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Variability of *Clostridium difficile* Surface Proteins and Specific Serum Antibody Response in Patients with *Clostridium difficile*-Associated Disease

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Pathogen attachment is a crucial early step in mucosal infections. This step is mediated by important virulence factors, such as surface proteins. *Clostridium difficile* surface proteins have been identified as (i) adhesins (the flagellar cap protein FliD; the flagellin FliC; and the cell wall protein Cwp66 with a two domain-structure [Cw66 N-terminal and Cwp66 C-terminal domains]) and (ii) protease (the Cwp84 protein). To address the roles of these proteins in the pathogenesis of *Clostridium difficile* and to identify vaccine antigen candidates, we analyzed the variability of the proteins and their immunogenicities in 17 patients with *C. difficile*-associated disease. PCR-restriction fragment length polymorphism analysis of amplified gene products revealed interstrain homogeneity with *fliC* and *fliD*, in contrast to *cwp66* genes. Immunoblot analysis showed that FliC and FliD were detected in the majority of isolates. The N-terminal domain of Cwp66 and Cwp84 were present in all strains tested, in contrast to the Cwp66 C-terminal domain, the expression of which was heterogeneous. The 17 sera from the corresponding patients were analyzed by enzyme-linked immunosorbent assay to detect antibodies directed against these proteins. Many patients developed antibodies to FliC, FliD, Cwp84, and the Cwp66 C-terminal domain, but not to the Cwp66 N-terminal domain. In conclusion, this study confirms the expression of these surface proteins of *C. difficile* during the course of the disease. In addition, the FliC, FliD, and Cwp84 proteins appeared to be good potential vaccine candidates.

The expression of virulence by bacterial pathogens often requires the production and actions of toxins and adhesins. Whereas toxins are generally released by the pathogens into the extracellular medium and can thus act at distant sites, surface proteins allow the microorganisms to adhere to host determinants (2, 10, 20).

Clostridium difficile is a gram-positive, spore-forming enteric pathogen. After disruption of the intestinal barrier by antibiotics, spores of *C. difficile*, acquired exogenously or endogenously, germinate, and bacteria multiply in the intestine. *C. difficile* synthesizes two major toxins, toxin A and toxin B, both of which are responsible for the clinical manifestations of the disease, which include diarrhea or, in the worst case, pseudomembranous colitis (18).

The colonization mechanism of *C. difficile* has recently been studied and is supposed to be a two-step process. The bacteria are initially able to interact with the apical microvilli of the intestinal epithelial cells and begin to release toxins A and B, which disrupt epithelial barrier function (16). The basolateral pole of epithelial cells thus becomes accessible, and a large number of bacteria are able to interact with receptors via their surface proteins (5). In addition to mediating the attachment of bacteria to host tissues, adhesins may have additional functions in the development of the infection. They may be biological effectors in vivo and thus influence the outcome of the host-pathogen interaction (9). Flagella contribute to the virulence of pathogenic bacteria through chemotaxis, as well as adhesion to and invasion of host surfaces (19)

Some of the surface proteins of *C. difficile* have been characterized: the proteins of the S-layer (4), the flagellin FliC, the major structural component of the flagellar filament, the flagellar cap protein FliD, and the cell wall proteins Cwp66 and Cwp84. FliD has been shown to have in vitro and in vivo adhesive properties and, in particular, to play a role in attachment to mucus (25). Cwp66 is a surface protein with a two-domain structure. The C-terminal domain (Cwp66-Cter) is exposed to the cell surface, displays repeated motifs, and has been described as an adhesin; the N-terminal domain (Cwp66-Nter), which shows homology to the CwlB autolysin of *Bacillus subtilis*, is supposed to anchor Cwp66 to the cell wall of *C. difficile* (26). Cwp84 is a protein with proteolytic activity which could have a role in the physiology of the bacteria (21).

The level of host immune response to toxins has been shown to correlate with the severity of the disease (13). Mulligan et al. showed that antibodies were also directed against surface proteins of *C. difficile* (15). In addition, it has been shown by Drudy et al. that a high level of immunoglobulin M (IgM) antibody to *C. difficile* S-layer proteins is associated with a markedly reduced risk of recurrent *C. difficile*-associated diarrhea (7).

In a previous study, we demonstrated that antibody levels against FliC, FliD, and Cwp66-Nter were significantly higher in a control group versus a group of patients with *Clostridium difficile*-associated-disease (CDAD), suggesting that these proteins are able to induce an immune response that could play a role in the defense mechanism of the host (17).

The aim of the present study was to analyze the genotypic

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TABLE 1. Primers used for PCR amplification and restriction enzymes used for RFLP

Target	Primers		Restriction enzymes
	Name	Sequence	
Ribotyping	RiboCD1	5′-GTGCGGCTGGATCACCTCCT-3′	
	RiboCD2	5′-CCCTGCACCCTTAATAACTTGACC-3′	
fliC	*fliC*-Nter	5′-ATGAGAGTTAATACAAATGTAAGTGC-3′	HindIII, HpaI, PvuII, HincII, DraI, HinfI
	fliC-Cter	5′-CTATCCTAATAATTGTAAAACTCC-3′	
fliD	*fliD*-Nter	5′-ATGTCAAGTATAAGTCCAGTAAG-3′	AccI, DraI, EcoRI, HinfI, HincII
	fliD-Cter	5′-TTAATTACCTTGTGCTTGTG-3′	
3′ part of *cwp66*	3′ - F	5′-GATAAAGTTACTCAAATTGGT-3′	RsaI, ScaI, SspI, DraI
	3′ - R	5′-TCTTCCCCATCTAGAAAC-3′	
5′ part of *cwp66*	5′ - F	5′-CGAAAGAATTAGGAGGTAAG-3′	RsaI, DraI, HindIII, KpnI
	5′ - R	5′-ACCAATTTGAGTAACTTTATC-3′	

and phenotypic variability of these surface proteins and their immunogenicities to confirm their expression in humans in order to identify novel antigens for active immunization.

MATERIALS AND METHODS

***C. difficile* strains and growth conditions.** Seventeen *C. difficile* strains were isolated from patients with CDAD (Microbiology Unit, Pr Delmée, Catholic University of Louvain, Brussels, Belgium). The diagnosis of *C. difficile* disease was confirmed by culture and detection of toxin B in fecal samples. *C. difficile* strains were grown under anaerobic conditions on Colombia cystein agar plates (Oxoid) supplemented with 5% horse blood (Biomerieux, Marcy l'Etoile, France) or in tryptone-glucose-yeast broth (Difco) for 48 h in aerobiosis. The *C. difficile* strain 79-685, isolated from a patient with pseudomembranous colitis, was a gift from the Department of Microbiology of the University of Strasbourg, Strasbourg, France, and was used as the reference strain.

Serum samples. Sera from patients infected by the 17 isolates studied were obtained 1 to 3 weeks after diagnosis (patients 1 to 17). Sera from 11 other patients suffering from CDAD were obtained from Jean Verdier Hospital (Assistance Publique-Hôpitaux de Paris, Bondy, France) and from the Centre Hospitalier Universitaire of Rouen (France) at different periods after diagnosis in order to follow antibody levels directed against the adhesins.

Comparison of the antibody level directed against Cwp84 was done by an enzyme-linked immunosorbent assay (ELISA) method as described previously (17). The control group was composed of seven sera from healthy women without a history of CDAD attending a maternity ward and three sera from children aged 1.5 months to 4.5 years with *C. difficile*-negative stool culture (culture and toxin negative) from Jean Verdier hospital, AP-HP group, France. The CDAD patient group was composed of nine sera from CDAD patients. The statistical analyses were done as described previously (17).

Restriction fragment length polymorphism (RFLP) and PCR ribotyping analyses. DNAs from the 17 clinical isolates and the 79-685 strain were extracted using the GFX genomic blood DNA purification kit (Amersham Biosciences). PCR amplifications were performed in a Thermocycler Biometra in a reaction volume of 25 μl using puRe*Taq* Ready-To-Go PCR beads (Amersham Bioscience). Each primer was designed based on reference strain 79-685 and was used at a final concentration of 2.4 ng/μl. Specific primers used for amplifications of each gene are described in Table 1. Amplifications were performed separately for the 5′ and the 3′ parts of the *cwp66* gene.

For PCR ribotyping, initial denaturation was carried out at 94°C, followed by 35 cycles of amplification: denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min. The amplified products were analyzed by electrophoresis in a 2% (wt/vol) agarose gel.

For PCR of the *fliC*, *fliD*, and *cwp66* genes, after initial denaturation, each cycle consisted of three steps: denaturation at 94°C (30 s), annealing for 30 s at 55°C for *fliC* and *fliD* or at 53°C for *cwp66*, 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.

For RFLP, 5 μl of amplified products was digested with different restriction enzymes (Table 1) and then analyzed by electrophoresis in a 1.2% (wt/vol) agarose gel. The restriction enzymes were designed according to the 630 or 79-685 *Clostridium difficile* gene sequences (Table 1).

Flagellar preparation. Flagella were isolated from the 17 isolates by the procedure described by Delmée et al. (6). Briefly, *C. difficile* strains were grown on blood agar plates under anaerobic conditions for 24 h. Bacteria were resuspended in 1.5 ml of sterile distilled water, and the suspension was strongly shaken for 2 min and centrifuged at 5,000 × *g* for 30 min at 4°C. The supernatants were centrifuged (25,000 × *g*; 1 h; 4°C). The pellets of flagella were suspended in 100 μl of phosphate-buffered saline at pH 7.4.

Surface protein extraction. *C. difficile* surface proteins were extracted from 24-h cultures as described by Wexler et al. (27) to investigate the presence of Cwp84 and Cwp66 in the 17 clinical isolates.

Obtaining recombinant proteins. Recombinant FliC, FliD, the two domains of Cwp66, and the catalytic N-terminal domain of Cwp84 from strain 79-685 were purified as previously described (11, 23, 25, 26). For cloning of the *C. difficile* 79-685 *cwp84* gene into an expression vector, two oligonucleotide primers, TG AGCTAGCGCAGAAAACCATAAAACTCTAGATG and GTGAATTCCTA TTTTCCTAAAAGAGTAT (incorporating NheI and EcoRI sites, respectively [underlined]), were used to amplify by PCR the full-length coding region of the *cwp84* gene with ThermalAceDNA Polymerase. The amplification product was purified with a High Pure PCR product purification kit (Roche). In order to attach a His tag to the N-terminal position of the fusion protein, pET-28a(+) plasmid DNA and the amplified PCR product were digested with NheI and EcoRI. The digested products were purified with the High Pure PCR product purification kit and then cloned in the pET-28a(+) plasmid and transformed first into *Escherichia coli* Top10 and then into *E. coli* BL21 DE3 Star. Subsequent protein expression and purification steps were performed by induction of protein expression with 1 mM of IPTG (isopropyl-β-D-thiogalactopyranoside), followed by single-step affinity chromatography employing a HIS-Select Nickel Affinity Gel, as described in protocols from Sigma.

Antibody production against 79-685 strain recombinant proteins. (i) Anti-FliC, anti-FliD, anti-Cwp66-Nter, and anti-Cwp66-Cter sera. Rabbit polyclonal antisera against the flagellin FliC, the cap protein FliD, and the Cwp66 N-terminal and Cwp66 C-terminal domains were raised as described previously (23, 26). Rabbit polyclonal monospecific Cwp66-N and Cwp66-C sera were prepared with purified C-terminal and N-terminal recombinant proteins by injecting lyophilized preparations (200 μg) in Freund's adjuvant into New Zealand White rabbits and then administering three boosters with 100 μg of protein in Freund's incomplete adjuvant on days 14, 28, and 42. The rabbits were sacrificed and bled 21 days after the last injection. Antibodies were purified on protein A-Sepharose as recommended by the supplier.

Antibodies were used at a 1/2,000 dilution in immunoblots.

(ii) Anti-Cwp84 sera. Mouse polyclonal antiserum was prepared by subcutaneously injecting the purified recombinant catalytic domain of the protein (8 μg) in Freund's complete adjuvant into C3H mice and then administering two boosters in Freund's incomplete adjuvant on days 14 and 28. The mice were sacrificed and bled 15 days after the last injection. Antibodies were used at a 1/500 dilution in immunoblots.

SDS-PAGE and immunoblotting. The flagellar proteins and the surface proteins were separated electrophoretically in a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto a nitrocellulose membrane (Amersham Biosciences) for immunoblotting. The membrane was incubated overnight at room temperature in blocking buffer (5% skimmed milk in TNT [10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween]), followed by 2 hours of incubation with antiFliC, antiFliD, antiCwp84, anti-Cwp66-Nter, and antiCwp66-Cter. The membrane was washed with TNT. Bound antibodies were detected using goat anti-rabbit IgG alkaline phosphatase conjugate for FliC, FliD, Cwp66-N, and Cwp66-C and with goat anti-mouse IgG

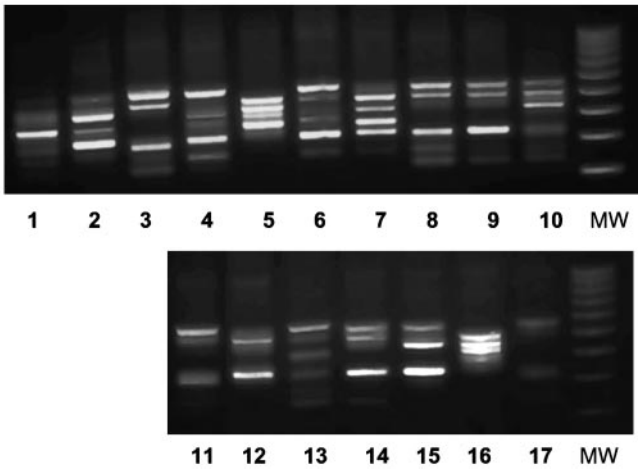

FIG. 1. PCR ribotyping profiles of the 17 *Clostridium difficile* isolates studied (1 to 17). Lane MW, 100-bp molecular size marker.

alkaline phosphatase conjugate for Cwp84 (1/2,000 dilution; Sigma). The substrates used were nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Invitrogen).

Detection of antibodies against the surface proteins in the patient sera. Anti-FliC, FliD, Cwp66-Nter, Cwp66-Cter, and Cwp84 antibodies were detected by an ELISA in patient sera as previously described (17). For each protein tested, the mean absorbance measured when the sample was replaced by phosphate-buffered saline–Tween–bovine serum albumin was defined as the background level. In all assays, samples yielding an absorbance 5 times the background absorbance were reported as positive, and those with a mean absorbance greater than 10 times the background were reported as strongly positive.

For the first 17 patients, only one serum sample each was analyzed. The mean absorbance and the corresponding standard deviation was calculated for each antigen. For the 11 other patients, several samples were successively analyzed. The first sample was analyzed in the 3 days after diagnosis, and a second sample and a third sample were analyzed 3 to 7 days and 7 to 12 days after diagnosis in order to follow the evolution of the antibody level against these proteins during the pathogenic process.

RESULTS

The first step of this work was to test the interstrain variability of the genes and the encoded proteins.

PCR ribotyping analysis. The distribution of the PCR ribotypes among the 17 clinical isolates is shown in Fig. 1. Fourteen genotypically different ribotypes were identified among the 17 clinical isolates. PCR ribotyping profiles of isolates 8, 9, and 14 on one hand and isolates 5 and 16 on the other hand seemed to be identical. The reference strain *C. difficile* 79-685 displayed the same ribotype as strain 3.

RFLP analysis of *fliC*, *fliD*, the 5′ part of *cwp66*, and the 3′ part of *cwp66*. The *fliC* and *fliD* genes from the 17 strains studied were able to be amplified with the selected primers with the same size amplified product. For *fliC*, two different restriction profiles were obtained. Among the 17 strains tested, 16 displayed the same profile, Ia, and only one showed a different profile, Ib (Table 2).

For *fliD*, three different restriction profiles were observed. Among the 17 strains tested, 14 displayed the same profile, Ia; two displayed profile Ib; and only one displayed profile Ic (Table 2).

For *cwp66*, the 3′ part of the gene was amplified for only 11 strains with the selected primers, and the amplified products displayed two different sizes. Four to six different restriction profiles were observed depending on the restriction enzyme used (Fig. 2A). The 5′ part of *cwp66* was amplified in 9 strains out of the 17 tested (Table 2). Among these nine amplified genes, six different RFLP profiles were observed (Fig. 2B and Table 2).

Detection of the FliC, FliD, Cwp66 (C-terminal and N-terminal), and Cwp84 proteins in *C. difficile* clinical isolates. We wanted to evaluate the presence of the different proteins in the isolates tested and their recognition by antibodies directed against the recombinant surface proteins from the 79-685 strain. Therefore, we searched for the presence of FliC, FliD, Cwp66-Nter, Cwp66-Cter, and Cwp84 proteins in the 17 *C. difficile* clinical isolates by immunoblotting using specific polyclonal antibodies corresponding to each recombinant protein from the 79-685 strain.

FliC and FliD were detected by SDS-PAGE and immunoblotting in 15 strains out of the 17 tested at the expected molecular mass (39 kDa for FliC and 56 kDa for FliD). These two proteins were simultaneously expressed even if FliC was present in greater quantity (Fig. 3A)

The N-terminal domain of Cwp66 was detected in all *C. difficile* isolates tested at the molecular mass of 50 kDa. Like the flagellar proteins, the native protein was recognized by rabbit antibodies produced with the recombinant protein of the 79-685 strain (Fig. 3B). In contrast, the C-terminal domain of Cwp66 was detected in only 12 isolates out of 17. For a few isolates, antibodies directed against this domain recognized bands at unexpected molecular masses from 15 to 40 kDa (Fig. 3C). Cwp84 was detected at the expected molecular mass in all isolates tested (Fig. 3D).

The second step of the work was to detect specific antibodies against these proteins in the sera of patients with CDAD.

TABLE 2. RFLP analysis of *fliC*, *fliD*, the 5′ part of *cwp66*, and the 3′ part of *cwp66*

RFLP group	Profile for *C. difficile* strain:																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	27	79–685
fliC	Ia[a]	Ia	Ia	Ia	Ia	Ia	Ib[b]	Ia	Ia	Ia	Ia	Ia	Ia	Ia	Ia	Ia	Ia	Ib
fliD	Ia	Ia	Ia	Ia	Ib	Ia	Ic	Ia	Ia	Ia	Ia	Ia	Ia	Ia	Ia	Ib	Ia	Ic
3′ part of *cwp66*	0[c]	0	0	Ia	Ia	IIc[a]	Ib	Ic	IIb	0	IIa	0	0	IIb	IIb	Ia	IIa	Ia
5′ part of *cwp66*	0	0	0	Ia	Ib	Ib	Ia	Ic	0	IIa	Id	0	0	0	0	Ib	IIb	IIb

[a] Groups I and II, amplified PCR products with different sizes.
[b] a, b, c, different RFLP profiles for same-size amplified product.
[c] Group zero, no PCR amplification.

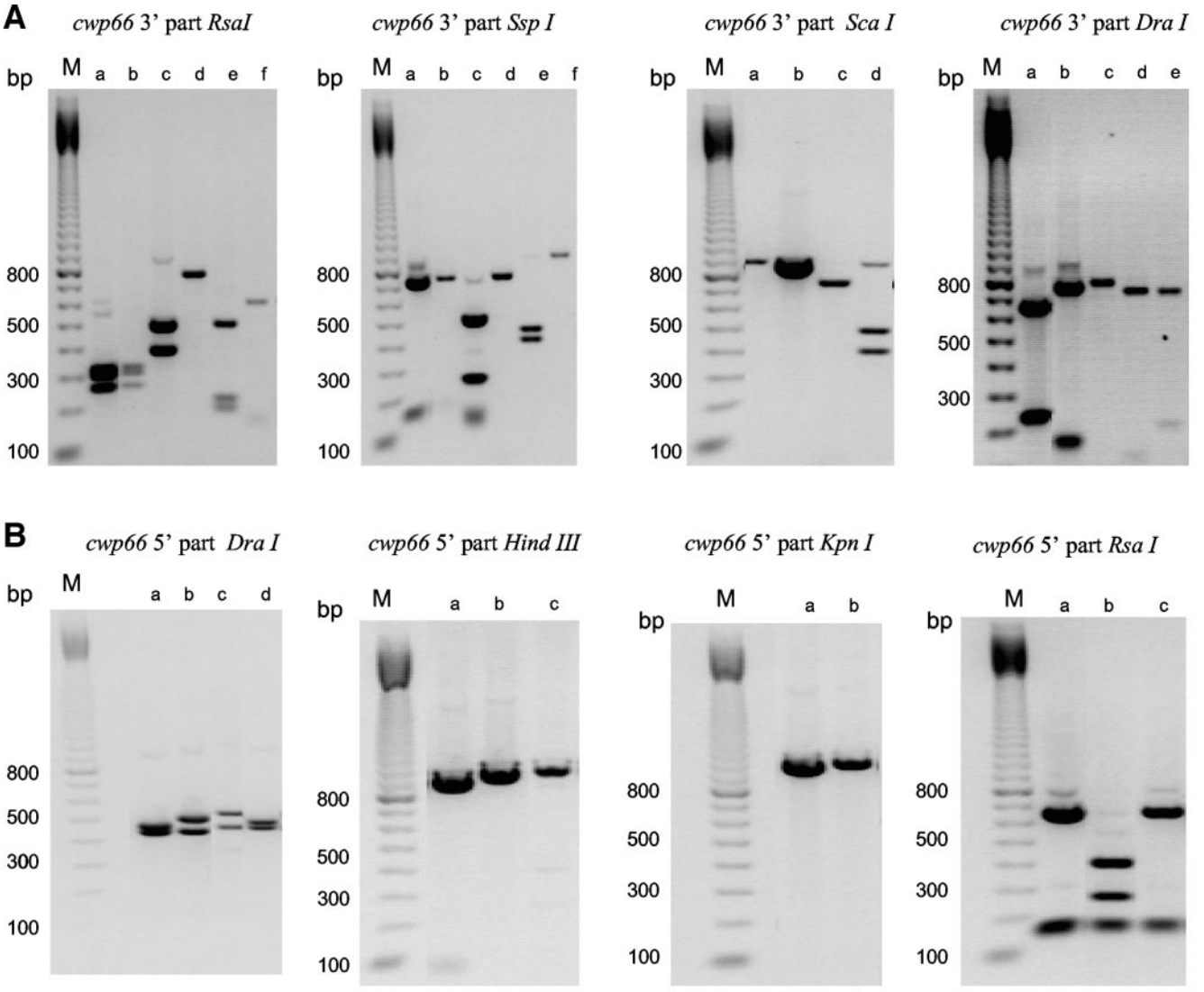

FIG. 2. PCR-RFLP profiles of *cwp66.* (A) The amplified 3′ part of the *cwp66* gene product was digested with RsaI, SspI, Sca I, and DraI. The different restriction profiles for the enzymes were designated a, b, c, d, e, and f. Lanes M, 100-bp ladders; lanes a, profile a; lanes b, profile b; lanes c, profile c; lanes d, profile d; lanes e, profile e; lanes f, profile f. The digested amplified products were subjected to electrophoresis in a 1.2% (wt/vol) agarose gel. (B) The amplified 5′ part of the *cwp66* gene product was digested with DraI, Hind III, KpnI, and RsaI. The different restriction profiles for the enzymes were designated a, b, c, and d. Lanes M, 100-bp ladders; lanes a, profile a; lanes b, profile b; lanes c, profile c; lanes d, profile d. The digested amplified products were subjected to electrophoresis in a 1.2% (wt/vol) agarose gel.

Detection of specific antibodies in sera from patients with CDAD. The cutoff absorbance levels for positive sera were 0.400, 0.329, 0.496, 0.980, and 0.380, respectively, for FliC, FliD, Cwp66-Nter, Cwp66-Cter, and Cwp84. The specificity of the ELISA was confirmed by immune absorption. Preincubation of positive samples with each protein at various concentrations (3 μg/ml to 50 μg/ml) resulted in a dose-dependent reduction in reactivity in the antiprotein ELISA system.

(i) FliC and FliD flagellar proteins. For FliC, among the 17 tested sera, 6 were defined as positive and 9 as strongly positive. For FliD, 13 were positive and 2 strongly positive. The two sera defined as negative for FliC were also negative for FliD.

(ii) Cwp66 N-terminal domain and Cwp66 C-terminal domain. Many patients developed antibodies directed to the C-terminal adhesin domain of the Cwp66 protein: 13 sera were positive and 1 strongly positive. Only a few patients developed antibodies recognizing the recombinant N-terminal domain of the Cwp66 protein in the 79-685 strain: only 2 out of the 17 tested were positive.

(iii) Cwp84. For Cwp84, 13 patient sera were considered strongly positive against Cwp84, 2 positive, and 2 negative. The difference in antibody levels against Cwp84 between the control group (A_{450} = 0.240) and the CDAD patient group (A_{450} = 0.170) was statistically significant, with the highest level in the control group (P = 0.03), which is similar to the other surface proteins, FliC, FliD, and the Cwp66 N-terminal domain (17).

The correlations of the detection of the native proteins in the clinical isolates by immunoblotting with the detection of specific antibodies to the recombinant proteins in sera from

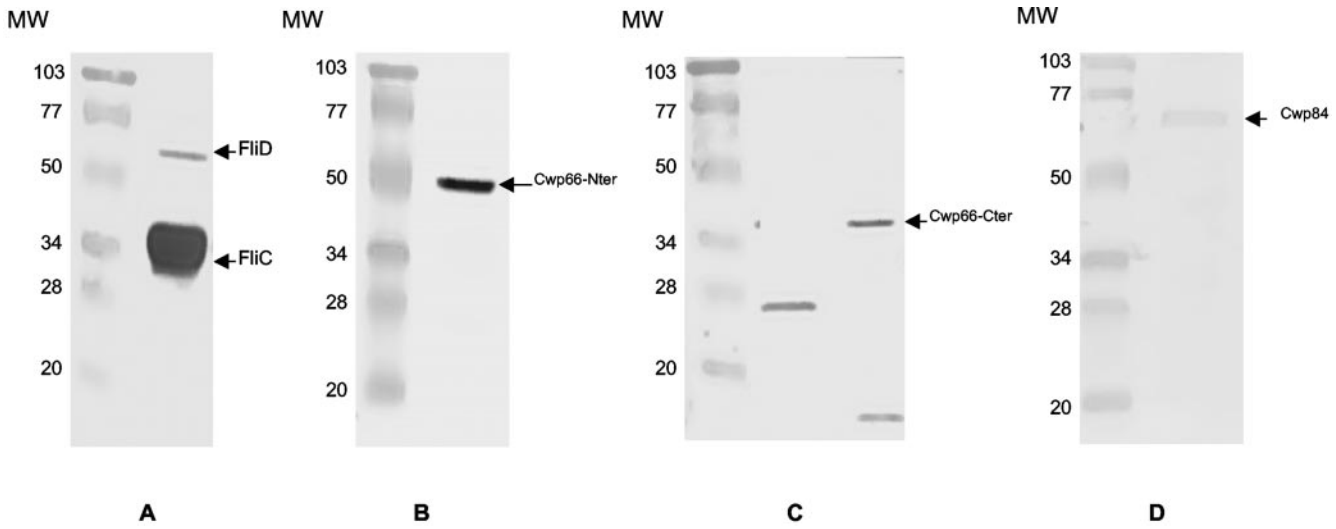

FIG. 3. Immunoblot analysis of flagellar preparations for FliC and FliD and cell wall protein extracts for Cwp66 and Cwp84. (A) Flagellar preparation revealed with anti-FliC and anti-FliD rabbit sera. (B) Protein extract revealed with anti-Cwp66 N-terminal rabbit serum (only one band was revealed at the same molecular mass of 50 kDa for all isolates). (C) Protein extract revealed with anti-Cwp66 C-terminal rabbit serum. Several bands at different molecular masses, depending on the strain tested, were revealed. (D) Protein extract revealed with anti-Cwp84 mouse serum. Only one band was revealed at the same molecular mass, 84 kDa, for all isolates. MW, molecular weight low-range standard (Bio-Rad).

patients infected by the corresponding strains are shown in Tables 3 and 4. It should be noted that two sera were negative for all the proteins tested (patients 5 and 8).

Finally, we followed the antibody responses during the disease in 11 other patients. For the FliC, FliD, and Cwp84 proteins, the antibody level was considered positive and remained stable during the analyzed period for the majority of the patients. The means of A_{450}s from the first to the last sample for these proteins were as follows: FliC, 1.58 ± 0.37; Cwp84, 1.08 ± 0.43; and FliD, 0.538 ± 0.121. In contrast, for one patient, the antibody levels appreciably increased between the fourth and the seventh days after diagnosis. For Cwp66-Nter, only three patients displayed positive results (mean A_{450}, 0.500 ± 0.22). As far as Cwp66-Cter is concerned (mean A_{450}, 0.9 ± 0.45), the antibody level decreased perceptibly 8 to 12 days after diagnosis for six patients.

TABLE 3. Detection of native proteins in clinical isolates and specific antibodies in sera from the corresponding CDAD patients

Patient no.	FliC		FliD	
	Protein detection[a,c]	Serum antibodies[b,c]	Protein detection	Serum antibodies
1	+	+	+	+
2	+	++	+	+
3	+	++	+	++
4	+	++	+	+
5	−	−	−	−
6	+	++	+	+
7	+	+	+	+
8	+	−	+	−
9	+	+	+	+
10	+	++	+	+
11	+	+	+	+
12	+	++	+	+
13	+	+	+	+
14	+	+	+	+
15	+	++	+	+
16	−	++	−	++
17	+	++	+	+

[a] Immunoblot detection of the native protein with the 79–685 strain recombinant protein antiserum.
[b] ELISA detection of antibodies directed against 79–685 recombinant protein in sera from CDAD patients.
[c] +, positive; −, negative; ++, highly positive serum antibodies.

DISCUSSION

Our laboratory has identified and characterized surface and flagellar proteins from *C. difficile* that could play a role in adherence to the intestinal mucosa and colonization of the digestive tract.

The aim of this work was to study the interstrain variability of these proteins and their immunogenicities in patients with CDAD to address their roles in the pathogenic process and to search for good vaccine antigen candidates. Infections by *C. difficile* increase morbidity and mortality and are a health expense. In spite of specific treatments, relapses are frequent due to the persistence of spores in the digestive tract. An antitoxin vaccine is under study (1), but it does not prevent the carriage of *C. difficile*. Mucosal vaccines, some of which target surface proteins as antigens, have been developed to protect against various bacteria (8). A mucosal vaccine directed against *C. difficile* colonization factors would be useful to prevent CDAD relapses.

PCR ribotyping is known to offer the best combination of advantages as an initial typing method for *C. difficile* (3), and it allowed us to determine that most of the strains studied were not genetically related. Isolates 5 and 16 on the one hand and 8, 9, and 14 on the other hand had the same ribotyping profile and could be related.

To analyze the variability of the genes studied, we performed PCR amplification with primers designed based on the refer-

TABLE 4. Detection of native proteins in clinical isolates and specific antibodies in sera from the corresponding CDAD patients

Patient no.	Cwp66 C-terminal domain		Cwp66 N-terminal domain		Cwp84	
	Protein detection[a,c]	Serum antibodies[b,c]	Protein detection	Serum antibodies	Protein detection	Serum antibodies
1	+	+	+	−	+	+
2	+	+	+	−	+	++
3	−	+	+	−	+	++
4	+	+	+	−	+	++
5	+	−	+	−	+	−
6	+	+	+	−	+	++
7	+	+	+	−	+	++
8	+	−	+	−	+	−
9	+	++	+	−	+	++
10	+	+	+	−	+	++
11	−	+	+	−	+	+
12	+	+	+	+	+	++
13	−	−	+	−	+	++
14	+	+	+	−	+	++
15	−	+	+	−	+	++
16	+	+	+	−	+	++
17	−	+	+	+	+	++

[a] Immunoblot detection of the native protein with the 79–685 strain recombinant protein antiserum.
[b] ELISA detection of antibodies directed against 79–685 recombinant protein in sera from CDAD patients.
[c] +, positive; −, negative; ++, highly positive serum antibodies.

ence strain 79-685 combined with RFLP analysis. The *fliC* and *fliD* genes showed little variability among the different isolates, with two main patterns observed, as previously described (24, 25). This low variability of the genes encoding the two flagellar proteins is confirmed by the protein expression. There is a good correlation between the presence of FliD and FliC, both proteins being present simultaneously depending on whether flagella were present.

FliC was found in greater quantity than FliD, reflecting the larger number of FliC subunits in the flagellar structure compared to FliD present in the flagellar tip (19). Thus, the rabbit antibodies, produced with the recombinant proteins FliC and FliD from strain 79-685, recognized the native proteins present in the flagellar preparations of the various isolates.

The N- and C-terminal parts of the flagellin have been described as responsible for secretion and polymerization of flagella, whereas the central region constitutes the surface-exposed antigenic part of the flagellar filament (28). This study showed that the exposed antigenic part of the flagella of *C. difficile* is highly immunogenic. As described for the flagellin of enteropathogenic *Escherichia coli* (29), FliC of *C. difficile*, localized at the bacterial surface, could effectively play a role in the interaction with the host. Therefore, the antibodies directed against this protein may play a role in the protection of patients at the first step of the pathogenic process.

FliC and FliD appear highly immunogenic in patients. In one patient displaying antibodies against FliC and FliD, the colonizing strain does not express the corresponding proteins in vitro. One hypothesis is that the flagella were not expressed in vitro but are expressed in vivo during the colonization process, as previously described by Tasteyre et al. (24, 25). Nevertheless, we cannot exclude the possibility that the antibodies correspond to preexisting antibodies in the patient serum.

The detection of antibodies directed against FliD can be explained by the presence of 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. FliC and FliD, because of their low interstrain variability and their high immunogenicity, could be interesting test antigens for active immunization. Moreover, flagellin has already been used as a vaccine antigen in other infections, such as salmonellosis. Oral or nasal immunization of mice with flagellin allowed a lower degree of infection in the immunized group than in the control group (22).

We have previously shown that antibodies raised against the two domains of Cwp66 partially inhibited the adherence of *C. difficile* to cultured cells, thus confirming that Cwp66 is an adhesin (26). These in vitro results suggest that antibodies directed against these microbial target molecules could similarly inhibit the adhesion process in humans, and they should be evaluated for their ability to block colonization and prevent *C. difficile*-associated disease.

The *cwp66* gene (the 5′ part or the 3′ part) displayed high variability among the different isolates. This suggests that the gene could be a useful biomarker for the study of strain diversity. Other surface proteins of *C. difficile*, such as the S-layer proteins, have been described as useful for phenotyping (14). In addition, the 3′ part of the *cwp66* gene, which encodes the adhesin domain of the protein, is known to be highly variable (21, 26). Therefore, genetic analyses of the surface protein could be used to complete actual typing methods.

The gene variability was confirmed by immunoblot analysis, which showed different bands at unexpected molecular masses for the Cwp66 C-terminal domain. Normally, the N-terminal and the C-terminal domains should be detected at the same molecular mass (66 kDa) by immunoblotting. Many surface proteins of gram-positive bacteria can undergo posttranscriptional modifications or may have features that cause a modified migration in SDS-polyacrylamide gels. The different bands in cell wall extracts are probably the result of a specific proteolytic cleavage in the C-terminal part of the protein. The immune absorption with Cwp66-Cter of the rabbit antiserum resulted in

significantly reduced bands, confirming its specificity (data not shown). In addition, we have previously shown that anti-Cwp66 N-terminal antibodies reacted in immunoblots with the purified 35-kDa Cwp66 N-terminal protein and in a *C. difficile* cell wall extract with a 50-kDa protein. Similarly, anti Cwp66 C-terminal antibodies recognized proteins of 50 kDa and 30 kDa in *C. difficile* cell wall extract. This protein of 30 kDa is also present in the final purification eluate of the Cwp66 C-terminal protein and is likely to represent a cleavage product of the purified 44-kDa Cwp66 C-terminal protein (26).

However, the high interstrain variability of the protein does not prevent antibodies directed against the 79-685 protein from recognizing Cwp66 in different isolates. By immunoblotting, the Cwp66 C-terminal domain was not detected by the 79-685 antibodies in only five isolates from patients 3, 11, 13, 15, and 17. Among the five patients, only one displayed no antibody directed against this C-terminal domain. This suggests that many strains share at least one common epitope within the Cwp66 C-terminal domain. Alternatively, the presence of specific antibodies in these patients could be due to preexisting antibodies. In addition, all but three patients developed antibodies directed against this C-terminal adhesin domain of the Cwp66 protein. Thus, this domain seemed to be highly immunogenic, confirming our previous results (17) and also confirming that this surface-exposed domain is subjected to immune selection (26).

Patients reacted to the protein Cwp66-Cter, which is immunogenic at the beginning of the infection, and then the level of antibodies decreased. This domain, which is highly variable according to strain, may be responsible for switch variation to escape the immune response of the host. In addition, in a previous study, we observed the antibody response against surface antigens in a CDAD patient group and in a control group. The difference was not significant for the C-terminal domain of Cwp66, in contrast to FliC, FliD, and the Cwp66 N-terminal domain. For all these reasons, this domain of the Cwp66 protein did not seem to be a suitable antigen for active immunization assay.

The 5′ part of the *cwp66* gene was as difficult to amplify as the 3′ part of the gene. Only 9 out of 17 isolates could be amplified. The PCR-RFLP results confirmed a high variability of this gene. It is noteworthy that the N-terminal domain of the protein could be detected in all isolates with antibodies directed against the recombinant Cwp66 N-terminal domain of the 79-685 strain, suggesting that even if the protein is highly variable, epitopes are conserved.

Only a few patients developed antibodies to this protein. The localization of this domain, which has been shown to be anchored in the cell wall, could be responsible for the absence of immune response (26). The domain does not have the qualities of a good vaccine antigen.

The variability of the functional region of the *cwp84* gene has been studied previously and appeared highly conserved (21). We confirmed this low variability by immunoblot analysis, which revealed the protein, with the expected molecular mass, in all isolates tested. This protease induced an intense immune response in most of the patients studied, except for two. In fact, these two patients (5 and 8), who were hospitalized in an aseptic unit and who were immunocompromised, were found to have no antibody response to any of the proteins analyzed. The characteristics of Cwp84, which are high immunogenicity as revealed by antibody level in patient sera, low variability (21), and putative involvement in bacterial virulence (11), suggest that it could be a potential vaccine candidate. Proteases, such as the ClpP protease of *Streptococcus pneumoniae*, have recently been implicated in vaccine development. Immunization of mice elicited a protective immune response against fatal systemic challenge with *S. pneumoniae*, making this protease a potential vaccine candidate for pneumococcal disease (12). Similarly, the Cwp84 protein of *C. difficile*, which is supposed to have a protease activity, could be tested as a candidate for a protein-based vaccine against *C. difficile* infection.

In our second survey of 11 additional patients, we followed an immune response during the pathogenic process. The stability of the antibody level for each patient observed for FliC, FliD, and Cwp84 may be explained by a delay in sampling the first serum, for which reason antibodies could have been already plateauing. In contrast, for one patient, who showed increasing levels of antibodies, the first serum was sampled at the beginning of the clinical signs of diarrhea. For all the patients, there was no history of previous CDAD. However, we cannot exclude the possibility that some of them may have had preexisting antibodies; however, in this case, the anamnestic response was not strong enough to prevent infection. For FliC, Cwp66 C-terminal domain, and Cwp84, the mean absorbances were variable among the 11 patients. This shows that there is higher interpatient variability for these proteins than for FliD and the Cwp66 N-terminal domain. Finally, this survey confirmed that the Cwp66 N-terminal domain displays a lower immunogenicity than the other surface proteins.

In conclusion, this study confirms that after diagnosis and for at least 2 weeks, antibodies directed against surface proteins of *C. difficile* are detected in patient sera, suggesting that these proteins are virulence factors. In addition, the study of the variability of the genes and the corresponding proteins, in combination with the immunogenicity of these proteins and the host response, helps to identify putative protective antigens. These antigens, alone or in combination, could be used to prevent intestinal colonization with *C. difficile*. Preliminary screening of the vaccine candidates is being tested in animal models.

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