Identification and characterization of a fibronectin-binding protein from *Clostridium difficile*

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A 68 kDa fibronectin-binding protein (Fbp68) from *Clostridium difficile* displaying significant homology to several established or putative Fbps from other bacteria was identified. The one-copy gene is highly conserved in *C. difficile* isolates. Fbp68 was expressed in *Escherichia coli* in fusion with glutathione-S-transferase; the fusion protein and the native Fbp68 were purified. Immunoblot analysis and cell fractionation experiments revealed that Fbp68 is present on the surface of the bacteria. Far-immuno dot-blotting demonstrated that Fbp68 was capable of fixing fibronectin. Indirect immunofluorescence and ELISA were employed to demonstrate that *C. difficile* could bind both soluble and immobilized fibronectin. With competitive adherence inhibition assays it was shown that antibodies raised against Fbp68 partially inhibited attachment of *C. difficile* to fibronectin and Vero cells. Furthermore, Vero cells could fix purified membrane-immobilized Fbp68. Thus Fbp68 appears to be one of the several adhesins identified to date in *C. difficile*.

**METHODS**

*C. difficile* strains. The *C. difficile* strain 79-685, isolated from a patient with pseudomembranous colitis, was a gift from the

**INTRODUCTION**

*Clostridium difficile*, a Gram-positive spore-forming anaerobic bacterium, is a major cause of pseudomembranous colitis and antibiotic-associated diarrhea (Bartlett *et al.*, 1978). The virulence of *C. difficile* is mainly due to toxins A and B, although the role of potential colonization factors has been elucidated to a certain extent. The latter include the capsule (Davies & Borriello, 1990), proteolytic enzymes (Poilane *et al.*, 1998; Seddon & Borriello, 1992), and the S-layer proteins P36 and P47 (Calabi *et al.*, 2001, 2002; Cerquetti *et al.*, 2000; Karjalainen *et al.*, 2001). Our laboratory has identified several adhesins potentially involved in colonization: Cwp66 (Karjalainen *et al.*, 2001; Waligora *et al.*, 2001), the flagellin FlC (Tasteyre *et al.*, 2000a, b, 2001b), the flagellar cap protein FlD (Tasteyre *et al.*, 2001a, b), and a member of the Hsp60 family of chaperones GroEL (Hennequin *et al.*, 2001a, b). Binding of *C. difficile* to colonic cells isolated from patients has also been shown (Drudy *et al.*, 2001).

The attachment of pathogenic micro-organisms to host cells and tissues is often mediated through the expression of surface receptors recognizing components of the extracellular matrix such as fibronectin. Fibronectin is a ubiquitous 450 kDa glycoprotein found in the body fluids and extracellular matrix of vertebrates (Ozeri *et al.*, 1998). Fibronectin-binding proteins (Fbps) have been described as possible adhesins mainly in streptococci and staphylococci, in which one bacterial species can express multiple ligands for fibronectin (Courtney *et al.*, 1994; van der Flier *et al.*, 1995). In Gram-positive cocci, Fbps are exposed on the bacterial surface (Navarre & Schneewind, 1999), generally through the LPXTG cell wall anchor (Fischetti *et al.*, 1990). *C. difficile* binds to extracellular matrix proteins such as fibronectin, fibrinogen, collagen and vitronectin (Cerquetti *et al.*, 2002). *C. difficile* surface layer proteins bind to collagen, thrombospondin and vitronectin, but not to fibronectin or laminin (Calabi *et al.*, 2002). Binding to fibronectin could be due to Fbps. However, little information concerning Fbps in anaerobic bacteria, including clostridia, is available.

In this study we undertook the isolation and characterization of the *C. difficile fbp68* gene encoding a putative Fbp as well as the expression and purification of the recombinant and native forms of the Fbp68 protein. PCR amplification of the *fbp68* gene coupled with RFLP analysis were used in an attempt to differentiate between clinical isolates. The surface localization of the protein was investigated as well as its role in fibronectin binding and Vero cell attachment.

**Abbreviations:** BCIP, 5-bromo-4-chloro-3-indolyl phosphate; Fbp, fibronectin-binding protein; NBT, nitro blue tetrazolium.

The GenBank accession number for the sequence determined in this work is AF394222.
Department of Microbiology of the University of Strasbourg, France, and is the reference strain of our laboratory. Eighteen clinical strains isolated from cases of *C. difficile*-associated diarrhoea in Brussels, Belgium, were investigated. The strains were grown under anaerobic conditions in Tryptone Glucose Yeast (TGY) infusion broth (Difco) at 37 °C unless indicated otherwise. *Escherichia coli* strain LB21 (Amersham Biosciences), used for recombinant Fbp68 expression, was grown in Luria–Bertani broth (Difco).

**Isolation of the fbp68 gene from *C. difficile* 79-685 by PCR amplification.** DNA was extracted from 10 ml of *C. difficile* culture according to the protocol provided in the Puregene Gram-positive and yeast DNA extraction kit (Genta systems) and resuspended in TE buffer at a concentration of 1 μg μl⁻¹. Amplification of a 2.3 kb DNA fragment that includes upstream and downstream sequences of the *fbp68* coding sequence was carried out using primers Fbp1 (GGCATGTCAGCTATGATCATC) and Fbp2 (TACATCTATA-TAAGAAGACTG). PCR was carried out in a PE Cetus Thermal Cycler 480 with 2 mM MgCl₂, 0·2 mM each dNTP and 1 U Taq polymerase (Promega). Cycling conditions were 34 cycles of 95 °C 1 min, 55 °C 1 min, and 72 °C 2 min. Amplified products were purified by the Wizard Gel Extraction Kit (Promega). The nucleotide sequences of both strands of the amplified products were obtained by using the Big Dye Terminator Cycle sequencing kit (Applied Biosystems) and analysed with an ABI PRISM 310 Genetic Analyser (Applied Biosystems). Additional primers were designed to obtain internal sequences. Protein sequence alignments were performed with CLUSTAL W (Thompson et al., 1994). Homology searches were conducted with FastA3 (European Bioinformatics Institute) or BLAST (National Institute for Biotechnology Information, USA).

**RFLP analysis and Southern blotting.** For PCR-RFLP analysis, the *fbp68* gene was amplified by PCR as described above on genomic DNA isolated from 18 *C. difficile* clinical isolates and reference strain 79-685. The DNA was digested with four restriction enzymes (Sphi, Drai, RadI and HindIII) (Biolabs) and electrophoretic profiles were compared in 1 % agarose gels.

For Southern blotting, 10 μg purified genomic DNA was digested with *AcdI* (Amersham Biosciences). The fragments were separated through a 0·8 % agarose gel and electrophoretically transferred to a positively charged nylon membrane (Roche). The PCR-amplified *fbp68* gene from *C. difficile* 79-685 was used as a probe and labelled with the BioProbe random primer labelling kit (Sigma), according to the manufacturer’s instructions. Hybridization was carried out overnight at 42 °C and washing of the membrane was performed under low stringency (0·5 % SSC at 42 °C). The subsequent washing steps and detection with streptavidin–alkaline phosphatase were carried out as recommended by the manufacturer (CDP-Star Universal detection kit, Sigma).

**Production, purification and identification of the Fbp68 proteins**

**Production of recombinant Fbp68.** The *fbp68* gene was cloned into the pGEX-6P1 expression vector (Amersham Biosciences): two oligonucleotides, FbpfusionEcoRI (TAATAGGTAATTCGATGGATTTATTATAC) and FbpfusionSalI (TAGTGTGAATTCGATGGATTTATTAC), incorporating an EcoRI and a SalI site, respectively (underlined), were used to amplify by PCR the full-length coding region of the *fbp68* gene of strain 79-685. The resulting 1·8 kb DNA fragment was digested with the two enzymes and ligated (1 U T4 ligase, Invitrogen) between the EcoRI and SalI sites of pGEX-6P-1. The plasmid carrying an in-frame fusion between *gst* and *fbp68* was transformed into *E. coli* LB21. Subsequent purification steps using single-step affinity chromatography employing glutathione–Sepharose 4B were performed as described in protocols from Amersham Biosciences.

**Production of Fbp68-specific antibodies.** Rabbit polyclonal antibodies were produced by the Agrobio Company (France) with the recombinant Fbp68. Antibodies were then purified on protein A–Sepharose according to the supplier’s recommendations (Amersham Biosciences).

**Immunoprecipitation of the native Fbp68.** *C. difficile* was cultured for 24 h in 100 ml TGY and harvested by centrifugation at 5000 g for 10 min at 4 °C. The pellet was washed twice with PBS (pH 7·2) and resuspended in 1·5 ml 0·1 M Tris/HCl (pH 8·6). Protein extracts were obtained by three successive freeze–thaw cycles, followed by 15 min centrifugation at 15000 g and recovery of the supernatant. One millilitre of protein A-Sepharose was centrifuged (15000 g, 5 min, 4 °C), the supernatant was removed and 1 ml of Fbp68-specific antibodies [1:10 dilution in buffer B (150 mM NaCl, 20 mM Tris/HCl pH 8·1, 1 % Triton X-100, 0·2 % BSA, 5 mM EDTA)] was added and incubated for 12 h at 4 °C with gentle rotation. After centrifugation (15000 g, 5 min, 4 °C), a 1:1 mixture of protease inhibitor cocktail (Sigma) and *C. difficile* protein extracts was added to the protein A–antibody pellet, followed by a 12 h incubation with gentle rotation at 4 °C. After centrifugation (15000 g, 5 min, 4 °C), the pellet was washed once with buffer B, three times with modified buffer B (0·5 % Triton X-100 plus 0·1 % SDS), three times with differently modified buffer B (500 mM NaCl, 0·5 % Triton X-100, no EDTA) and twice with 50 mM Tris/HCl (pH 8·1). After centrifugation, 10 μl 10 % SDS was added to the pellet and left for 10 min at room temperature. After heating at 100 °C for 5 min, 250 μl of the second washing buffer without BSA was added. The solution was centrifuged and the supernatant containing the protein was recovered.

**Reconfiguration of native and recombinant Fbp68.** Because of aberrant migration through SDS-PAGE, native and recombinant Fbp68 (rFbp68) were reconfigured. The protein bands were visualized by soaking the SDS-polyacrylamide gel in cold 0·25 M KCl and 1 mM DTT for 5 min. The visible bands were sliced out from the gel, crushed, resuspended in elution buffer [0·1 % SDS, 0·05 M Tris/ HCl (pH 7·9), 0·1 mM EDTA, 5 mM DTT, 0·1 mg BSA ml⁻¹ and 0·15 M NaCl] and incubated at 37 °C for 4 h. The gel fragments were removed from the elution buffer by filtering through a 0·45 μm filter (Millipore). The proteins in the filtrate were precipitated by adding 4 vols cold acetone (−20 °C) and placing the tube in an ethanol/dry ice bath for 30 min. The precipitated proteins were pelleted by a 10 min centrifugation, and the pellet was allowed to dry for 10 min at 42 °C. The proteins were resuspended in 20 μl 6 M guanidine.HCl and incubated at room temperature for 10 min. The guanidine.HCl solution was diluted 50-fold with dilution buffer (0·05 M Tris/HCl, 20 %, v/v, glycerol, 0·10 mg BSA ml⁻¹, 0·15 M NaCl, 1 mM DTT, 0·10 mM Na₂EDTA), and the proteins were allowed to reconfigure in this solution for 12 h at room temperature.

Only rFbp68 was used in the binding assays described below, since the specific antibodies were obtained with the recombinant protein.

**Fbp68 localization: SDS-PAGE and immunoblotting with cell fractioning.** Bacterial proteins were separated into three fractions – cell wall, membrane and cytoplasm – as previously described (Hennequin et al., 2001a), using a modification of the method described by Jonquères et al. (1999). Twenty micrograms of the total protein extracts, measured by the Bio-Rad DC protein assay kit, were separated by SDS-PAGE using a 7·5 % SDS-polyacrylamide gel and then transferred to nitrocellulose. The nitrocellulose membrane was incubated for 1 h at room temperature in blocking buffer [5 % skimmed milk in TNT (10 mM Tris/HCl, pH 8·0, 150 mM NaCl, 0·05 % Tween)] and then for 1 h in a 1:2500 dilution of Fbp68-specific antibodies. The membranes were washed in TNT and bound antibodies were detected with goat anti-rabbit IgG alkaline

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phosphatase conjugate (1:2500 dilution; Sigma) with NBT-BCIP (Invitrogen) as substrates. The purity of the fractions was verified by determining the presence or absence of Cowp66 (a peptidoglycan-attached protein) (Walogila et al., 2001), GroEL (a surface-exposed heat-shock protein) (Hennequin et al., 2001a) and PepC (a cytoplasmic-membrane-associated protein) (Hennequin et al., 2001a) revealed by specific antibodies.

Binding of Fbp68 to extracellular matrix proteins by far-immuno dot-blotting. To study the binding of Fbp68 to extracellular matrix protein, 10 µg fibronectin (Roche), fibrinogen (Sigma), collagen type VI (Sigma) and vitronectin (Sigma) were deposited onto a nitrocellulose membrane and air-dried. Similarly, 10 µg BSA (Sigma) was deposited onto the membrane (negative control). The membrane was incubated for 1 h at 37°C in blocking buffer (5 % skimmed milk in TNT) and then in 10 ml of the same buffer containing 10 µg rFbp68. The rFbp68, adsorbed by the different proteins, was subsequently reacted with a 1:2500 dilution of rabbit anti-rFbp68 antibody. Detection was carried out with goat anti-rabbit IgG–alkaline phosphatase conjugate (1:2500 dilution; Sigma) with NBT-BCIP as substrates.

Binding of C. difficile to soluble and immobilized fibronectin

Binding to soluble fibronectin. The adsorption of fibronectin by C. difficile 79-685 was examined by indirect immunofluorescence. Bacteria were cultured anaerobically for 16 h at 37°C. After centrifugation and washing with PBS (pH 7.2), the bacteria were incubated for 2 h in 10 ml PBS in the presence of 0.5 µg fibronectin ml⁻¹. After centrifugation and washing with PBS (pH 7.2), a drop of the culture was deposited onto a microscope slide and dried. The slide was subsequently immersed in PBS (pH 7.2) for 1 h at room temperature, with rabbit anti-fibronectin monoclonal antibody (Sigma) (dilution 1:2500). Controls were carried out similarly: rabbit anti-C. difficile antibody was used as a positive control, the primary antibody omitted or bacteria incubated without fibronectin with anti-serum raised against fibronectin were used as negative controls. The slide was washed three times with PBS (pH 7.2) for 15 min in total and incubated for 30 min with FITC-conjugated mouse anti-rabbit IgG antibody (Immunotech; 1:160 dilution in PBS). After washing, the specimens were examined with a Leitz Aristoplan microscope with epifluorescence coupled to an Image Analyser Visiolab 1000 (Biocon).

Binding to immobilized fibronectin. The technique used to examine binding of C. difficile to immobilized fibronectin was a modification of the ELISA described by Chia et al. (2000). The experimental part with C. difficile was carried out in anaerobic conditions. Purified human fibronectin was immobilized on microtitre plates (Nunc) by adding 100 µl of the protein solution [10 µg ml⁻¹] in 0.05 M sodium carbonate buffer (pH 9.5)] to each well and incubating the plates for 1 h at 37°C. Subsequently, 200 µl of blocking buffer [1 % BSA in PBS (pH 7.2)] was added per well and the incubation was continued for 2 h at 37°C. The wells were washed three times with PBS (pH 7.2) before use. C. difficile was grown to late stationary phase and harvested by centrifugation for 10 min at 5000 g. The bacterial pellet was washed with PBS and resuspended in PBS at 10⁷ c.f.u. ml⁻¹ and then serially diluted twofold. The different bacterial dilutions were then applied to the fibronectin-coated assay plates (100 µl per well) and incubated at 37°C for 30 min. Non-adherent bacteria were removed with five washes in PBS. Subsequently, a 1:2500 dilution of a rabbit antiserum against C. difficile was added to each well, followed by incubation for 30 min at 37°C. Plates were washed with PBS, and a 1:2500 dilution of alkaline phosphatase-labelled goat anti-rabbit IgG was added, followed by three washings and addition of 200 µl p-nitrophenyl phosphate (Sigma) substrate per well [1 mg ml⁻¹ dissolved in 0.1 M carbonate buffer (pH 9.6) with 1 mM MgCl₂] for 15 min at room temperature. The reactions were stopped by the addition of 50 µl 3 M NaOH. The absorbance was measured at 405 nm in an ELISA plate reader (model Anthos HTII, Anthos Labtec Instruments). Wells without bacteria or without the first or second antibodies served as negative controls. The adhesion index is given at the mean A₄₀₅ of triplicate test wells of three different assays.

Fibronectin adherence inhibition assays. Ten⁴ bacteria were incubated with different dilutions (1:100, 1:500, 1:1000) of anti-Fbp68 in PBS for 30 min before binding to immobilized fibronectin on ELISA plates as described above. The positive control was bacterial adhesion in the presence of a 1:500 dilution of preimmune serum and the negative control adhesion to wells without bacteria. The adhesion index was determined as described above. The significance of differences between the positive assay and the inhibition assays was assessed by Student’s t-test.

Attachment of C. difficile to Vero cells mediated by Fbp68 and fibronectin

Maintenance and preparation of Vero cells as well as adherence assays were performed as previously described (Karjalainen et al., 1994).

Binding of radiolabelled Vero cells to fibronectin and Fbp68. Five micrograms of human fibronectin and rFbp68 were separately deposited on a nitrocellulose membrane and dried. The membrane was blocked at 37°C for 4 h with 5 % BSA in TNT, washed with PBS, and incubated for 90 min at 37°C under 5 % CO₂ with Vero cells (10⁵ cells cm⁻²) metabolically labelled with 1-¹⁴C)methionine (Amersham-Biosciences) and resuspended in minimum essential medium (Life Technologies). After washing with PBS, protein-bound cells on the blotted membrane were detected by exposure of the membrane to Kodak Biomax photography film (Sigma).

Cell adherence inhibition assays. Bacteria, washed twice in PBS, were incubated with anti-Fbp68 (1:10, 1:100 or 1:500 dilution in PBS) for 30 min before contact with Vero cells (1 h at 37°C under anaerobic conditions). Five washings in PBS eliminated non-adherent bacteria and the cells were fixed and stained with May-Grunwald-Giemsa (Sigma). The adhesion index was taken as the mean number of adhering bacteria per cell (counted at a magnification of ×1000) from at least three different assays. The positive adherence controls consisted of bacterial adhesion with a 1:10 dilution of preimmune serum in PBS or with PBS alone. The results of the inhibition assays were compared to the positive controls (considered as 100 % adhesion) and expressed as relative adherence (%). The significance of differences between the positive assay and the inhibition assays was assessed by Student’s t-test.

RESULTS

Isolation and analysis of the fbp68 gene from C. difficile

A putative gene encoding a Fbp has been identified on the Bacillus subtilis genome (GenBank accession number G69877). Homology searches with this sequence using TBLASTN revealed the presence of a similar gene on the sequenced genome of C. difficile 630 (http://www.sanger.ac.uk). PCR amplification was used to isolate the corresponding gene from C. difficile 79-685, using primers derived from the gene sequence of the 630 strain.

The 1773 bp fbp68 gene of C. difficile 79-685 (GenBank accession no. AF394222) has the capacity to encode a
68 kDa protein. The G+C content of fbp68 is 27 mol%, close to that of the C. difficile genome. The 591 amino acid protein carries 31% charged residues and is predominantly hydrophilic. Similar to the putative Fbp from B. subtilis, Fbp68 does not appear to possess a classical signal peptide (von Heijne, 1985) as do most Fbps from other bacteria (Navarre & Schneewind, 1999). Fbp68 displays the best homology (44% identity) with the putative Fbp of B. subtilis mentioned above and 38% and 39% identity, respectively, with the adherence and virulence protein A (PavA) of Streptococcus pneumoniae (AAF05332) and FBP54 of Streptococcus pyogenes (AAA57236), for which a clear role in adhesion to target cells has been established (Courtney et al., 1994; Holmes et al., 2001). In addition, Fbp68 displays 66% identity with the fibronectin-binding domain of FBP54 (Courtney et al., 1994).

Eight degenerate repeats r1 to r8 are present in the Fbp68 protein sequence (Fig. 1a). Analysis using ProDom software (Corpet et al., 2000; http://protein.toulouse.inra.fr/...)

![Fig. 1.](image-url)
Expression and purification of Fbp68

In order to purify the recombinant protein, the \textit{fbp68} gene of strain 79-685 was cloned into an expression vector, expressing it in fusion with glutathione S-transferase (GST). The recombinant protein (rFbp68) was cleaved from the GST protein. As shown in Fig. 2, lane A, one band corresponding to the recombinant protein with a molecular mass of 60 kDa was observed in the final eluate after SDS-PAGE. However, frequently a second band with an approximate molecular mass of 35 kDa was also observed (Fig. 2, lane B). Neither of the two bands had a molecular mass identical to that deduced from the nucleotide sequence. Because of these aberrant migrations in SDS-PAGE, native Fbp68 was extracted. A \textit{C. difficile} total protein extract was immunoprecipitated using protein A-Sepharose together with antibodies raised against rFbp68. As shown in Fig. 2, lane C, the native protein migrates in a denaturing gel faster than would be expected from the amino acid sequence. Since the aberrant migration could be due to an unusual conformation of the protein, reconfiguration of the native protein (lane C) as well as the two bands of the purified rFbp68 (lane B) was carried out by dilution of a denaturing agent (guanidine.HCl) in an appropriate buffer containing the reducing agent DTT and EDTA. As shown in Fig. 2, lanes D, E and F, after reconfiguration all three proteins migrate in a similar fashion according to their calculated molecular mass (68 kDa).

Conservation and expression of the \textit{fbp68} gene in \textit{C. difficile} isolates

We examined inter-strain variability of the \textit{fbp68} gene by PCR-RFLP. Identical digestion profiles were obtained for all the isolates tested (data not shown). Thus the gene structure of \textit{fbp68} of \textit{C. difficile} appears highly conserved. Moreover, the \textit{fbp68} gene sequences from strains 79-685 and 630 are nearly identical (only 9 nucleotide differences) (data not shown).

The results of Southern hybridization experiments showed, for all strains tested, the presence of a 1-7 kb band corresponding to the \textit{fbp68} gene (not shown), indicating that only one copy of the gene is present on the bacterial chromosome.

Surface localization of Fbp68

Fbp68 localization in whole bacteria was studied by cell fractionation. As shown in Fig. 3(a, b), Fbp68 was localized mainly in the cytoplasmic fraction and to a lesser extent in the cell wall fraction.

Binding of Fbp68 to fibronectin and other extracellular matrix proteins

Judging from amino acid sequence homology to several established Fbps, such as FBP54 of \textit{S. pyogenes} (Meehan \textit{et al.}, 2000), Fbp68 should be able to bind to fibronectin. Therefore, the fibronectin-binding properties of Fbp68 were characterized by far-immuno dot-blotting as well as by binding to fibrinogen, collagen and vitronectin. Fbp68 was able to bind to fibronectin, fibrinogen and, to a lesser extent, vitronectin. Binding to collagen was as low as that to BSA (data not shown).

Binding of \textit{C. difficile} to soluble and immobilized fibronectin

Binding of \textit{C. difficile} to soluble and immobilized fibronectin was studied by two different methods. Further evidence for a specific role of Fbp68 in fibronectin adherence was obtained by adherence inhibition ELISA assays using polyclonal antibodies to Fbp68.

Binding to soluble fibronectin. As shown in Fig. 4, whole \textit{C. difficile} bacteria showed fluorescence with antisera raised against fibronectin when bacteria were preincubated
with fibronectin, suggesting that *C. difficile* can bind to soluble fibronectin.

**Binding to immobilized fibronectin.** Binding of *C. difficile* to fibronectin fixed to microtitre wells was determined by ELISA. As shown in Fig. 5, a dose-dependent bacterial binding was observed for densities between $10^5$ and $10^7$ c.f.u. ml$^{-1}$. Therefore, *C. difficile* can bind to immobilized fibronectin.

**Fibronectin adherence inhibition assay.** Co-incubation of bacteria with anti-Fbp68 at different dilutions partially inhibited adherence on fibronectin-coated plates. The

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**Fig. 3.** (a) Surface localization of *C. difficile* Fbp68 investigated by fractionation experiments. Clostridial cell wall proteins were extracted. Twenty micrograms of the cell wall fractions were separated by PAGE, transferred to nitrocellulose and immunodetected using: lane 1, Cwp66 antibody recognizing a peptidoglycan-attached protein from *C. difficile* (positive control); lane 2, GroEL antibody recognizing a surface-exposed heat-shock protein from *C. difficile* (positive control); lane 3: Fbp68 antibody; lane 4, PepC antibody recognizing a cytoplasmic membrane-associated protein from *C. difficile* (negative control). (b) Comparison of Fbp68 localization in the cytoplasmic and the cell wall fractions. Twenty micrograms of each fraction were separated by PAGE, transferred to nitrocellulose and immunodetected using Fbp68 antibody. Lane 1, cytoplasmic fraction; lane 2, cell wall fraction.

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**Fig. 4.** Binding of *C. difficile* 79-685 to soluble fibronectin investigated by indirect immunofluorescence. Top: adsorption of fibronectin by *C. difficile* was visualized by using as first antibody a mouse fibronectin antiserum and then FITC-labelled anti-mouse immunoglobulin. Antiserum raised against whole bacteria (positive control) gave the same image as that shown. Bottom: negative control, using antisera raised against fibronectin with bacteria incubated without fibronectin. When the primary antibody was omitted, the same image as that shown was obtained.

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**Fig. 5.** Effect of concentration of bacteria on adherence to fibronectin. Wells were coated with fibronectin (10 µg ml$^{-1}$), and incubated with various concentrations of bacteria at 37°C for 30 min. Each data point represents the mean value of triplicate assays. A dose dependence of bacterial binding was observed for densities between $10^5$ and $10^7$ c.f.u. ml$^{-1}$. [Graph showing the relationship between log (No. of bacteria ml$^{-1}$) and Absorbance ($A_{	ext{opt}}$)]
highest inhibition was obtained with the dilution 1 : 1000. An $A_{405}$ of 0·88 was obtained after incubation of bacteria pre-incubated with pre-immune serum, whilst the $A_{405}$ was 0·60 with anti-Fbp68 antibody at a dilution of 1 : 1000, a statistically significant decrease ($P \leq 0·05$).

**Attachment of *C. difficile* to Vero cells mediated by Fbp68 and fibronectin**

The fact that Fbp68 can bind to fibronectin and is located in the cell wall fraction suggests a possible role for this protein in cell adherence. Therefore, the capacity of Vero cells to bind fibronectin was studied, and involvement of Fbp68 in adhesion of *C. difficile* was investigated in competitive inhibition assay using polyclonal antibodies to Fbp68.

**Binding of radiolabelled Vero cells to fibronectin and Fbp68.** Radiolabelled Vero cells were able to bind to immobilized fibronectin and, to a lesser extent, directly to the purified rFbp68 (data not shown). This suggests that Vero cells carry fibronectin on their surface and that Fbp68 could mediate *C. difficile* attachment to Vero cells.

**Cell adherence inhibition assays.** Further evidence for a role of Fbp68 in cell adherence was obtained by cell adherence inhibition assays. Bacteria co-incubated with anti-Fpb68 antibody at a dilution of 1 : 10 and 1 : 100 demonstrated a relative adherence of 60% and 70% respectively, compared with the controls (incubation with preimmune serum and PBS considered as 100% adherence), indicating that Fpb68 may indeed be involved in the adherence process. This decrease was statistically significant ($P \leq 0·05$). Co-incubation with anti-Fbp68 at a dilution of 1 : 1000 gave a relative adherence of 90%; this decrease was not statistically significant.

**DISCUSSION**

The objective of the studies in our laboratory is to identify and characterize surface proteins from *C. difficile* which could play a role in adherence and colonization of the digestive tract. Since the genome sequence of a virulent strain is now available, we decided to employ genome mining to identify genes encoding potential surface proteins. In numerous cocci, Fbps are surface associated and serve a functional role as adhesins. Therefore, we decided to search for Fbp homologues in the *C. difficile* genome. Only one candidate gene was identified, named *fbp68*, encoding a protein with features shared by surface proteins of Gram-positive bacteria, as follows. (i) Fbp68 carries repeated sequences throughout the protein. Such repeats have been postulated to play a role in immune evasion through antigenic variation or alterations in substrate-binding properties (Whatmore, 2001) and could explain an aberrant migration in SDS-PAGE (Meehan *et al.*, 2000). (ii) Fbp68 appears to be organized in domains, which carry highly conserved residues present in other Fbps. The functional significance of these domains is not clear, but we suspect that the first domain could be involved in fibronectin binding. Indeed, the fibronectin-binding domain of FBP54 of *S. pyogenes* has been mapped to the first 89 residues of the protein (Courtney *et al.*, 1994) and the putative fibronectin-binding domain of Fbp68 displays 66% identity with these 89 residues. As FBP54 contains a possible coiled-coil structure, the Fbp68 sequence was analysed for the presence of predicted coiled-coil segments. One such motif was identified, having four leucines, separated by six or seven amino acid residues (residues 311, 319, 326 and 333). The peculiar putative structure of Fbp68 suggests that its repeated regions could function as a conserved, partially coiled-coil mechanical module to expose different functional domains on the surface, as suggested for streptococci (Manganelli & van de Rijn, 1999). Predicted coiled-coil regions can also be involved in either homo- or heteromerization.

The homology of Fbp68 to other Fbps and the presence of domains including a coiled-coil domain and repeat structures in Fbp68 suggest that the protein is surface-localized. Furthermore, known functions for Fbps are mediation of adherence to fibronectin and eukaryotic cells. Far-immuno dot-blotting established clearly that Fbp68 is capable of binding to fibronectin and some other extracellular matrix proteins. If a protein is to serve as an adhesin, surface localization is frequently observed. Surface proteins of Gram-positive bacteria frequently carry the cell wall anchor LPXTG (Fischetti *et al.*, 1990; von Heijne, 1985), which, however, is absent from Fbp68, as it is from FBP54 of *S. pyogenes* (Courtney *et al.*, 1994). It is known that not all surface proteins of Gram-positive bacteria contain these motifs (Navarre & Schnewind, 1999). Immunoblot analyses of cell wall fractions revealed that Fbp68 is associated with the cell surface. How Fbp68 of *C. difficile* ends up on the bacterial surface is unclear at the moment as the protein carries no classical signal peptide. Pathways of protein secretion in Gram-positive bacteria are not fully understood. The alkaline phosphatase reporter transposon (TnFuZ) developed by Gibson and Caparon allowed the study of proteins exported from Gram-positive bacteria; in *S. pyogenes*, two exported proteins without signal peptide have been described. In addition, it is known that some well-characterized proteins of *S. pyogenes* (z-enolase, glyceraldehyde-3-phosphate dehydrogenase) which are surface-exposed lack a defined export signal (Gibson & Caparon, 2002).

When presented with both soluble and immobilized forms of fibronectin, some bacteria selectively react with only one form (Courtney *et al.*, 1994). Therefore, two types of experiments were performed to assess the capacity of *C. difficile* to bind fibronectin: indirect immunofluorescence to investigate binding to soluble fibronectin, and an ELISA to investigate binding to immobilized fibronectin. *C. difficile* could bind both soluble and immobilized fibronectin, and anti-Fbp68 partially inhibited this binding. Group A streptococci also react with both forms (Courtney *et al.*, 1994). The capacity of *C. difficile* to bind immobilized fibronectin...
in vitro may translate to its capacity to bind to fibronectin present on the surface of numerous eukaryotic cells in vivo. Binding to soluble fibronectin, which is ubiquitous in body fluids, could allow the bacteria to attach directly to tissues (Chia et al., 2000). C. difficile may also be able to bind to tissues through fibronectin–fibrin, fibronectin–collagen or fibronectin–fibronectin interactions (Vercellotti et al., 1985). In addition, binding to fibronectin could facilitate migration of bacteria to distant regions of the body (liver abscesses due to C. difficile have been described: Sakurai et al., 2001) and coating of the micro-organism with soluble fibronectin may facilitate escape from host immune surveillance.

We showed that Vero cells are capable of binding Fbp68. These cells produce fibronectin (dos Santos & Wada, 1999) and our results showed that Vero cells can strongly bind fibronectin. Thus fibronectin could serve as a bridge between C. difficile and certain cell types, as has been described for S. pyogenes cell attachment mediated by protein F (Ozeri et al., 1998).

The capacity of Vero cells to bind C. difficile Fbp68 and the adherence inhibition by Fbp68-specific antibodies suggest a role for this protein in cell adherence. Adherence assays to fibronectin confirmed the ability of C. difficile to fix fibronectin. Adherence may be optimally induced and expressed in response to various environmental conditions.

Anti-Fbp68 partially inhibited binding of C. difficile to fibronectin, suggesting a specific but partial role for this protein in adherence to fibronectin. The partial adherence inhibition of C. difficile to Vero cells with anti-Fbp68 antibodies confirms that cell adhesion of C. difficile is mediated by multiple adhesins through several extracellular matrix proteins.

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