INFECTION AND IMMUNITY, Oct. 1994, p. 4347–4355
0019-9567/94/$04.00+0

Vol. 62, No. 10

Cloning of a Genetic Determinant from *Clostridium difficile* Involved in Adherence to Tissue Culture Cells and Mucus

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Received 9 March 1994/Returned for modification 2 May 1994/Accepted 12 July 1994

Our laboratory has previously shown that *Clostridium difficile* adherence to Caco-2 cells is greatly enhanced after heat shock at 60°C and that it is mediated by a proteinaceous surface component. The experiments described here show that *C. difficile* could adhere to several types of tissue culture cells (Vero, HeLa, and KB) after heat shock. The type of culture medium (liquid or solid, with or without blood) had little effect on adhesion. To clone the adhesin gene, polyclonal antibodies against *C. difficile* heated at 60°C were used to screen a genomic library of *C. difficile* constructed in λZapII. Ten positive clones were identified in the library, one of which (pCL6) agglutinated several types of erythrocytes in the presence of mannose. In Western blots (immunoblots), this clone expressed in *Escherichia coli* a 40- and a 27-kDa protein; a 27-kDa protein has been previously identified in the surface extracts of heat-shocked *C. difficile* as a possible adhesin. The clone adhered to Vero, Caco-2, KB, and HeLa cells; the adherence was blocked by anti-*C. difficile* antibodies, by a surface extract of *C. difficile*, and by mucus isolated from axenic mice. Furthermore, the clone could attach ex vivo to intestinal mucus isolated from axenic mice. Preliminary studies on the receptor moieties implicated in *C. difficile* adhesion revealed that glucose and galactose could partially block adhesion to tissue culture cells, as did di- or trisaccharides containing these sugars, suggesting that the adhesin is a lectin. In addition, *N*-acetylgalactosamine, a component of mucus, and gelatin partially impeded cell attachment.

Clostridium difficile is recognized as the major etiologic agent in pseudomembraneous colitis and antibiotic-associated colitis and diarrhea (1, 15, 17). Several potential and putative virulence factors that could play a role in *C. difficile* pathogenicity have been identified. At least five toxic factors have been described, but only toxin A and toxin B have been extensively studied (19). The two toxins are indispensable for disease development in the hamster model. Of the two toxins, toxin B exhibits more potent cytotoxic activity, whereas toxin A induces intraluminal fluid accumulation in the intestine without activation of adenylate cyclase (18). The capsule of *C. difficile* may serve as an antiphagocytic factor (9). The production of proteolytic enzymes, such as collagenase or hyaluronidase, is thought to play a role in releasing suitable substrates from available protein sources for metabolism (30–32). There is no direct correlation between toxin production and enzyme synthesis; however, the strains that are highly virulent in the hamster model are the most proteolytic (31).

For a large variety of microorganisms, mucosal association is an important virulence determinant. Although a minority of strains of *C. difficile* have been found to carry a small number of fimbriae, no definitive role for these structures can be ascribed at the moment (3). It is clearly established that *C. difficile* can associate with the intestinal mucosa in humans and hamsters (2, 4), although the structures mediating this process remain to be elucidated. There appears to be an association between virulence and adherence: the highly virulent strains attach to the mucosa better than poorly virulent or avirulent strains (4).

Recent work performed in our laboratory has shown that *C. difficile* adhesion to the cultured intestinal cell lines Caco-2 and HT29-MTX increases after heat shock treatment of the bacteria (14). The adhesion implicated two surface proteins of *C. difficile*, since polyclonal antibodies raised against the adhesins could block attachment to cultured cells. In this communication, we describe the cloning in *Escherichia coli* of a genetic determinant coding for a 27-kDa adhesin of *C. difficile*. We examined the properties of adherence of the recombinant clone to cultured epithelial cells and mucus isolated from axenic mice. In addition, we describe the identification of carbohydrates that could serve as receptors for *C. difficile* attachment to the intestinal mucus and epithelial cells.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. difficile* was cultivated in an anaerobic chamber at 37°C (twice for 24 h each). The liquid culture media used were brain heart infusion broth (Pasteur Diagnostic, Paris, France), TGY (tryptone glucose yeast broth; Difco, Detroit, Mich.), and GAPTT (Gelose-Autolysat-Peptone-Tryptone-Tween broth; Centre National de la Recherche Scientifique-Institut National de la Recherche Agronomique, Jouy en Josas, France). The solid medium for plates was Columbia cysteine agar supplemented with 7% horse blood or serum (BioMérieux, Charbonnières les Bains, France). All growth media were prereduced prior to use.

The *E. coli* strain HB101 (5) was used as a host for recombinant plasmids in hemagglutinations and adhesion assays; XL1Blue *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}ZΔM15 Tn*10*(Tet^r)], XL1Blue-MR Δ(*mrcA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1*, and SURE e14$^-$ (*mcrA*) Δ(*mcrCB-hsdSMR-mrr*)*171 sbcCrecB recJ umuC*::Tn*5*(Kan^r) *supE44 lac gyrA96 relA1 thi-1 endA1* [F′ *proAB lacI*^{q}ZΔM15 Tn*10*(Tet^r)] (Stratagene, La Jolla, Calif.) were used as hosts for λZapII and in cloning. *E.*

coli was grown in Luria broth (LB) or 2× YT liquid medium (enriched Luria broth) at 37°C with aeration or on LB agar (28). When carrying plasmids, the strains were cultivated in the presence of ampicillin (10 mg/ml). For infection with λZapII, the LB medium was supplemented with 10 mM $MgSO_4$ and 0.2% maltose.

Axenic mice. Axenic mice were obtained from the Centre National de la Recherche Scientifique breeding center of laboratory animals (Orléans, France) and from our own breeding. The mice were maintained in sterile isolators (Isoconcept, Orléans, France) and received standard nutrition sterilized by irradiation.

Recombinant DNA techniques. Plasmid isolations were performed by the alkaline lysis procedure with a kit from Qiagen (Hilden, Germany). Ligations, restriction endonuclease digestions, and nucleotide fill-ins were done by the method of Sambrook et al. (28) and according to protocols provided by the vendors. The tryptic soy broth method was used for transformation of *E. coli* (7). Stratagene protocols were followed in the cloning using the λZapII vector.

Isolation of *C. difficile* genomic DNA. *C. difficile* DNA was isolated from the toxigenic strain 79-685 as follows. Fifty milliliters of a 24-h *C. difficile* culture was pelleted and washed with 30 ml of TNE (10 mM Tris-HCl [pH 7.5], 10 mM NaCl, 1 mM EDTA) and 10 ml of TNE containing 25% glucose. A 1.25-ml volume of 0.25 M Tris-HCl containing 10 mg of lysozyme per ml was then added, and the mixture was incubated for 30 min at 37°C, after which 1.25 ml of 0.5 M EDTA was added (with incubation for 20 min at 37°C) and then 1 ml of 10% sarcosyl and 50 ml of RNase (10 mg/ml) were added (with incubation for 30 min at 37°C). Fifty milliliters of proteinase K (20 mg/ml) was added, and the mixture was incubated for 12 h at 37°C. The DNA was purified by phenol-chloroform extraction, ethanol precipitated, and resuspended at 1 mg/ml in Tris-HCl–EDTA.

Construction of a genomic library of *C. difficile*. Ten milligrams of *C. difficile* DNA was partially digested by *Hin*dIII; to render the ends compatible with the vector, they were filled in with 2 mM dATP and dGTP (Boehringer, Mannheim, Germany). Fragments (2 to 10 kb) were isolated from a gel; 0.2 mg of the insert DNA was ligated with 1 mg of vector. The *cos* sites of the λZapII expression vector (Stratagene) were first protected from fill-in by rendering the vector circular by ligation; the vector was then digested with *Xba*I and the ends were filled in with 2 mM dCTP and TTP. These manipulations produced 2-base compatible overhangs between the insert and vector DNAs. After ligation and in vitro packaging (Gigapack; Stratagene), the titer of the genomic library thus obtained was determined and the library was amplified by using *E. coli* XL1Blue-MR cells. For determination of the recombinant/nonrecombinant ratio in the library and for in vivo excision of pBluescript from the λ vector, XL1Blue cells were employed.

Preparation of antibodies. *C. difficile* whole cells cultivated on Columbia cysteine agar were washed twice with phosphate-buffered saline (PBS), heated at 60°C, rewashed, and killed with 0.1% Formol. Rabbits were injected subcutaneously with 2×10^{10} bacteria emulsified in Freund's complete adjuvant and then given three additional injections in Freund's incomplete adjuvant. The rabbits were bled 1 week after the last immunization. The presence of anti-*C. difficile* antibodies in the serum was verified by agglutination with *C. difficile*. The antibodies were absorbed against an *E. coli* lysate as described elsewhere (28).

Screening of the library. Approximately 50,000 PFU were plated on 150-mm-diameter petri dishes; after a 3.5-h incubation at 42°C, the phages were transferred onto an IPTG (isopropyl-β-D-thiogalactopyranoside)-impregnated nitrocellulose membrane (28). After a 4-h incubation at 37°C, the membranes were removed and washed in TNT (10 mM Tris-Cl [pH 8.0], 150 mM NaCl, 0.05% Tween 20), blocked in TNT plus 5% defatted milk, incubated with a 1:1,000 dilution of adsorbed anti-*C. difficile* antibodies, rewashed, reincubated with anti-rabbit immunoglobulin G alkaline phosphatase conjugate (1:2,500 dilution; Sigma), and rewashed, and color was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates (28). Positive plaques were picked, and the phages were eluted from the agar and used to reinfect *E. coli* XL1Blue-MR. After an additional two or three rounds of screening as described above, all phages were positive. In vivo excision of pBluescript containing insert DNA from λZapII was accomplished by infecting the strain XL1Blue simultaneously with λZapII containing insert DNA and helper phage R408, as described by Short et al. (33).

Hemagglutination assays. Erythrocytes collected from humans (O^+, O^-, A^+, and B^+), sheep, rabbits, and horses were washed twice in PBS and used as a 1% suspension in PBS (containing 2% D-mannose for *E. coli* HB101).

For hemagglutination assays, the bacteria were grown on LB agar (*E. coli*) or Columbia cysteine agar (*C. difficile*). Slide agglutination was performed as previously described (13). 3+ indicates an immediate strong agglutination at room temperature, 2+ indicates a pronounced but not immediate agglutination, 1+ indicates weak agglutination after 5 min of incubation, and 0 indicates an absence of agglutination.

Cell culture. Vero (monkey kidney epithelial cells), HeLa (human uterine epithelial cells), KB (human tongue epithelial cells), and MDCK (canine kidney epithelial cells) cell lines were routinely grown in Dulbecco's modified Eagle's minimal essential medium (Eurobio, Paris, France) supplemented with 10% fetal calf serum, 1% nonessential amino acids, 1% glutamine, 200 U of penicillin per ml, and 40 mg of streptomycin per ml. All experiments and maintenance were carried out at 37°C with a 5% CO_2 atmosphere. Monolayers were prepared on glass coverslips, which were placed in six-well tissue culture plates (Corning Glass Works, Corning, N.Y.). Caco-2 cells were grown as previously described (14).

Cell adherence assay. Adherence of bacteria to cultured cells was assessed as previously described (14). Bacteria adhering to cells other than Caco-2 were stained with May-Grünwald-Giemsa stain; bacteria adhering to Caco-2 cells were revealed by Gram staining. The adhesion index is given as the average number of adhering bacteria per cell (counted at a magnification of ×1,000) from at least three different assays.

Adherence to mucus ex vivo. Ceca of axenic mice 5 to 8 weeks of age were excised, turned over, and emptied of their contents by being rinsed with PBS. The ceca were then expanded by injection with buffer and ligated. The number of *E. coli* organisms, washed with PBS, was adjusted to 2×10^8/ml; 2% D-mannose was added to inhibit mannose-sensitive adherence due to type 1 fimbriae. The bacterial suspension was incubated with the exposed cecal mucosa for 1 h at 37°C. The cecum was then washed eight times with PBS to eliminate nonadhering bacteria and then homogenized in 3 ml of PBS. These bacteria as well as those in the last three rinses were enumerated on petri dishes (*E. coli*) or in tubes (*C. difficile*).

Inhibition of adhesion. In assays of inhibition of adhesion to cultured cells, the bacteria were incubated for 1 h with the diluted anti-*C. difficile* antibodies (1:1,000 dilution in TNT plus 5% defatted milk), receptor analogs (50 mM in PBS), *C. difficile* surface extract (200 mg/ml), or mucus (100 mg/ml) at 37°C before addition onto the monolayer.

The surface extract of *C. difficile* was prepared as follows. *C.*

difficile grown in 200 ml of TGY was washed once and resuspended in PBS. After a 30-min incubation in PBS at 40°C, the bacteria were centrifuged at 20,000 × *g* for 20 min. The pH of the supernatant containing membrane-associated proteins was adjusted to 7.0.

SDS-PAGE and Western blot (immunoblot) analysis of proteins. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method of Laemmli (16) was used with 12% (vol/vol) acrylamide gels. Molecular mass standards of 15.4, 18.1, 28.3, 43.3, 69.8, 105.1, and 215.5 kDa were obtained from Bethesda Research Laboratories (Cergy Pontoise, France). The gels were stained with Coomassie brilliant blue (Bio-Rad, Ivry sur Seine, France). Protein concentrations were measured with the Bio-Rad protein assay kit.

Proteins were electrophoretically transferred to nitrocellulose membranes (Céralabo, Aubervilliers, France) by the method of Towbin et al. (34). The membranes were screened for signals as described above ("Screening of the library").

Transmission and immunoelectron microscopy. Cultures (24 h) of *C. difficile* 79-685 and *E. coli* were centrifuged and the pellets were resuspended in distilled water. The bacteria were negatively stained with a phosphotungstic acid solution for 5 min and adsorbed on carbon-stabilized nitrocellulose film copper specimen grids (1-min contact) by placing each grid on a drop of the bacterium-staining solution. After drying, the grids were observed with an EM 301 Philips transmission electron microscope (TEM).

For immunoelectron microscopy, after adsorption of bacteria to the grids for 5 min, the grids were placed on a drop of 10 mM Tris (pH 7.5)–150 mM NaCl–1% bovine serum albumin for 30 min. The grid was then floated on a solution of adsorbed anti-*C. difficile* antibodies (1:500 dilution) for 1 h, washed three times (5 min each time) with 10 mM Tris (pH 7.5)–150 mM NaCl, and then incubated for 1 h with a 1:20 dilution of anti-rabbit immunoglobulin G labelled with 10-nm-diameter colloidal gold particles (Sigma). Nonspecifically bound gold particles were removed by three washings as described above, and the grids were stained with phosphotungstic acid before observation by TEM.

RESULTS

The *C. difficile* strain 79-685, isolated from a case of pseudomembranous colitis, was chosen for the genetic studies because of its toxigenic, enzymatic, and adherent properties (14).

Adherence of *C. difficile* 79-685 to tissue culture cells. When the enterocyte-like Caco-2 cells were used in culture, a 10-min heat shock at 60°C of *C. difficile* 79-685 was sufficient to allow maximal adherence to these cells. At 100°C, no bacteria remained viable and no adhesion to Caco-2 cells occurred, whereas at 60°C 10% of the bacteria remained viable and only viable bacteria could adhere to the cultured cells (not shown). As shown in Table 1, *C. difficile* adhered equally well to the Caco-2 cell line whether grown on solid (Columbia cysteine) or liquid (TGY or brain heart infusion) medium. Heating of the bacteria at 60°C increased adhesion to this cell line up to 11-fold compared with 37°C (Columbia cysteine plus serum). The presence of blood in the bacterial culture medium had little effect on adhesion.

The relatively low level of attachment and the uneven distribution of heat-shocked *C. difficile* organisms over the Caco-2 cell monolayer prompted us to test adhesion to other types of cell lines. As shown in Table 1, after heat shock *C. difficile* could adhere also to Vero, KB, and HeLa cells to the same extent as or to a greater extent than to Caco-2 cells.

TABLE 1. Adhesion of *C. difficile* 79-685: effects of culture medium and tissue culture cell line

Medium or cell line[a]	Heating temp (°C)	Adhesion[b]
Medium		
Columbia cysteine + serum	37	0.25 ± 0.12
+ washing[c]	60	2.7 ± 0.25 (10.8)
Columbia cysteine + blood	37	0.92 ± 0.03
	60	2.34 ± 0.37 (2.5)
Columbia cysteine + blood	37	0.62 ± 0.31
+ washing[c]	60	2.7 ± 0.48 (4.4)
TGY broth[c]	37	0.44 ± 0.06
	60	2.14 ± 0.34 (4.9)
BHI broth[c]	37	0.22 ± 0.06
	60	1.34 ± 0.25 (6)
Cell line		
Vero	37	0.42 ± 0.08
	60	13.7 ± 3.79 (32.6)
HeLa	37	0.01 ± 0.00
	60	4.40 ± 1.84 (440)
KB	37	0.01 ± 0.01
	60	11.9 ± 5.13 (1,190)
MDCK	37	0.18 ± 0.05
	60	0.59 ± 0.29 (3.27)

[a] The effect of culture medium was tested by using the Caco-2 cell line.
[b] Mean numbers of *C. difficile* organisms adhering to a cell (± standard errors). At least 100 cells were counted, and each adhesion assay was conducted at least twice. Values in parentheses are fold increases over values for bacteria kept at 37°C.
[c] The bacteria were collected and washed twice with PBS before the adherence assay. BHI, brain heart infusion.

Because of the relative facility of culturing Vero cells compared with Caco-2 cells and the apparently more even distribution of receptors for *C. difficile* over these cells (Fig. 1), Vero cells were used in most adhesion assays, although some results were confirmed by using the Caco-2 cells.

Screening of the *C. difficile* genomic library. To clone the *C. difficile* adhesin in *E. coli*, a genomic library of *C. difficile* was constructed in the λZapII expression vector. The library had a titer of 7×10^4 PFU/ml with 50% recombinants. An amplified library was determined to have a titer of 7×10^7 PFU/ml.

The genomic library was screened with antibodies prepared against heat-shocked bacteria in toto, since it is likely that the adhesin is present in a significant amount on the bacterial surface after heat shock. As little adhesion to cultured cells occurred at 37°C, adsorption of the anti-*C. difficile* antibodies to non-heat-shocked *C. difficile* created an antiserum containing antibodies against the adhesin, while eliminating those unrelated to adhesion. By immunoelectron microscopy, it became evident that these antibodies recognized a multitude of proteins which were not intimately associated with the bacterial surface but were rather loosely associated with it (Fig. 2). In addition, in Western blots, these antibodies recognized a number of proteins in *C. difficile* lysate (see Fig. 3A, lane 1) and culture supernatant (not shown), the latter probably as a result of shedding of surface proteins into the medium and lysis of bacteria.

The screening allowed us to identify 35 positive plaques among 100,000 plaques screened. Ten of the positive plaques were plaque purified and chosen for further study. In vivo excision of the pBluescript plasmids from λZapII resulted in 10 recombinant plasmids potentially carrying the gene for the adhesin of *C. difficile*.

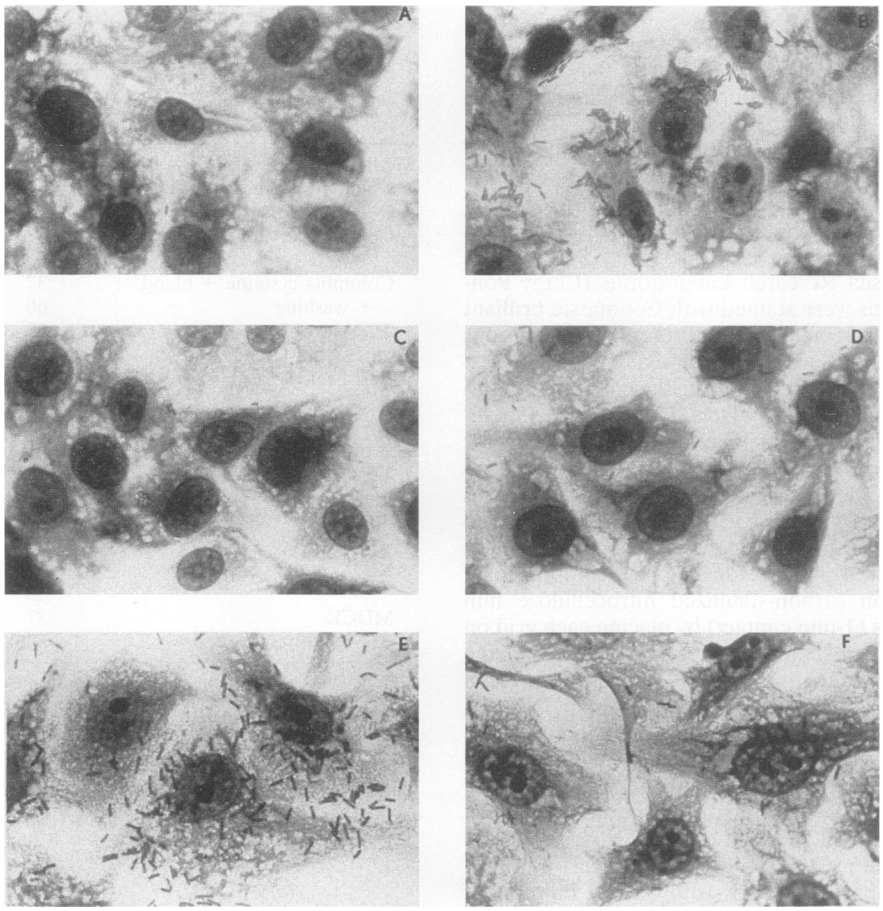


FIG. 1. Patterns of bacterial adherence to Vero cells examined by light microscopy (magnification, ×1,000). (A and B) *E. coli* HB101/pBluescript and HB101/pCL6, respectively; (C and D) *E. coli* HB101/pCL6 incubated with mucus isolated from axenic mice or with anti-*C. difficile* antibodies (1:1,000 dilution), respectively, before adhesion assay; (E and F) *C. difficile* heated at 60°C for 20 min or heat shocked and incubated with anti-*C. difficile* antibodies, respectively, before adhesion assay.

Identification of a hemagglutinating clone. The recombinant clones were first tested for hemagglutination with human O^+ and O^-, horse, and sheep erythrocytes in the presence of mannose, which inhibits type 1 pilus-mediated adherence. For this purpose, the plasmids were transformed into *E. coli* HB101, which does not carry fimbriae, as verified by TEM (Fig. 2). The results are presented in Table 2. Clones 5, 6, 7, and 10 showed a low degree of agglutination with one or more of the erythrocytes tested; however, clone 6 (HB101/pCL6) displayed the most rapid and complete agglutination in the presence of mannose of human O^+, horse, and sheep erythrocytes. This clone did not express fimbriae, as assessed by TEM (Fig. 2). Clone 6 also agglutinated human A^+ and B^+ and rabbit erythrocytes (not shown).

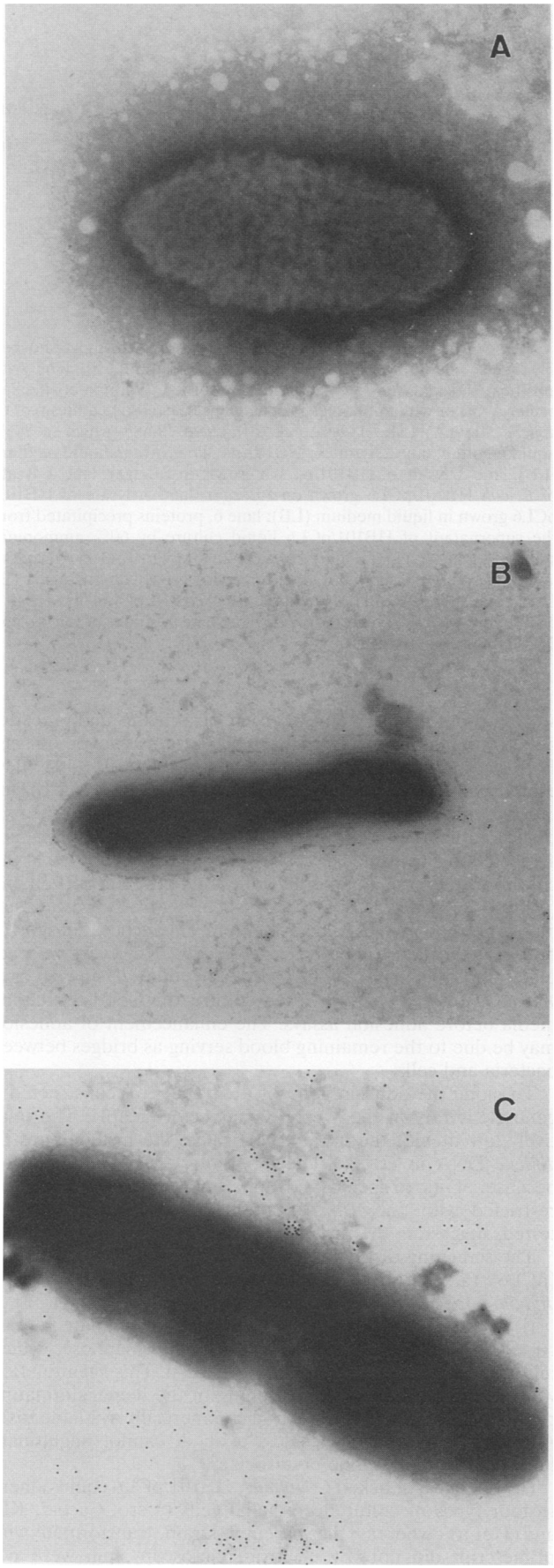
We also examined the hemagglutinating properties of eight isolates of *C. difficile* available in our laboratory. The toxigenicity of these isolates ranged from highly toxigenic to nontoxigenic. All the strains agglutinated human O^+, A^+, and B^+, sheep, rabbit, and horse erythrocytes to a low degree (the most pronounced hemagglutination was with the rabbit erythrocytes) in the presence of mannose, regardless of the toxigenicity of the strain tested (not shown).

Identification and characterization of an adherent clone. We tested *E. coli* HB101 carrying the 10 recombinant plasmids for adherence to Vero cells. As shown in Table 2, *E. coli* HB101 or HB101/pBluescript with no insert did not adhere to this cell line. The same clones that displayed hemagglutination also showed various levels of adhesion to this cell line, the highest levels being those of clones 6 and 10.

Clone 6 was chosen for further study because of its pronounced hemagglutinating and adhesive properties. Upon adherence, clone 6 exhibited formation of small aggregates of bacteria (Fig. 1); it could also adhere in a mannose-resistant fashion to HeLa, KB, and Caco-2 cells but not to MDCK cells (not shown). When grown in liquid medium, clone 6 neither hemagglutinated nor adhered to Vero cells (Table 2).

The adherence of *E. coli* HB101 containing pCL6 or the pBluescript without an insert and *C. difficile* 79-685 to intestinal mucus isolated from axenic mice was tested. As evident from Table 2, clone 6 showed a 100- to 1,000-fold increase in adherence to the mucus ex vivo compared with the controls (HB101/pBluescript and HB101, respectively).

The insert carried by pCL6 is 10.5 kb. Since the insert DNA is resistant to digestion by several restriction enzymes, it was not possible to obtain a complete restriction map of the insert. Digestion of the insert with *Sac*I yielded, among other fragments, a 4.3-kb subfragment which was cloned into pBluescript (p4.3kb). *E. coli* HB101 carrying p4.3kb adhered to Vero cells at a much higher level than clone 6 (mean, 28 bacteria per cell). It also adhered to mucus at a level 10,000-fold higher



than that of HB101/pBluescript (not shown). Subcloning of the 4.3-kb insert is in progress.

The expression of proteins by the recombinant clones was studied by Western blotting (Fig. 3). The proteins transferred onto nitrocellulose were revealed with anti-*C. difficile* antibodies adsorbed to *E. coli* and non-heat-shocked *C. difficile*. The antibodies reacted with a multitude of *C. difficile* proteins present in the lysate of strain 79-685 grown in TGY liquid medium (Fig. 3A, lane 1). The antibodies recognized a 40-kDa and a 27-kDa protein in the *E. coli* lysate carrying pCL6 grown on solid medium (Fig. 3A, lane 4); these proteins were absent from the supernatant of this clone when it was grown in liquid medium (Fig. 3A, lane 6). When HB101/pCL6 was grown in liquid medium, the 27-kDa protein was no longer expressed (Fig. 3A, lane 5). In the lysates of HB101/p4.3kb cultivated on solid medium, the antibodies recognized the 27-kDa protein (lanes 3); when the strain was grown in liquid medium, this protein was no longer expressed (Fig. 3A, lane 2). The antibodies raised against a 27-kDa surface-associated *C. difficile* protein, previously identified by our laboratory as a possible adhesin (14), recognized a 27-kDa and a 40-kDa protein in the lysates of HB101/pCL6 and HB101/p4.3kb cultivated on solid medium (Fig. 3B, lanes 1 and 2). The other adherent clones (5, 7, and 10) did not express the 27-kDa adhesin (data not shown).

By immunoelectron microscopy, it was evident that clone 6 expressed a surface-associated protein recognizable by antibodies raised against the 27-kDa adhesin (Fig. 2).

Adherence inhibition. Incubation of clone 6 or *C. difficile* with a 1:1,000 dilution of anti-*C. difficile* antibodies raised against heat-shocked bacteria or with a surface extract of *C. difficile* for 1 h inhibited the adherence of the clone to the Vero cell monolayer (Table 2; Fig. 1). Likewise, a surface extract of clone 6 could partially impede *C. difficile* attachment (Table 2). In addition, mucus isolated from axenic mice could diminish adherence of clone 6 and *C. difficile* to the same cell line (Table 2; Fig. 1).

A number of monosaccharides [D-(−)-arabinose, D-(+)-arabitol, D-(−)-fructose, D-(+)-galactose, D-(+)-glucose, D-(+)-glucosamine, D-(−)-mannitol, D-(+)-mannose, mesoinositol, D-(−)-ribose, α-L-rhamnose, and D-(+)-xylose], disaccharides (α-lactose, maltose, melibiose, and sucrose), and a trisaccharide [D-(+)-raffinose] were tested for inhibition of adhesion to Vero cells of clone 6 and heat-shocked *C. difficile* (Table 3). Partial inhibition of adherence was obtained with the following sugars (in approximate order of inhibition): glucose > melibiose > raffinose > galactose > lactose > ribose. In addition, the inhibitory effects of mucus components and gelatin were tested in the same model; partial inhibitions were obtained with galactose and *N*-acetylgalactosamine as well as with gelatin.

DISCUSSION

Our studies of *C. difficile* binding to epithelial cells and mucins are motivated by the widely accepted principle that adhesion is an important factor in bacterial pathogenicity,

FIG. 2. Transmission electron micrographs of *E. coli* HB101 (magnification, ×17,000 (A), *E. coli* HB101/pCL6 (magnification, ×17,000) (B), and heat-shocked *C. difficile* (magnification, ca. ×29,000) (C). Revelation was done with anti-heat-shocked *C. difficile* antibodies adsorbed to non-heat-shocked bacteria and then with anti-immunoglobulin G labelled with 10-nm-diameter gold particles. Note the absence of fimbriae from HB101 and HB101/pCL6. For HB101/pCL6, the same results were obtained with anti-27-kDa antibodies.

TABLE 2. Hemagglutination and adherence of recombinant clones and *C. difficile* 79-685

Recombinant clone(s) (culture medium)	Hemagglutination with erythrocytes[a]				Adherence[b]		Inhibition of binding by:			
	Human O^+	Human O^-	Horse	Sheep	Vero cells	Mucus ex vivo	Anti-*C. difficile* antibody[c]	*C. difficile* surface extract	Surface extract of pCL6	Mucus
1, 2, 3, 4, 8, and 9 (LB agar)	0	0	0	0	0.72 (0.14–1.91)	ND[d]	ND	ND	ND	ND
5, 7, and 10 (LB agar)	1+	0	1+	0	6.47 (4.6–8.1)	ND	ND	ND	ND	ND
6 (LB agar)	2+	0	2+	3+	9.4 (5.2–16.9)	4.5×10^7 (4.5×10^7 to 5.0×10^7)	0.56 (0.02–1.1)	1.68 (0.91–2.8)	ND	1.54 (1.3–1.78)
6 (LB broth)	0	0	0	0	0.95 (0.54–1.10)	ND	ND	ND	ND	ND
HB101 (LB agar)	0	0	0	0	0.08 (0.04–0.10)	1.4×10^4 (1.5×10^4 to 3.0×10^4)	ND	ND	ND	ND
HB101/pBR[e] (LB agar)	0	0	0	0	0.05 (0.03–0.25)	3.8×10^5 (3.0×10^5 to 4.1×10^5)	ND	ND	ND	ND
C. difficile at 60°C (TGY broth)[f]	1+	0	1+	1+	13.7 (8.8–17.6)	2.0×10^4 (1.5×10^4 to 3.0×10^4)[g]	0.03 (0.01–0.2)	0.13 (0.06–0.2)	2.70 (2.07–3.34)	0.52 (0.28–0.84)

[a] Scored as described in Materials and Methods.
[b] All adherence assays were conducted in the presence of 2% mannose. Adhesion to tissue culture cells is expressed as the average number of bacteria adhering per cell (ranges are given in parentheses). Each clone was tested for adhesion at least twice. Adhesion to mucus is expressed as the average number of bacteria adhering to mucus determined by plate counts.
[c] 1:1,000 dilution in TNT plus 5% defatted milk.
[d] ND, not determined.
[e] *E. coli* HB101 carrying pBluescript with no insert.
[f] All eight strains of *C. difficile* tested gave the same results in the hemagglutination assay.
[g] Nonheated *C. difficile* did not adhere to mucus of axenic mice (not shown).

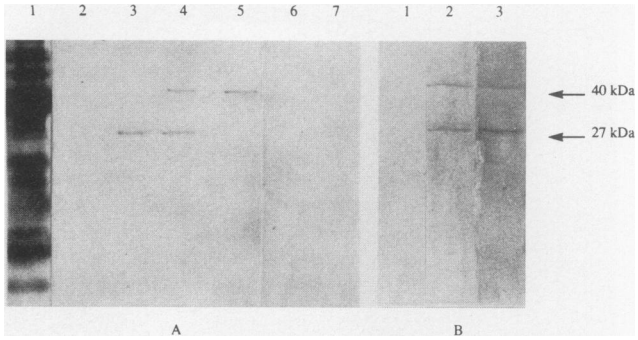


FIG. 3. Immunoblot analysis of proteins expressed by heat-shocked *C. difficile* and the recombinant clones. Revelation was done with anti-heat-shocked *C. difficile* antibodies adsorbed to non-heat-shocked bacteria (A) or with antibodies raised against the 27-kDa adhesin of *C. difficile* (B). (A) Lane 1, lysate of *C. difficile* 79-685 grown in TGY liquid medium; lane 2, lysate of HB101/p4.3kb grown in liquid medium (LB); lane 3, lysate of HB101/p4.3kb grown on LB agar; lane 4, lysate of *E. coli* HB101/pCL6 grown on LB agar; lane 5, lysate of HB101/pCL6 grown in liquid medium (LB); lane 6, proteins precipitated from the supernatant of HB101/pCL6 liquid culture by 60% ammonium sulfate (4 h at 4°C) and dialyzed against 20 mM Tris (pH 7) overnight; lane 7, lysate of *E. coli* HB101/pBluescript grown on LB agar. (B) Lanes 1 to 3, lysates of HB101/pBluescript, HB101/pCL6, and HB101/p4.3kb, respectively, all grown on LB agar. The positions of the 40- and 27-kDa proteins are indicated.

since it promotes the delivery of bacterial toxins and precedes penetration of target epithelial cells by invasive organisms. Previous studies by Borriello et al. (4) and us (14) have established that *C. difficile* is capable of adhering to hamster mucosa and tissue culture cells, respectively. Adherence to tissue culture cells is greatly enhanced if the bacteria are subjected to a heat shock at 60°C. The presence of blood in the culture medium did not modify adhesion to the Caco-2 cell line (Table 1). In contrast, our previous study (14) suggested that blood present in the culture medium enhanced adhesion of non-heat-shocked bacteria to Caco-2 cells. However, we speculate that this increase was probably due to nonvigorous washings of the bacteria after culture on blood-containing media before adhesion assays. The enhancement of adhesion may be due to the remaining blood serving as bridges between bacteria and cells.

To clone the adhesin gene of *C. difficile*, we constructed a genomic library in the λ expression vector λZapII. The relatively low titer of the library may reflect the instability of *C. difficile* DNA in *E. coli*. The DNA of strain 79-685, chosen because of its toxigenic and adherent properties, could be restricted with only *Hin*dIII among a number of enzymes tested, suggesting that it is methylated.

The screening of the library allowed us to obtain a plasmid (pCL6) that carries a genetic determinant coding for a *C. difficile* adhesin for the following reasons.

(i) *E. coli* HB101 carrying pCL6 could agglutinate several types of erythrocytes in the presence of mannose, which inhibits type 1 pilus-mediated agglutination. This agglutination was not due to an accidental cloning of the hemagglutinating portion of toxin A, as verified by a commercially available toxin A detection test (10). Besides, toxin A cannot agglutinate human, sheep, or equine erythrocytes.

(ii) Like heat-shocked *C. difficile*, HB101/pCL6 could adhere to four types of cultured epithelial cells (Vero, Caco-2, KB, and HeLa), whereas the parental strain transformed with pBluescript did not. By electron microscopy, pili were not

TABLE 3. Vero cell adherence of clone 6 and heat-shocked *C. difficile* 79-685 treated with carbohydrates[a]

Substrate	Adherence (% of control level)	
	E. coli HB101/pCL6	*C. difficile* at 60°C
PBS (control)[b]	100	100
SUGARS		
D-(−)-Arabinose, D-(+)-glucosamine, D-(−)-fructose, D-(−)-mannitol, D-(+)-mannose, mesoinositol, and D-(+)-xylose	100[c]	100[c]
D-(+)-Glucose	16	25
α-Lactose (4-*O*-β-D-galactopyranosyl-α-D-glucose)	36	58
Melibiose (6-*O*-D-galactopyranosyl-D-glucose)	38	24
D-(+)-Raffinose (*o*-α-D-galactopyranosyl [1-6]-α-glucopyranosyl-β-D-fructofuranoside)	33	38
D-(−)-Ribose	58	57
MUCUS COMPONENTS		
D-(+)-Galactose	30	58
N-Acetylgalactosamine	70	44
N-Acetylglucosamine and α-L(−)-fucose	100[c]	100[c]
OTHERS		
Gelatin (0.2%)	43	46
D-(+)-Arabitol, maltose, α-L-rhamnose, sucrose, and *N*-acetylneuraminic acid (sialic acid)	—[d]	—[d]

[a] Receptor analogs were incubated with bacteria at a concentration of 50 mM for 1 h before being added to the cell monolayer. Each inhibition assay was performed at least twice.
[b] Bacteria were incubated with PBS before the adhesion assay (level of adhesion, 100%).
[c] 100% (same as control) ± 25%.
[d] —, inconsistent results from assay to assay.

detected on either *E. coli* HB101 or HB101/pCL6 (Fig. 2); therefore, the adhesin is not likely to be fimbrial but, rather, an outer membrane protein.

The morphological patterns of adhesion of clone 6 and *C. difficile* were somewhat different: the bacteria were more evenly distributed over the monolayer in the case of heat-shocked *C. difficile*, whereas clone 6 exhibited a tendency to form clusters (Fig. 1). In general, the receptors for the adhesin seem to be more evenly distributed among Vero cells than among Caco-2 cells.

A subclone of pCL6 carrying a 4.3-kb *Sac*I fragment (HB101/p4.3kb) adhered to the tissue culture cells and mucus at a much higher level than clone 6. The insert carried by this subclone may be in such an orientation in the pBluescript vector that a vector-carried promoter increases the expression of the adhesin gene.

(iii) Adherence of HB101/pCL6 to tissue culture cells was specifically blocked by anti-*C. difficile* antibodies, even at a 1:1,000 dilution. Furthermore, a surface extract prepared from *C. difficile* was likewise capable of inhibiting adhesion (Table 2), suggesting that this extract contains the adhesin. Conversely, the surface extract of clone 6 was capable of inhibiting *C. difficile* adherence.

(iv) In a Western blot (Fig. 3), when grown on solid medium, HB101/pCL6 expressed two proteins with molecular masses of 40 and 27 kDa; the two proteins are cell associated, since they were absent from the culture supernatants. Under similar growth conditions, the subclone p4.3kb also expressed the 27-kDa protein (Fig. 3, lanes 3). The relationship between the two proteins remains to be elucidated, but it seems possible that the 40-kDa protein is a precursor of the 27-kDa one, since the two proteins are recognized by the anti-27-kDa antibodies (Fig. 3B). A 27-kDa surface-associated protein was previously identified by us as a possible *C. difficile* adhesin (14). Alternatively, the two proteins may share common epitopes which are recognizable by the antibodies. The fact that the 27-kDa protein was not synthesized when the recombinant clones were grown in liquid medium (under this condition, there is neither hemagglutination nor cell adhesion) lends evidence to the role of this protein in adhesion. By immunoelectron microscopy, the recombinant clone was also found to express a protein recognizable by anti-*C. difficile* antibodies as well as by anti-27-kDa adhesin antibodies (Fig. 2). Interestingly, the other adherent clones (5, 7, and 10) did not express the 27-kDa protein as determined by Western blotting; it is possible that these clones synthesize an adhesin different from that expressed by clone 6.

(v) Identical inhibition of clone 6 and *C. difficile* adhesion to tissue culture cells by carbohydrates, mucus, and mucus constituents was observed (see below).

The fact that the gut mucosa of humans is normally covered with mucus necessitates studies of the role of the mucus lining in the adherence and subsequent colonization of *C. difficile*, since it is difficult to imagine bacterial adherence to enterocytes without the bacteria first adhering to the mucus. This model envisions two receptors for adherence, one in the mucus and the other on the epithelial cell surface, as has been proposed for *Pseudomonas cepacia* (27), K88 antigen-positive enterotoxigenic *E. coli* (24, 35), and *Campylobacter jejuni* (22, 23).

We have previously observed that heat-shocked *C. difficile* can adhere to the mucus-producing cell line HT29-MTX; adherent bacteria were observed in the mucus gel covering the monolayer (14). We show here that heat-shocked *C. difficile* and HB101/pCL6 could adhere ex vivo to the mucus isolated from axenic mice (Table 2). The significant level of adhesion by strain HB101 alone to mucus could be explained by the fact that the lipopolysaccharide of gram-negative bacteria can bind mucus components (8, 23). Alternatively, it is well established that hydrophobic interactions alone between mucus and bacteria are sufficient to allow a basal level of binding (12, 26, 27).

Incubation of *C. difficile* and clone 6 with a crude mucus preparation from axenic mice before adhesion to cultured cells essentially abolished attachment to the Vero cells (Table 2; Fig. 1). Gastrointestinal mucus has previously been reported to inhibit the binding of organisms, such as *Entamoeba histolytica*

(6), *Shigella flexneri* (11), *Yersinia enterocolitica* (20, 21), and *C. jejuni* (23). The inhibitory effect has been reported to be mediated by nonspecific bacterial aggregation by the mucin (36). In the case of *C. difficile*, however, it appears that the inhibition of cell and mucus association is due to specific binding of carbohydrates of intestinal mucin to the adhesin of the bacterium, since the adhesion was partially inhibited by galactose and *N*-acetylgalactosamine found in the mucus.

In parallel, since the attachment of *C. difficile* and HB101/pCL6 to tissue culture cells was partially inhibited by galactose and glucose and sugars containing these monosaccharides (Table 3), it is probable that the epithelial cell receptor contains these carbohydrate moieties. The fact that the binding of the bacteria was not completely abolished by the monosaccharides implies that *C. difficile* attachment to cells involves a receptor with more than one sugar residue. Furthermore, clone 6 and, to a lesser degree, *C. difficile* could agglutinate erythrocytes carrying the determinants of A or B specificity. The specificities of the human blood group antigens A and B are determined by oligosaccharide chains terminated with *N*-acetylgalactosamine and galactose, respectively.

Since the aforementioned sugars capable of inhibiting bacterial adherence to tissue culture cells are present on epithelial cell surfaces and erythrocytes and in the mucus, it is possible that the receptors in these locations contain identical sugar residues. We have initiated experiments in vivo to confirm the identity of the receptors.

On the basis of our results, we propose the following hypothesis for *C. difficile* pathogenesis. Under normal conditions, the enterocytes are protected by a layer of mucus to which *C. difficile* may bind, since the adhesin is expressed at a low level, but is sloughed off with the mucus into the intestinal lumen. After induction of antibiotherapy or other, unknown stimuli, the adhesin is overexpressed, allowing firm attachment to the mucus layer via specific receptors. After its degradation by other bacteria, the mucus layer may initially provide nutrients for *C. difficile* thus allowing rapid proliferation of *C. difficile*. *C. difficile* can then adhere, using the same adhesin, to the receptors on the intestinal cells, with ensuing toxin production and disease. Still another level of adhesion may be adhesion to collagen, which may play a role in persisting infection by allowing bacterial attachment to connective-tissue components after destruction of the epithelial cell lining.

Interestingly, recent studies have found that a DNA insert cloned from the *Chlamydia trachomatis* chromosome conferring an adhesive phenotype on *E. coli* codes for a 28-kDa protein and an 82-kDa protein which show homology to the GrpE and DnaK heat shock proteins, respectively (25, 29). The antisera raised against the two proteins are cross-reactive. Not only is our heat shock-induced 27-kDa adhesin similar in size to the 28-kDa protein, but also the anti-27-kDa antibodies sometimes recognize a high-molecular-mass protein corresponding to the 82-kDa protein in *E. coli* lysates, suggesting that the adhesion mechanisms of these two bacteria may share common features. Sequencing data for the gene coding for the 27-kDa adhesin will aid in elucidating this possibility.

ACKNOWLEDGMENTS

We thank A. Servin for providing advice concerning the Caco-2 cells and D. Terza for assistance with electron microscopy.

This work was supported in part by a postdoctoral fellowship awarded to T.K. by Ministère de l'Éducation Nationale et de la Culture.

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