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Characterization of Flagella of *Clostridium difficile* and Their Role in Serogrouping Reactions

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Slide agglutination with rabbit antisera allows the differentiation of 10 serogroups of *Clostridium difficile*, namely, A, B, C, D, F, G, H, I, K, and X. Each serogroup displays a specific protein profile in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, except for A, which displays 12 different protein profiles (A1 to A12). In the present work, electron microscopy revealed the presence of uniformly distributed flagella in the reference strains of serogroups G and K and in all strains representative of the 12 subgroups within serogroup A. No flagella were observed in the other reference strains. Flagella sheared from the cell surface were partially purified by differential centrifugation. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of these preparations revealed one distinct band with an apparent molecular mass of approximately 39 kilodaltons. Antiserum was prepared by immunizing a rabbit with the serogroup A flagellin, which had been eluted from the gel. In immunoblotting, this antiserum cross-reacted with the flagellin of the other strains. When the cells were deflagellated by a short sonication, the cross-reactions observed by slide agglutination with A, G, and K antisera were suppressed. Similarly, shearing of flagella allowed specific slide agglutination of the 12 subgroups of serogroup A.

Serogrouping by slide agglutination with rabbit antisera allows the differentiation of 10 serogroups of *Clostridium difficile*, namely A, B, C, D, F, G, H, I, K, and X. This typing scheme has proven useful in clinical and epidemiological studies (6–8). By using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), we showed that each serogroup displayed a specific protein profile, except for A. Among strains belonging to serogroup A, 12 different patterns were observed (A1 to A12) (7). When protein profiles were analyzed by immunoblotting with specific rabbit antisera, specific antigenic determinants were identified for each serogroup (4). However, the localization of the determinants at the cell surface was not investigated.

In the present work, we studied the characteristics of the flagellar antigen of *C. difficile* and its influence on the serogrouping scheme.

MATERIALS AND METHODS

Strains of *C. difficile*. The reference strains of the 10 serogroups were strain W1194 (ATCC 43594) of serogroup A, strain 1351 (ATCC 43593) of serogroup B, strain 545 (ATCC 43596) of serogroup C, strain 3232 (ATCC 43597) of serogroup D, strain 1470 (ATCC 43598) of serogroup F, strain 2022 (ATCC 43599) of serogroup G, strain 2149 (ATCC 43600) of serogroup H, strain 7322 (ATCC 43601) of serogroup I, strain 4811 (ATCC 43602) of serogroup K, and strain 5036 (ATCC 43603) of serogroup X. They have been described elsewhere (6, 7).

Strains 8643, 8676, 6516, 7701, 6517, 7773, 8737, 8785, AM222, SE005, and 8480, all belonging to serogroup A, were used as references for protein profiles A2 to A12 observed by SDS-PAGE (in the order cited); reference strain W1194 displays the A1 profile.

Additionally, 140 strains from various clinical and geographical origins were investigated for the presence of flagella; they had been either isolated in our hospital or received from other laboratories. They were distributed among all serogroups and SDS-PAGE profiles.

Electron microscopy. Bacteria were inoculated into liquid medium containing 3% (wt/vol) proteose peptone no. 2 (Difco Laboratories), 0.5% (wt/vol) yeast extract (Difco), 1% (wt/vol) glucose, 0.5% (wt/vol) NaCl, and 0.05% L-cysteine hydrochloride (pH 7.2) and were incubated aerobically for 18 h at 36°C. They were harvested by centrifugation (1,000 × *g* for 15 min) and suspended in 1 ml of saline. The bacterial suspensions were applied to carbon-coated Formvar grids for 30 s. Excess suspension was removed, each grid was washed three times with 1 drop of distilled water, and 1 drop of stain (3% phosphotungstic acid at pH 6) was added to each grid. After the removal of excess stain, each grid was allowed to air dry and was examined in a Philips 300 electron microscope at 80 kV.

Bacterial flagella were also revealed by the staining procedure described by Kodaka et al. (12). The stained preparations were microscopically examined under an oil immersion objective at a magnification of ×1,000.

Partial flagellum purification. The strains were grown anaerobically on four blood agar plates for 48 h. Bacteria were harvested in 5 ml of distilled water. The suspensions were strongly shaken for 1 min with a Vortex mixer (The Vortex Manufacturing Co.) and centrifuged at 5,000 × *g* for 30 min at 4°C. The supernatants were centrifuged at 25,000 × *g* for 1 h at 4°C. The pellets were suspended in 100 μl of phosphate-buffered saline (PBS) (pH 7.4).

SDS-PAGE. SDS-PAGE was performed as previously described (7) by mixing 2 volumes of the test suspension with 1 volume of SDS buffer (25 mM Tris hydrochloride, 15% 2-mercaptoethanol, 30% glycerol, 10% SDS, 0.1% bromophenol blue), heating the mixture at 100°C for 5 min, and loading the mixture onto a 10% polyacrylamide gel (20 cm wide; 25 cm high). Electrophoresis was conducted at room temperature for 15 h at 60 V. Low-molecular-mass standards were from Bio-Rad Laboratories. Gels were stained with Coomassie blue or used for transfer onto

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nitrocellulose. The molecular mass of flagellin was calculated by reference to standard markers.

Immunoblotting. Polypeptides separated in gels were transferred electrophoretically onto nitrocellulose membranes (102 by 133 mm; pore size, 0.45 μm; Schleicher & Schuell, Dassel, Federal Republic of Germany) by the method of Towbin et al. (26) at 4°C for 240 min at 60 V. Transfer was performed in a transblotting chamber containing 20% (vol/vol) methanol, 25 mM Tris hydrochloride, and 150 mM glycine buffer (pH 8.6). Free protein-binding sites were saturated by overnight incubation at 4°C in blocking buffer containing 5% (wt/vol) skim milk powder and 0.01% (wt/vol) Merthiolate in PBS (pH 7.4).

The nitrocellulose was incubated at 20°C for 4 h with rabbit antiserum diluted 1/100 in blocking buffer under moderate agitation. Incubation was continued overnight at 4°C. After the nitrocellulose was washed three times at 20°C with 0.1% Tween 20 in PBS (pH 7.4) for 10 min, it was incubated for 1 h at 20°C in 0.4% peroxidase-conjugated swine anti-rabbit immunoglobulin antiserum in blocking buffer. After the nitrocellulose was washed as described above and then once with PBS without Tween 20, it was incubated for 15 min at 20°C in a freshly prepared mixture of 100 ml of PBS–20 ml of 0.06% 4-chloro-1-naphthol in methanol–60 μl of hydrogen peroxide. The membrane was washed four times with distilled water and dried.

Protein elution. Gel slices were electroeluted in dialysis bags with 0.1% SDS–25 mM Tris hydrochloride–192 mM glycine buffer (pH 8.6). After centrifugation, the supernatant was dialyzed against 13 mM ammonium bicarbonate buffer (pH 7.8) and concentrated by lyophilization.

Specific protein antisera. Antiserum was raised in a rabbit by subcutaneous injection of 2 ml of an emulsion consisting of 1 ml of eluted protein solution (0.5 mg/ml) and 1 ml of incomplete Freund adjuvant. Identical amounts were injected into 10 subcutaneous sites on the back of the animal every week for 6 weeks, after which the rabbit was bled by cardiac puncture.

Slide agglutination. Twenty-one rabbit antisera (A to X and A2 to A12) were obtained as previously described (6). In brief, bacterial antigen for immunization as well as for serogrouping was prepared from a 6-h culture in liquid medium. Bacteria were washed twice with saline and suspended in 0.4% Formol–saline. Rabbits were injected intravenously with 0.5, 1, 2, and 2 ml of the Formol-treated suspension at 4-day intervals and bled 10 days after the last injection by cardiac puncture. Agglutination was performed on slides by mixing 1 drop of the diluted antiserum with 1 drop of the bacterial suspension. Nonflagellated cells were obtained by the following technique. After 6 h of culturing in liquid medium, cells were harvested by centrifugation, suspended in 2 ml of PBS, subjected to 20 s of sonication at 30 W with a Sonifier (Branson Sonic Power Co.) equipped with a microtip, washed twice with saline, and suspended in 0.4% Formol–saline.

RESULTS

Electron microscopy of the 21 reference strains revealed the presence of numerous flagella distributed in a uniform manner on all strains representative of the 12 SDS-PAGE subgroups of serogroup A. Both strains of serogroups G and K also had flagella, but they were much less numerous. The other reference strains had no flagella. Figure 1 illustrates the flagella observed on the serogroup A reference strain. Similar observations were made when the staining technique of Kodaka et al. was used.

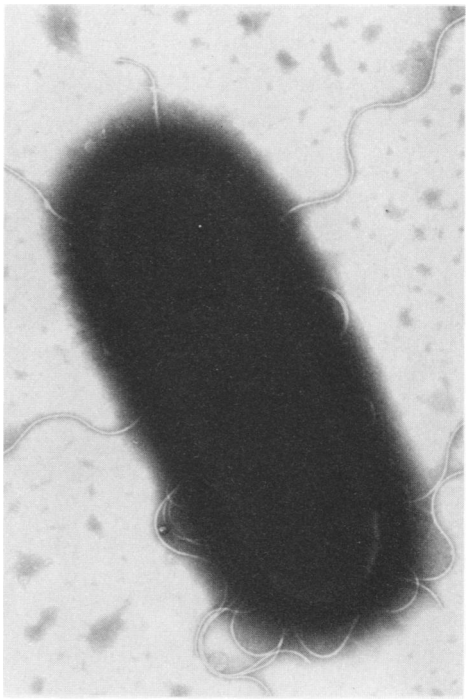

FIG. 1. *C. difficile* W1194 (ATCC 49594) of serogroup A negatively stained with phosphotungstate.

The precipitates obtained by ultracentrifugation of flagella sheared from the cell surface were seen to be an almost homogeneous flagellar mass. Figure 2 shows an electron micrograph of partially purified flagella from strain W1194 of serogroup A. Once again, no flagella were seen in the serogroup B, C, D, F, H, I, and X preparations. On the other hand, flagella from all positive preparations were morphologically indistinguishable.

Flagellar preparations were electrophoretically separated by SDS-PAGE and stained with Coomassie blue. Those from the flagellated strains (G, K, and A1 to A12) displayed a single band with an apparent molecular mass of 39 kilodaltons (kDa). Only minor variations in the molecular masses of the bands were observed in serogroups A4, A5, and G. The flagellar band did not correspond to any of the major bands observed by SDS-PAGE of the corresponding whole-cell preparations. As an example, Fig. 3 shows the comparative electrophoretic profiles of flagellar and whole-cell preparations of strain 7773 (A7).

A rabbit antiserum was raised against the flagellin of strain W1194, which had been eluted from the gel. At a low titer (1:10), this antiserum agglutinated the Formol-treated cells of the 14 flagellated strains but not the others. In immunoblotting it reacted strongly with the 39-kDa band of the putative flagellin in all the flagellated strains (Fig. 4).

To determine whether the presence or absence of flagella was constant within a serogroup, we examined a series of 140 strains from various clinical origins by using the staining technique of Kodaka et al. (12). Table 1 gives the number of flagellated strains among the different serogroups. With one exception, the 68 strains of serogroup A all expressed abundant flagella. The exception was strain T005, received from Sandu Toma (Ministry of Health, Toronto, Canada), which displayed an A10 profile in SDS-PAGE. Interestingly, this strain was agglutinated by the A10 antiserum but not by any of the other 20 antisera or by the flagellin antiserum.

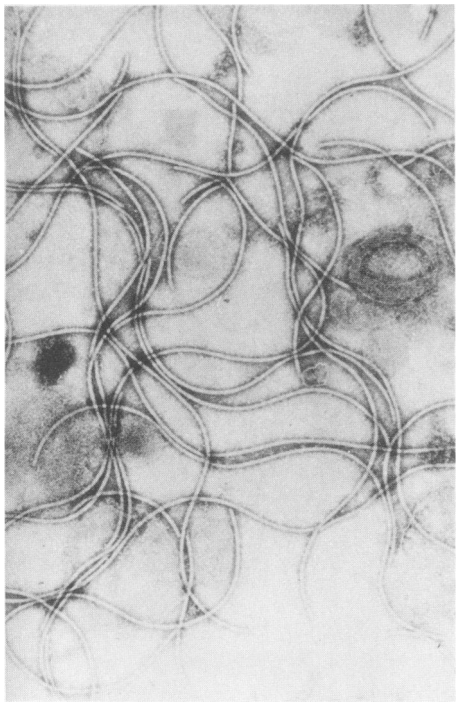

FIG. 2. Electron micrograph of partially purified flagella from strain W1194 of serogroup A.

Not all strains of serogroups G and K were flagellated, whereas some strains of serogroups D and H had flagella, in contrast to the reference strains. Clearly, a correlation appeared between the presence of flagella and cross-agglutination with each of the antisera raised against serogroups with flagellated strains as well as with the flagellin antiserum. For example, strains of serogroup H which had flagella were agglutinated by antisera to serogroups H, G, K, and A1 to A12, whereas nonflagellated strains of the same serogroup were only agglutinated by antiserum to serogroup H.

Furthermore, the role of flagella in cross-agglutination was

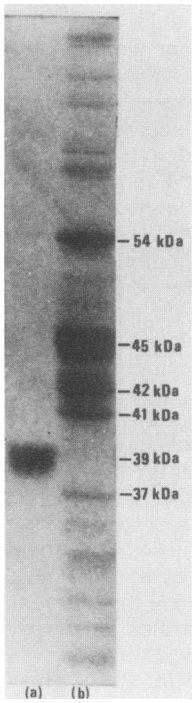

FIG. 3. Comparative SDS-PAGE profiles of a partially purified flagellar preparation (lane a) and a whole-cell preparation (lane b) of strain 7773 belonging to serogroup A, SDS-PAGE subtype A7.

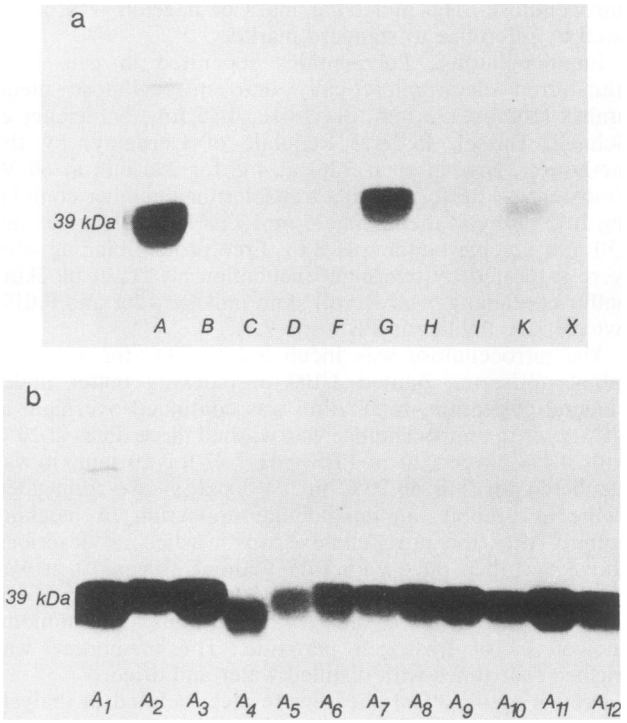

FIG. 4. Immunoblots of the putative flagellin in all of the reference strains with antiserum raised against the eluted 39-kDa flagellin of strain W1194. (a) A to X, Serogroup A to X reference strains. (b) A1 to A12, Twelve SDS-PAGE subtypes within serogroup A.

TABLE 1. Proportions of flagellated strains among 140 *C. difficile* strains from various clinical and geographical origins

Serogroup	SDS-PAGE profile	Total no. of strains	No. of flagellated strains
A	A1	20	20
A	A2	15	15
A	A3	2	2
A	A4	1	1
A	A5	5	5
A	A6	2	2
A	A7	4	4
A	A8	10	10
A	A9	1	1
A	A10	4	3
A	A11	1	1
A	A12	3	3
B	B	3	0
C	C	12	0
D	D	8	4
F	F	5	0
G	G	12	6
H	H	16	9
I	I	3	0
K	K	10	6
X	X	3	0

confirmed by the fact that nonflagellated cells obtained by sonication could no longer cross-agglutinate. For example, strain W1194 of serogroup A1 was agglutinated only by its homologous antiserum after short sonication. Of particular interest was the resulting possibility to differentiate the 12 subgroups within serogroup A by slide agglutination, with a perfect correlation with SDS-PAGE profiles. It is worth noting that the pellet of bacterial cells harvested after the first centrifugation in the flagellum purification procedure still displayed cross-agglutination. Electron microscopy demonstrated that the flagella were not completely sheared at this step, in contrast to the results of sonication.

DISCUSSION

This work provides further information about the antigenic basis of the slide agglutination technique that we have used for typing *C. difficile*. From previous studies, we knew that serogrouping correlated with the protein profiles obtained by SDS-PAGE and that one of the specific bands in SDS-PAGE gels was a serogroup-specific antigenic determinant involved in agglutination (4, 7). However, immunoblotting did not allow us to understand the cross-agglutinations that occur among strains displaying different SDS-PAGE profiles, especially for serogroup A, in which 12 different SDS-PAGE subtypes cross-agglutinate. The present work demonstrates that (i) flagella are present on *C. difficile* strains belonging to five serogroups, (ii) the flagellin seems to be identical in all flagellated strains that have been studied, and (iii) this flagellin is responsible for cross-agglutinations in the serogrouping procedure.

Peritrichous cells are common in the genus *Clostridium*. However, the flagella of *C. difficile* have not yet been studied. Electron micrographs revealed that peritrichous cells are only found in serogroups A, D, G, H, and K. They are almost always present and are more numerous in serogroup A. The apparent molecular mass of 39 kDa is within the range of values that are usually observed for other bacterial flagellins (11, 18, 23). All flagellins isolated in this study seem similar: despite minor differences in molecular masses observed in serogroups A4, A5, and G, the flagellins are all antigenically related, as shown by immunoblotting with a specific antiserum raised against the 39-kDa protein. As expected, this antiserum agglutinates flagellated cells on slides. Thus, our serogrouping scheme, which used rabbit antisera raised against whole Formol-treated bacteria, involved at least two kinds of antibodies: the first kind is directed against the flagellar antigens and the second kind is directed against a somatic determinant which best correlates with SDS-PAGE profiles. Comparison with flagellins of other *Clostridium* spp. was not done in this work. It would be of interest to study whether this flagellin is implicated in the serologic cross-reactivity that is observed between *C. difficile* and other species, such as *C. sordellii*.

The 39-kDa band was not present in the whole-cell profile (Fig. 3), probably because of an insufficient protein concentration. It is reasonable to suppose that our procedure for whole-cell electrophoresis, which includes two washes with low-speed centrifugation before sonication, might cause the loss of most of the flagella.

The presence of flagella does not seem to be linked to any virulence determinant, as it is for other bacterial species. We previously established a clear correlation between serogroup and pathogenicity. For example, almost all cases of pseudomembranous colitis are caused by toxigenic strains of serogroup A, C, or H; the first always has flagella, the second never has them, and the third sometimes has them. Moreover, there is no correlation between toxin production and the presence or absence of flagella.

From all these data it was obvious that somatic antigens were superior to flagellar antigens for typing purposes. The elimination of flagellar cross-agglutinations was conceivable by two means: agglutinin absorption or suppression of flagellar antigens. We reported in our first description of the serogrouping technique that absorption could eliminate some cross-agglutinations (6). However, we never succeeded in absorbing antisera to serogroup A by using strains of nonhomologous serogroup A SDS-PAGE subtypes. Moreover, repeated absorptions eventually resulted in the suppression of all agglutinations, even those of the homologous strain. This result was probably due to the fact that the somatic antigens are hidden by the abundant flagella in serogroup A. An argument for this hypothesis is that an antiserum raised against strain T005, the only nonflagellated strain that we found within serogroup A, did not agglutinate all flagellated strains of the same SDS-PAGE subtype, A10, unless these strains were deflagellated by sonication (unpublished results).

The elimination of flagellar antigens by short sonication gave us more satisfying results. This procedure allows a better correlation between serogrouping and SDS-PAGE typing and consequently provides a more comprehensive approach to the typing of *C. difficile*. Tabaqchali et al. were the first to use SDS-PAGE profiles (22). So far they have identified 16 different types. Both SDS-PAGE typing and serogrouping were successfully applied by several authors in epidemiological studies (5, 8, 9, 15, 17, 24, 25). Other methods, such as phage typing (1, 3), plasmid profile typing (2, 21), immunoblotting (14, 16, 20), and restriction endonuclease DNA analysis (13, 19, 27), have all been proven useful for epidemiological purposes. Some correlations between these different approaches were established but, obviously, that between serogrouping and SDS-PAGE typing appears to be the most consistent. Mulligan et al. first reported the excellent correlation between both systems (15), and we confirmed it later (7). In both studies, however, SDS-PAGE typing was more discriminating than was serogrouping. Furthermore, Heard et al. demonstrated that the strain-specific proteins which are responsible for the distinctive patterns in SDS-PAGE are also immunogenic (10), and we later showed that these determinants are involved in bacterial agglutination (4). However, as mentioned by Mulligan et al. and also by us, SDS-PAGE and immunoblotting distinguished more groups than did serogrouping (7, 16). We have shown here that flagellin is responsible for cross-agglutinations and that these cross-agglutinations can be suppressed by simple shearing of the flagella. With shearing, the 10 serogroups could be extended to 21, each corresponding to a specific SDS-PAGE profile. This perfect correlation between both typing schemes should lead to a consensus for one designation for the groups in both systems, which would be of great value for improving our knowledge of *C. difficile* epidemiology.

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