various *C. difficile* isolates, the specific primers used were Nter (5′-ATGAGAG TTAATACAAATGTAAGTGC-3′) and Cter (5′-CTATCCTAATAATTGTAA AACTCC-3′) corresponding to the 5′- and 3′- end sequences of the *fliC* gene of the *C. difficile* 79-685 strain. DNA amplification by PCR was performed in a reaction volume of 100 μl consisting of 1 μl of a bacterial suspension washed twice with phosphate buffer, primer Nter (1 mM), primer Cter (1 mM), deoxynucleoside triphosphates (0.2 mM), $MgCl_2$ (2 mM), 1 U of *Taq* polymerase, and 1× polymerase buffer (Promega, Madison, Wis.). The reaction mixture was overlaid with mineral oil. Initial denaturation was carried out at 94°C for 5 min. Thirty-five cycles of amplification were performed in a Thermocycler 480 (Perkin-Elmer, Norwalk, Conn.). Each cycle consisted of three steps: denaturation at 94°C (30 s), annealing at 55°C (30 s), and extension at 72°C (1 min). An additional step of extension for 10 min at 72°C was performed at the end of the amplification to complete the extension of the primers. Samples (5 μl) of amplified products were digested with the restriction enzymes *Hin*dIII, *Dra*I, *Hpa*I, *Pvu*II, *Hin*cII, *Hin*fI (Amersham-Pharmacia Biotech), and *Rsa*I (Life Technologies) according to the vendor's recommendations. The digested amplified products were analyzed by electrophoresis in a 1.2% (wt/vol) agarose gel with a 100-bp ladder (Amersham-Pharmacia Biotech) as the molecular size marker.

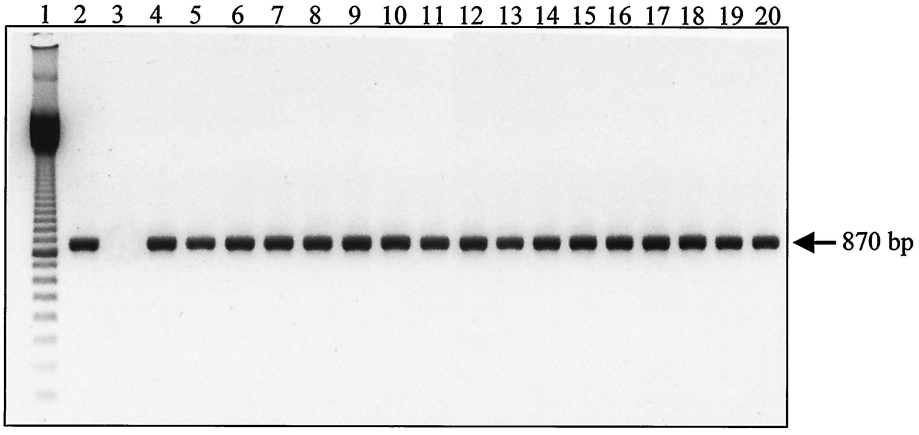

FIG. 1. RT-PCR products obtained with primers Nter and Cter and RNA isolated from nonflagellated *C. difficile* strains. Lane 1, 100-bp ladder (Amersham-Pharmacia Biotech); lane 2, strain 79-685 (positive control); lane 3, RNA from *C. sordellii* (negative control); lane 4, EX560; lane 5, ATCC 43596 (serogroup reference C); lane 6, 54637; lane 7, 54828; lane 8, 51936; lane 9, 1075; lane 10, ATCC 43597 (serogroup reference D); lane 11, 55944; lane 12, 6058; lane 13, SE956; lane 14, ATCC 43600 (serogroup reference H); lane 15, ATCC 43601 (serogroup reference I); lane 16, 54823; lane 17, 55684; lane 18, 52356; lane 19, 57207; lane 20, 20356.

DNA sequencing. PCR products were purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany). DNA sequencing was carried out with the BigDye terminator DNA sequencing kit (PE Applied Biosystems, Warrington, England). The samples were analyzed with the automated DNA sequencer ABI PRISM 310 genetic analyzer (Perkin-Elmer). The sequencing was initiated on both strands with the primers Nter and Cter and was finished with internal primers designed from the sequences obtained.

RNA extraction and RT-PCR. RNA was extracted from 10 ml of 8-h *C. difficile* anaerobic culture. Bacteria were harvested by centrifugation at 5,000 × *g* for 5 min at 4°C and then resuspended in 0.5 ml of cold TE buffer (10 mM HCl, 1 mM EDTA, pH 7.4) and kept on ice. Glass beads (0.6 g; 425 μm < diameter < 600 μm; Sigma Chemical Co., St. Louis, Mo.) were added in a solution containing 0.17 ml of 4% (wt/vol) Bentone rheological additive (Rheox Ltd., Livingston, Scotland), 0.5 ml of acid phenol (Sigma Chemical Co.), and 0.05 ml of 10% (wt/vol) sodium dodecyl sulfate solution. The solution was mixed three times by vortexing for 1 min each, interrupted by 1-min pauses. The aqueous phase was recovered by centrifugation at 12,000 × *g* at 4°C for 15 min and then extracted three times with a phenol-chloroform (1:1, vol/vol) solution and precipitated with ethanol. The RNA pellet was washed with 75% (vol/vol) cold ethanol, vacuum dried, and resuspended in 50 μl of TE buffer. The RNA was treated with DNase I (Amersham-Pharmacia Biotech) and stored at −20°C.

The RT-PCR was carried out with the SuperScript one-step RT-PCR system (Life Technologies) in a 50-μl mixture containing 1 μg of RNA template, a 1 mM concentration each of primers Nter and Cter, 1.2 mM $MgSO_4$, and the reaction cocktail according to the manufacturer's instructions. The RNA of *C. difficile* 79-685 was used as a positive control, and the RNA of *C. sordellii* was used as a negative control. The reaction mixture was overlaid with mineral oil. 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 480 (Perkin-Elmer). Each cycle consisted of three steps as described above. The amplified products were subjected to electrophoresis on a standard 1% (wt/vol) agarose gel.

Serogrouping and toxigenicity. Serogroups were determined by slide agglutination with rabbit antisera (12) and were confirmed by typing by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (13). Toxin A production was determined by the *C. difficile* toxin A test (Oxoid). In vitro cytotoxin (toxin B) determination was performed with HeLa cells cultured in minimum Eagle medium with Earle's salts (Life Technologies) supplemented with 10% fetal calf serum (Life Technologies), 1% nonessential amino acids (Life Technologies), and 200 mM L-glutamine (Life Technologies) in microtiter plates (3×10^4 cells per well). Fivefold serial dilutions of filtrates of 48-h TGY liquid cultures of *C. difficile* were incubated for 18 h with the cells at 37°C in a 5% CO_2 atmosphere. After fixation and coloration of the culture cells with methylene blue, cytotoxic effect was observed by inverse microscopy.

Computer analyses. Nucleotide and protein sequence alignments were performed with DNA Strider software and the Multalin program (9).

Nucleotide sequence accession numbers. The nucleotide sequence of the *fliC* locus of strains 79-685, 545, 3232, and 5036, corresponding to serogroups S3, C, D, and X, respectively, were assigned GenBank numbers AF065259, AF095236, AF095237, and AF095238.

RESULTS

Detection of *fliC* gene and flagella by EM and *fliC* gene expression. As observed by EM, 30 out of 47 strains showed visible flagella, whereas 17 were nonflagellated (Table 1). All strains from serogroup A were flagellated; in contrast, no strain from serogroup C carried flagella. In other serogroups both flagellated and nonflagellated strains were observed. The number and length of flagella also varied considerably among strains.

PCR amplification using *fliC*-specific oligonucleotide primers Nter and Cter was employed to investigate the presence of the gene in *C. difficile* isolates. The amplification gave a single product in all 47 *C. difficile* strains studied (not shown), whereas no amplified product was obtained from the negative control *C. sordellii* strain. An 870-bp fragment was obtained from 46 strains, whereas the serogroup X reference strain revealed an 850-bp amplified fragment.

The lack of flagella on the bacterial surface could be due to the absence of transcription of the *fliC* gene. Therefore, to investigate expression of the flagellin gene in flagellated and nonflagellated strains, DNA transcription was investigated by detecting flagellin mRNA by RT-PCR. The results (Fig. 1) show that a single 870-bp amplified fragment was obtained from all the 17 nonflagellated strains, including four nonflagellated serogroup C, D, H, and I reference strains.

The absence of genomic DNA contamination in the RNA samples was verified by PCR using Nter and Cter primers. Furthermore, a counterexperiment was carried out with an RNA sample treated with RNase and subjected to an RT-PCR as described above. No amplified products were detected in these two control experiments, thus confirming the purity of the RNA preparation and the specificity of the target RNA.

Detection and copy number of *fliC* in *C. difficile* isolates. In some bacteria, *fliC* can be present in multiple copies on the bacterial chromosome. To assess whether *fliC* is present in mono- or multicopy, the amplified DNA from strain 79-685 was used as a probe in Southern hybridization of chromosomal DNA of strain 79-685 and the 10 reference serogroups (A, B, C, D, F, G, H, I, K, and X). Hybridization under low-stringency conditions showed that DNA of all isolates hybridized with the *fliC*-specific probe. Only one copy of the gene was present in each strain. Some strains carry a *Hin*dIII site and therefore

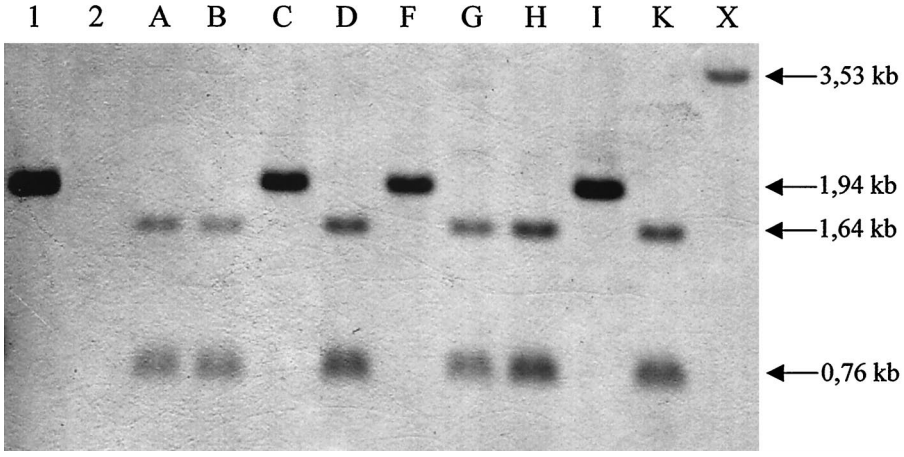

FIG. 2. Southern blot of chromosomal DNA isolated from *C. difficile* reference strains belonging to Delmée serogroups hybridized under low stringency with the *fliC* probe. DNA was digested with *Hin*dIII, electrophoresed, and transferred to a nylon membrane. Lane 1, strain 79-685 (positive control); lane 2, *C. sordellii* (negative control); lane A, ATCC 43594 (serogroup A); lane B, ATCC 43593 (serogroup B); lane C, ATCC 43596 (serogroup C); lane D, ATCC 43597 (serogroup D); lane F, ATCC 43598 (serogroup F); lane G, ATCC 43599 (serogroup G); lane H, ATCC 43600 (serogroup H); lane I, ATCC 43601 (serogroup I); lane K, ATCC 43602 (serogroup K); lane X, ATCC 43603 (serogroup X). The two bands in lanes A, B, D, G, H, and K are produced by the *Hin*dIII site near base 560 of the flagellin gene.

show the presence of two bands (Fig. 2). The presence of a *Hin*dIII site was confirmed by subjecting the amplified *fliC* gene product to *Hin*dIII digestion. The digestion with *Hin*dIII allows the classification of the strains in three groups: the first group exhibits a single 1.94-kb single band (79-685, C, F, and I), and the second group displays two bands of 1.64 kb and 0.76 kb (A, B, D, G, H, and K), while there is a single 3.53-kb band for the serogroup X reference strain (Fig. 2).

DNA sequence analysis. In order to confirm that the 870-bp amplified product described above was the flagellin gene, PCR fragments obtained from the 10 reference serogroup strains were partially sequenced with the Nter primer. Sequencing revealed an N-terminal sequence identical to the *fliC* gene of the *C. difficile* 79-685 strain in all PCR products (data not shown). To investigate the conservation of the *fliC* gene coding region in strains representing the different profiles obtained by Southern blotting, the *fliC* gene of the serogroup C, D, and X reference strains was amplified by PCR using specific primers (Nter and Cter) as described in Materials and Methods. DNA and deduced amino acid sequence analysis revealed an open reading frame composed of 873 nucleotides (290 amino acids) for the 79-685 strain and our C and D reference strains, while the open reading frame was 846 nucleotides, corresponding to 281 amino acids, for the serogroup X reference strain. The latter strain carried a short deletion in the central region of flagellin (Fig. 3).

The degree of the identity between the deduced amino acid sequences for the four strains was analyzed and showed 100% identity between FliC of 79-685, the gene of which was previously sequenced by us (45), and the serogroup C reference strain, while the identity was 90 and 77% between serogroup C and serogroup D and X reference strains, respectively, and 76% between serogroup D and X reference strains.

At the protein level, the identity between FliC of the serogroup C reference strain and of the serogroup D and X reference strains was 86 and 72%, respectively, and was 71% between serogroup D and X reference strains. It is evident from the analysis that flagellin proteins of *C. difficile* strains exhibit conservation in the N and C termini, while the central region is more diverse. The presence of a number of conserved alanine residues suggests a secondary structure in α-helical conformation (alanine is a helix-forming residue); this conservation could reflect the functional importance of the terminal regions in forming the tertiary structure of the flagellin protein monomers.

RFLP analysis of flagellin genes. As sequence analysis of *fliC* from three strains suggested significant variability of gene structure, we decided to examine gene structure by PCR-RFLP analysis in all the isolates studied. The amplified *fliC* gene was

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          1                                                  50
79-685    MRVNTNVSAL IANNQMGRNV NGQSKSMEKL SSGVRIKRAA DDAAGLAISE
C         MRVNTNVSAL IANNQMGRNV NGQSKSMEKL SSGVRIKRAA DDAAGLAISE
D         MRVNTNVSAL VANNQMGRNV NAQSKSMEKL SSGVRIKRAA DDAAGLAISE
X         MRVNTNVSAL IANNQMGRNV SGQSKSMEKL SSGLRIKRAA DDAAGLAISE
          ++++++++++  +++++++++   ++++++++ +++ ++++++ ++++++++++

          51                                                 100
79-685    KMRAQIKGLD QAGRNVQDGI SVVQTAEGSL EETGNILQRM RTLSLQSANE
C         KMRAQIKGLD QAGRNVQDGI SVVQTAEGSL EETGNILQRM RTLSLQSANE
D         KMRAQIKGLD QAGRNVQDGI SVVQTAEGAL EETGNILQRM RTLSLQSSNE
X         KMRAQLKGLD QAGRNVQDGI SVVQTAEGAL EETGNILTRM RTLAVQASNE
          +++++ ++++ ++++++++++ ++++++++ + +++++++ ++ +++  +  ++

          101                                                150
79-685    INNTEEREKI ADELTQLKDE IERISSSTEF NGKKLLDGTS STIRLQVGAS
C         INNTEEREKI ADELTQLKDE IERISSSTEF NGKKLLDGTS STIRLQVGAS
D         TNTAEERQKV ADELLQLKDE VERISSSIEF NGKKLLDGSS TEIRLQVGAN
X         TNSKDERAKI AGEMEQLRSE VDRIADSTKF NGENLLS-SD KKIALQVGAE
           +   ++ +  + +  ++  +   ++  +  + ++  ++       + +++++

          151                                                200
79-685    YGTNVSGTSN NNNEIKIQLV NTASIMASAG ITTASIGSMK AGGTTGTDAA
C         YGTNVSGTSN NNNEIKIQLV NTASIMASAG ITTASIGSMK AGGTTGTDAA
D         FGTNVAGTTN NNNEIKVALV NTSSIMSKAG ITSSTIASLN VDGASGTDAA
X         --------AV SNNVIEVSLI NTKGVLTTRN VNSANIDAMS VSGSIGTEAA
                   ++ +   +  ++             +       +  ++ ++

          201                                                250
79-685    KTMVSSLDAA LKSLNSSRAK LGAQQNRLES TQNNLNNTLE NVTAAESRIR
C         KTMVSSLDAA LKSLNSSRAK LGAQQNRLES TQNNLNNTLE NVTAAESRIR
D         KQMVSSLDMA LKELNTSRAK LGAQQNRLES TQNNLNNTIE NVTAAESRIR
X         SKMIVNLDSS LADINSARAL LGAQQNRLES TQNNLNNTVE NVTAAESRIR
                ++   +   +  ++  ++++++++++ ++++++++ + ++++++++++

          251                                          291
79-685    DTDVASEMVN LSKMNILVQA SQSMLAQANQ QPQGVLQLLG Z
C         DTDVASEMVN LSKMNILVQA SQSMLAQANQ QPQGVLQLLG Z
D         DTDVASEMVN LSKMNILVQA SQSMLAQANQ QPQGVLQLLG Z
X         DTDVASEMVN LSKMNILVQA SQSMLSQANQ QPQGVLQLLG Z
          ++++++++++ ++++++++++ +++++ ++++ ++++++++++
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FIG. 3. Sequence alignment of the deduced amino acid sequence of FliC of *C. difficile* strains 79-685, serogroup reference C, D and X strains. Identical residues are indicated with an asterisk (+). Deletions are indicated with a dash (—). The alignment was performed with the MULTALIN program (10).

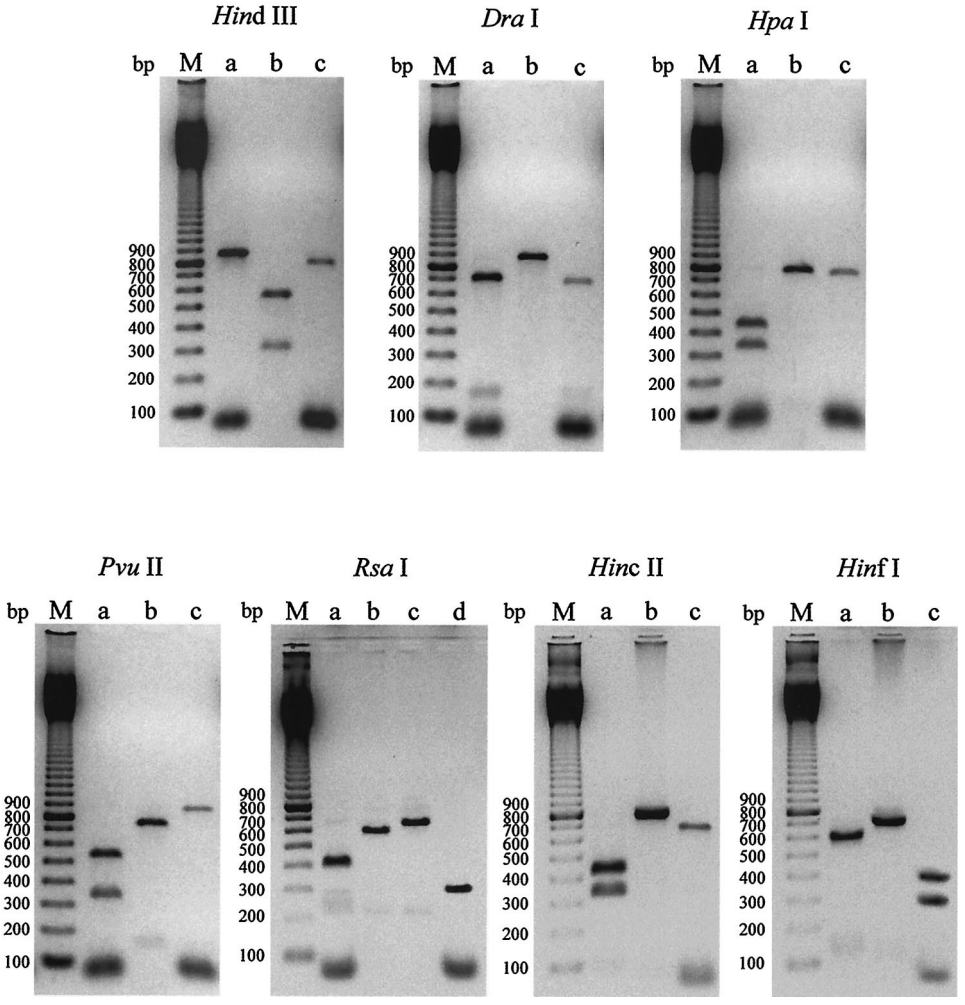

FIG. 4. RFLP patterns of PCR-amplified flagellin genes. The amplified *fliC* gene of *C. difficile* isolates was digested with *Hin*dIII, *Dra*I, *Hpa*I, *Pvu*II, *Rsa*I, *Hin*cII, and *Hin*fI. The different restriction profiles obtained with each enzyme were designated a, b, c, and d. Lanes M, 100-bp ladder (Amersham-Pharmacia Biotech); lanes a, profile a; lanes b, profile b; lanes c, profile c; lanes d, profile d.

digested with *Hin*dIII, *Dra*I, *Hpa*I, *Pvu*II, *Rsa*I, *Hin*cII, and *Hin*fI. According to the restriction map (not shown), these restriction sites are distinct and therefore can be used to perform RFLP analysis.

The results (Fig. 4) show the different restriction patterns obtained from the *C. difficile* strains. Three different restriction profiles were obtained with *Hin*dIII, *Dra*I, *Hpa*I, *Pvu*II, *Hin*cII, and *Hin*fI enzymes, and four restriction profiles (designated a, b, c, and d) were obtained with *Rsa*I endonuclease. As far as the serogroup X reference strain is concerned, the *fliC* RFLP analysis revealed a unique and different profile with each enzyme: profile c with *Hin*dIII, *Dra*I, *Hpa*I, *Pvu*II, *Hin*cII, and *Hin*fI restriction enzymes and profile d with *Rsa*I.

The strains can be classified into nine RFLP groups (Table 2). The most frequent RFLP types are I (15 strains) and VII (16 strains). RFLP type I encompasses mostly nonflagellated strains that are either toxin positive or negative, whereas RFLP type VII encompasses mostly flagellated strains that are toxin positive; types V and IX are rare (one of each) and include only toxin-negative strains. In addition, types III, VI, and VIII are also characterized by toxin-negative strains. The single isolate TO005 is the only one to be characterized as RFLP profile IV.

Serogroups C, D, F, G, and I are homogeneous in terms of RFLP profile, with all strains tested here displaying the same pattern. In contrast, profiles of serogroups, A, B, H, K, S3, and X are variable. Serogroup A is divided in four RFLP groups (I, IV, V, and VII), serogroup B is divided in three groups (I, III,

TABLE 2. Classification of restriction enzyme patterns into RFLP groupgroups

RFLP group	Restriction enzyme pattern generated by digestion with[a]:						
	*Hin*dIII	*Dra*I	*Hpa*I	*Pvu*II	*Rsa*I	*Hin*cII	*Hin*fI
I	a	a	a	a	a	a	a
II	a	a	b	a	a	b	a
III	a	a	a	a	a	b	b
IV	a	a	b	b	b	b	b
V	a	a	b	b	c	b	b
VI	b	b	a	a	a	a	a
VII	b	b	b	b	b	b	b
VIII	b	b	b	b	c	b	b
IX	c	c	c	c	d	c	c

[a] Restriction enzyme patterns are shown in Fig. 4.

TABLE 3. Repartition of serogroups into the nine RFLP groups

Serogroup	No. of strains studied	No. of strains belonging to the following RFLP group:								
		I	II	III	IV	V	VI	VII	VIII	IX
A	6	1			1	1		3		
B	4	1		2				1		
C	5	5								
D	3								3	
F	4		4							
G	4							4		
H	3							2	1	
I	4	4								
K	5	1						4		
S3	5	2						2	1	
X	4	1					2			1

and VII), serogroup H is divided in two groups (VII and VIII), serogroup K is divided in two groups (I and VII), serogroup S3 is divided in three groups (I, VII, and VIII), and serogroup X is divided in three groups (I, VI, and IX). The serogroup X reference strain has a separate profile and forms the RFLP group IX (Table 3).

DISCUSSION

For numerous pathogens, the capacity to adhere, invade, and destroy the colonic mucosal cells appears to be an essential aspect of the first stage of their pathogenicity. *C. difficile* is responsible for the most-frequent hospital-acquired infection consequent to antibiotic therapy. It causes diarrheal disease, which can lead to an intense acute response: pseudomembranous colitis. *C. difficile* releases two exotoxins into the colon which are responsible for the disease: toxin A and toxin B. Toxin A (an enterotoxin) elicits fluid secretion, mucosal damage, and intestinal inflammation; toxin B (a cytotoxin) is completely devoid of enterotoxicity.

Before these events take place, *C. difficile* entering the gut must find pathways to reach suitable epithelial cells, which are naturally protected by a layer of dense mucus. *C. difficile* can adhere to the mucous layer (23). Afterwards the bacterium could penetrate the mucous layer with the aid of its proteases (38) and flagella; finally, *C. difficile* attaches to enterocytes by means of specific adhesins (23, 48); the role of flagella in this process has yet to be defined. At these different steps, the presence of complex and specialized chemotaxis and flagellar systems may play a role.

The studies undertaken here revealed that with EM certain strains of *C. difficile* are characterized by their inability to produce in vitro visible flagella. We have shown by PCR that the flagellin gene was present in both flagellated and nonflagellated *C. difficile* strains. The specificity of the amplification was confirmed by the fact that the flagellin gene of *C. sordellii*, highly flagellated and genetically very close to that of *C. difficile*, was not amplified with these primers. Gene amplification by PCR has been frequently used as a rapid method for detection and identification of pathogenic bacteria including *Clostridium perfringens* (16), *Campylobacter* spp. (36), *Listeria monocytogenes* (19), and *Bordetella bronchiseptica* (22). Therefore, amplification of the *fliC* gene could be used as a rapid method to detect and identify *C. difficile*.

We showed here that the flagellin gene was expressed in flagellated and nonflagellated *C. difficile* strains by detection of flagellin mRNA with the one-step method RT-PCR. According to Macnab (27) and Manson (28), the *Escherichia coli* flagellin gene was only expressed when both the basal body and the hook of the flagellum were fully formed through the membrane of bacterium. From that, the flagellin, the cap protein, and the junction hook-flagellum proteins were synthesized and assembled to achieve flagellar filament formation from the external membrane (5, 42). In nonflagellated *C. difficile* strains, the cap and/or junction hook-flagellum proteins could be inactivated by mutation, thus preventing transport of flagellin subunits through the bacterial membrane and polymerization. To confirm this hypothesis, it would be interesting to verify the presence of the flagellin protein in nonflagellated strains.

The nonflagellated *C. difficile* strains possess a cryptic flagellin gene. We cannot rule out, however, that in vivo all strains could be flagellated, and we intend to study the in vivo expression of *fliC* in our mouse model. Cryptic genes have been characterized in nonmotile bacteria. Indeed, the expression of surface flagella in some pathogenic bacteria may be induced only by factors related to a specific biological microenvironment or under certain in vitro growth conditions. Holt and Chaubal (21) showed that the carbon source, the viscosity of the medium, and the temperature of incubation can induce the motility of *S. enterica* Pullorum, thought to be nonmotile and nonflagellated. *Shigella* spp. are described as nonflagellated and nonmotile organisms. However, Giron (17) detected motility and flagella by EM in four strains and two clinical isolates, depending on the culture conditions under which temperature, salt, glucose, oxygen, or agar concentrations were modified. Tominaga et al. (46) showed the presence of intact cryptic flagellin genes in nonflagellated *Shigella flexneri* and *Shigella sonnei* strains. These genes produced normal-type flagella in an *E. coli* Δ*fliC* strain. Their results suggested a loss of the expression potential of flagellar genes, probably by various mutations and/or gene rearrangements. It would be interesting to investigate the role of mucus as an inducing factor for flagellal expression.

In order to study the variability of flagellin genes, the *fliC* gene was sequenced in three strains representing different profiles obtained by Southern blotting. Sequencing showed high conservation in the N-terminal and C-terminal regions and pronounced variability in the central domain of the flagellin protein (Fig. 3). The N- and C-terminal parts are responsible for secretion and polymerization of flagella, whereas the central region constitutes the surface-exposed antigenic part of the flagellar filament as described by Winstanley and Morgan (52), but flagellin may vary considerably among species in both amino acid sequence and size (49, 52). The deletion of amino acids in the variable domain of the serogroup X reference strain suggests, analogous to other bacteria, that the central region plays no role in the structure of flagella since this strain possesses flagella. Mutations of the flagellin gene do not account for the absence of flagella since the flagellin gene of the flagellated 79-685 strain is strictly identical to that of the nonflagellated serogroup C reference strain.

Different methods have been developed to study the epidemiology of *C. difficile* or to identify or type strains. Analysis of restriction patterns of DNA of clinical isolates has been used for investigations of epidemiology and typing of *C. difficile*-associated diarrhea (24). Pantosti et al. (37) used the electrophoretic patterns of extracted proteins to characterize *C. difficile* strains from various sources and showed correlation between certain electrophoretic patterns and virulence. Delmée et al. (13) compared serogrouping of *C. difficile* by slide agglutination with rabbit antisera and polyacrylamide gel electrophoresis of whole-cell proteins, permitting correlation between the two typing systems and establishment of a single classification. Recently, new molecular techniques have been devel-

oped to type *C. difficile* strains based on DNA polymorphism. DNA pattern profiles have been obtained by PCR amplification of a specific chromosomal region such as the rRNA gene (4) or the 16S-23S rRNA gene intergenic spacer region (44). Another molecular method, based on DNA polymorphism, has been found to be useful to distinguish strains of *C. difficile*, namely, arbitrary primed PCR, also called random amplified polymorphic DNA analysis. Arbitrary primed PCR or random amplified polymorphic DNA analysis has been used as an efficient discriminative method for investigation of nosocomial outbreaks of *C. difficile*-associated diarrhea (2, 3, 5; F. Barbut, N. Mario, J. Frottier, and J. C. Petit, Letter, Eur. J. Clin. Microbiol. Infect. Dis. **12:**794–795, 1993).

In our study, we have used the PCR-RFLP method to study genetic diversity among *C. difficile* strains. With this molecular technique, correlation between RFLP groups and serogroups was clear for certain serogroups. Serogroups C and I are represented by a single RFLP group, group I, and serogroups D, F, and G are represented by RFLP groups VIII, II, and VII, respectively. We noticed that serogroup F exclusively possesses pattern II, which is not shared by any other strain in this study. Similar data were shown by Rupnik (41) concerning the toxinotype of strains belonging to serogroup F. Other serogroups (A, B, H, K, S3, and X) were subdivided into several RFLP groups. However, six RFLP groups, corresponding each to a single serogroup, could be used to confirm some strains. Indeed, RFLP groups II, III, IV and V, and VI and IX were correlated to serogroups F, B, A, and X, respectively. Some conclusions could also be drawn concerning the state of flagellation and toxigenesis, but a larger number of strains need to be investigated to draw more-definitive conclusions.

The study of flagellin gene diversity has been also carried out with other bacteria, such as *C. jejuni* (33, 35, 43), *P. aeruginosa* (32, 50), *S. enterica* (10), *Vibrio parahaemolyticus* (29), *H. pylori* (34), or *Burkholderia cepacia* (47, 51). The results have clearly demonstrated the pronounced genetic diversity of the flagellar gene of various bacteria. The PCR-RFLP method has sometimes been used with success to differentiate several bacterial flagellal types from isolates. However, in certain cases, this procedure does not appear sufficient to type bacterial species.

The flagellin genes are excellent biomarkers with which to study strain diversity. The particular structure of the flagellin gene, with terminal conserved regions allowing gene amplification by PCR, allows analysis by RFLP and sequencing to study the variations in the central region. The PCR-RFLP procedure is rapid, highly specific, and reproducible. If a vaccine is to be developed for *C. difficile* disease based on different proteins, the preparations should include a mixture of flagellin proteins from major RFLP groups to allow the best possible protection. *C. difficile* flagella could play a role in intestinal colonization during the first stage of pathogenesis. Colonization is induced in response to environmental conditions. It is likely that production of flagella could be under the control of a system which may be turned on or off by various factors in the gut environment. Important questions remain to be explored as to the identity of these factors and what role flagella and motility play in the pathogenic scheme.

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