Role of fibronectin-binding protein A in Clostridium difficile intestinal colonization

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INTRODUCTION

Clostridium difficile is an emerging nosocomial pathogen of increasing importance and virulence, especially with the appearance of hypervirulent strains in the last few years. It is the major cause of pseudomembranous colitis and causes 15–20% of antibiotic-associated diarrhoea cases associated with the use of antibiotic treatment (Cartman et al., 2010; Poxton et al., 2001). Antibiotics disrupt the normal intestinal microbiota, allowing C. difficile to colonize the gut. The pathogenesis of C. difficile infections has been attributed to two toxins, TcdA and TcdB, which act as glycosyltransferases and modify small GTPases of the Rho protein family within the host cell, resulting in alterations in the cytoskeleton (Genth et al., 2008; Voth & Ballard, 2005). Apart from these two toxins, a binary toxin is also produced by a few strains but little is known about the other virulence factors which are involved in the colonization process. Presently, only a few cell surface proteins have been identified and characterized. These proteins include the S-layer proteins (Calabi et al., 2002; Cerquetti et al., 2000), the cell wall protein Cwp66 (Waligora et al., 2001), the protease Cwp84 (Janoir et al., 2007) and the heat-shock protein GroEL (Hennequin et al., 2001). This study focuses on one of the adhesive proteins, the fibronectin-binding protein (annotated FbpA in the 630 strain and Fbp68 in the 79-685 strain) (Hennequin et al., 2001).

Fibronectin is a dimeric glycoprotein (~440 kDa) which is present in a soluble form in plasma and in an immobilized form on cell surfaces and in extracellular matrix (Joh et al., 1999). It is an important target for bacterial attachment in many pathogens, such as Streptococcus pyogenes, Streptococcus pneumoniae and Listeria monocytogenes, where fibronectin-binding proteins are important virulence factors (Courtney et al., 1999; Dramsi et al., 2004; Holmes et al., 2001; Molinari et al., 1997; Terao et al., 2001). In a previous study, a fibronectin-binding protein, Fbp68 from C. difficile strain 79-685, was identified as one of its several adhesins. It was reported that Fbp68 is able to adhere to soluble and to immobilized fibronectin, as well as to fibrinogen. Competitive adherence inhibition assays have shown that antibodies raised against Fbp68 partially inhibit the attachment of C. difficile to fibronectin and Vero cells (Hennequin et al., 2003).
Thanks to the new ClosTron gene knockout system which has been successfully developed by Heap et al. (2007), we constructed a mutant in the fbpA gene. The mutant was characterized in vitro and in vivo and compared to the isogenic wild-type strain in order to further understand the role of fibronectin-binding protein FbpA in the pathogenesis of C. difficile.

METHODS

Bacterial strains and growth conditions. The C. difficile 630Δerm strain and the CDAFbpA mutants generated in 630Δerm were grown in brain–heart infusion (BHI) broth and in Columbia cysteine agar supplemented with 0.4 % C. difficile selective supplements (Oxoid) and 4 % horse blood (CC) at 37 °C under anaerobic conditions. Escherichia coli TOP10 (Invitrogen) was used for gene cloning and E. coli HB101 RP4 was used for conjugation. Both strains were grown in Luria–Bertani medium under agitation at 37 °C. Kanamycin and ampicillin were used for E. coli at 25 and 100 µg ml⁻¹, respectively.

Construction of the C. difficile ΔfbpA mutant. The ClosTron gene insertional knockout system was used for the construction of the CDAFbpA mutant (Heap et al., 2007). Different insertion sites within the fbpA gene were identified using the Targetron Design Site program (http://www.sigma-genosys.com/targetron). Two different antisense sites were chosen (640 bp and 1370 bp from the start codon). A 350 bp fragment was generated by SOEing PCR with primers designed by inputting the targeted sequence into the web-based program (Table 1). The PCR product was excised and purified from a 1 % agarose gel. It was then cloned into pMTL007 based program (Table 1). The PCR product was excised and purified with specific pMTL007 primers (Table 1). The derivative pMTL007 plasmid construct was transformed by electroporation into the conjugative donor E. coli HB101 RP4 and transferred by conjugation into C. difficile 630Δerm. Successful transconjugants were selected onto CC medium supplemented with 15 µg thiamphenicol ml⁻¹. The thiamphenicol-resistant colonies were then restreaked onto CC medium supplemented with 5 µg erythromycin ml⁻¹ to select for the presence of spliced ErmRAM, which indicates intron integration. The obtained integrants were screened by colony PCR using primers specific for fbpA and the inserted RAM (retrotransposition-activated marker) (Table 1). Sequencing of these PCR products allowed us to confirm the insertion in the targeted gene.

Western blot analysis. C. difficile was grown overnight in BHI broth and washed twice with PBS. The pellet was resuspended in Tris/HCl (62 mmol l⁻¹; pH 6.8) supplemented with 60 µg mutanolysin ml⁻¹ and incubated at 37 °C for 1 h 30 min. The suspension was then mixed for 5 min. Protein extracts were separated by 10 % SDS-PAGE and electrophoresed to Hybond-P membranes (GE Healthcare). After blocking with BSA, the blot was incubated with a 1/10 000 dilution of an anti-FbpA polyclonal antibody (Hennequin et al., 2003), followed by incubation with an anti-rabbit IgG alkaline phosphatase conjugate (Sigma; 1/20 000). The blot was then washed and reacted with the substrate solution BCIP/NBT (Sigma).

Immobilized fibronectin-binding assay. Binding of C. difficile to immobilized human fibronectin (Sigma) on microtitre plates (Nunc) was measured as described previously (Hennequin et al., 2003). In brief, wells were coated overnight at 4 °C and for 1 h at 37 °C with 10 µg fibronectin ml⁻¹. After blocking with 1 % BSA in PBS for 2 h, the plates were incubated for 30 min with 100 µl of various concentrations of a late stationary phase C. difficile culture. After washing five times with PBS, the bound bacteria were detected using an anti-C. difficile polyclonal antibody diluted to 1/2 500. Antibodies were then detected by alkaline phosphatase-conjugated anti-rabbit IgG (Sigma; 1/2 500) and by p-nitrophenyl phosphate substrate (Sigma). The resulting colour development was measured at A595.

Adhesion to Caco-2 and HT29 cell lines. In vitro adhesion of C. difficile was studied on human colonic enterocyte-like Caco-2 cells (passage 71) and on mucus-secreting HT29-MTX cells (passage 45) in culture. Epithelial cells were cultured in 24-well plates in Dulbecco modified Eagle’s medium (DMEM; Gibco) supplemented with 10 % (v/v) fetal bovine serum and 1 % (v/v) non-essential amino acids (only for Caco-2 cells) and incubated at 37 °C in a 5 % CO₂ incubator. The culture medium was changed daily. Differentiated monolayers were used 15 days (Caco-2) and 21 days (HT29-MTX) after seeding. Overnight cultures of C. difficile were pelleted, washed and resuspended in DMEM without fetal bovine serum and without non-essential amino acids. After counting in a Salumbini chamber, the bacterial culture was adjusted to 10⁵ bacteria ml⁻¹. The real density of the inoculum was determined subsequently from viable c.f.u. counts. Bacterial suspension (0.5 ml) was added to each well and plates were then incubated for 1 h 30 min at 37 °C under

| Table 1. Primers used in this study for mutant generation |
|---------------------------------|---------------------------------|
| Primer                      | Sequence (5’→3’)                             |
| IBS₃₄₀                  | AAAAAAGCTTATAATTATCCCTACTGCTGTAGCGTGGTGCCGACGATAGGTTG |
| EBS₁₁₄₀                 | CAGATGTCACAATATTGCGTATACAGATAATGCTCTGAGACATAATCTACCTTCCTGT |
| EBS₂₆₄₀                 | TGACGCCGAGTTCTTAATTTCTGGTGACACGATGAGAGGAAGTGTC |
| EBS₃₇₀                  | AAAAAAGCTTATAATTATCCCTACTGCTCTGAGACATAATCTACCTTCCTGT |
| EBS₁₁₄₀                 | CAGATGTCACAATATTGCGTATACAGATAATGCTCTGAGACATAATCTACCTTCCTGT |
| EBS₂₆₄₀                 | TGACGCCGAGTTCTTAATTTCTGGTGACACGATGAGAGGAAGTGTC |
| EBSu                    | CGAAATTTAGAAACTTGGCTTGCAAGAAC |
| FbpA₃₄₀-F               | TCTAATTGCCACAGACAAAA |
| FbpA₃₄₀-R               | AGAAATCTTCCAGAATACAGAAC |
| FbpA₁₁₄₀-F              | CACCATCTGAAATTGCTCAAAA |
| FbpA₁₁₄₀-R              | GGGAGTCTTCCATACAGACACA |
| ErmRAM-F                | AGCCGTTATATTTGATAAAAAATATAATAGTGAGG |
| ErmRAM-R                | AGCCGTTGCGACTATACGCTTTATTTCTCCTCCG |
anaerobic conditions. The non-adherent bacteria were removed by washing five times with PBS and the bound bacteria were detached by adding 0.5 ml 1% saponin per well. Serial dilutions were plated on BHI agar plates and c.f.u. were counted after 48 h of incubation. For each plate, three wells were used to count the number of cultured cells in order to express the results as c.f.u. per cell. Briefly, cells were treated with 200 μl trypsin for 10 min and 300 μl DMEM was added. Cells were scraped and homogenized for subsequent enumeration.

The assays were done in triplicate in three independent experiments. Statistical analyses were performed using the Mann–Whitney test with StatEL software. All conclusions were based on P<0.05 as significant.

Measurement of bacterial hydrophobicity

接触角测量。接触角测量采用G1/G40 (Krüss) 自动接触角仪，该仪器是用于测定Drape Shape Analysis DSAv1.80软件。一种含有活的和死的细菌的悬液（2×10^9 每毫升细菌在0.15 M PBS）被沉积到一个显微镜片上，然后被放在37℃干燥。细菌的接触角的测量是通过相差显微镜扫描显微镜（Zeiss LSM 510）与Plan-Apochromat ×63/1.40油镜头进行的。超声波水被沉积到表面，并且接触角是在直接测量的。每组实验是用三只实验品来独立培养的。实验结果是根据三组独立实验的平均值来计算的。

Bacterial adherence to hexadecane. Adherence to hexadecane was tested by the method of Bellon-Fontaine et al. (1996). The washed bacterial pellet was diluted in 0.15 M PBS to a concentration of about 10^8 bacteria ml^-1. The A605 of this suspension (Ao) was measured using a spectrophotometer (UltraSpec 1000; Amersham Pharmacia Biotech). Each bacterial suspension (2.4 ml) was vortexed for 90 s with 0.4 ml n-hexadecane (Sigma). The mixture was allowed to stand for 15 min to ensure complete separation of the two phases. The absorbance of the water phase was then measured (A). The percentage bacterial adherence to hexadecane is subsequently calculated by the following equation: % adherence=(1−A/Ao)×100, where Ao is the A605 of the bacterial suspension before mixing and A is the absorbance after mixing. Each experiment was carried out five times with bacterial originating from independent cultures. Statistical analyses were performed using the Mann–Whitney test with StatEL software. All conclusions were based on P<0.05 as significant.

In vivo experiments. C3H/HeN germ-free and human microbiota- associated mice from INRA of Jouy-en-Josas (ANAXEM, France) were used. To prepare C. difficile for challenge, C. difficile was grown in BHI broth at 37°C in an anaerobic chamber overnight. Bacteria were harvested by centrifugation, suspended in PBS and diluted to 2×10^7 cells ml^-1 after counting with a Salumbini chamber. The real density was determined subsequently from viable c.f.u. counts. Groups of 6-week-old male mice (n=6) were orally challenged with 10^10 bacteria ml^-1. The number of bacteria present in faeces was determined by using serial dilutions in PBS and by seeding them on BHI agar. Seven days after C. difficile challenge, mice were sacrificed and dissected in order to obtain the entire caecum of each mouse. Each caecum was washed eight times by gentle shaking in PBS buffer, weighed and placed in PBS to a final concentration of 10 mg ml^-1. The caecum was then homogenized with an Ultra-Turrax apparatus (IKA-Labortechnik) for 1 min at 13 500 r.p.m. Serial dilutions were seeded and cultured in appropriate media.

Competition experiments were performed by co-infecting the same animal with equal amounts of wild-type and mutant strains. The amount of each bacterium in faeces was obtained by plating on selective plates, according to the erythromycin resistance of the CDADbpA mutant. Experiments on human microbiota-associated mice were performed 2 days after antibiotic treatment. Mice were treated with 3 mg amoxicillin/clavulanic acid (8/1, v/v) (Augmentin; Teva Classics) for 8 days by intra-gastric gavage. The amount of C. difficile in faeces was obtained by plating on C. difficile selective plates (CC).

RESULTS AND DISCUSSION

C. difficile ΔFbpA mutant does not express FbpA and does not bind fibronectin

The C. difficile ΔFbpA mutant was constructed by Clostron technology. This technique consists of retargeting the mobile group II intron from the ltrB gene of Lactococcus lactis (L1.ltrB), which provides stable insertional inactivation of specific genes and which confers erythromycin resistance via a retrotransposition-activated marker. Two different mutants were obtained in the fbpA gene with two different insertion sites, 640 bp (CDΔFbpA_640) and 1370 bp (CDΔFbpA_1370). Disruption of fbpA was confirmed by PCR and by direct sequencing of the junction points. Western blot analysis confirmed that the two mutants did not express FbpA (Fig. 1). It should be noted that FbpA migrates faster (at about 35 kDa) than expected (68 kDa) in a denaturing gel. It was previously observed that native Fbp68 of the C. difficile 79-685 strain had an aberrant migration on SDS-PAGE (Hennequin et al., 2003). This could be explained either by an unusual conformation of the full-length protein or by another post-translational processing of the native FbpA in C. difficile.

Analysis of the fibronectin-binding properties of the

![Fig. 1. Lack of FbpA expression by the CDΔFbpA_640 mutant in Western blot analysis.](http://jmm.sgmjournals.org)
Various concentrations of CD630 protein was no longer expressed in the two mutants. CDΔFbpA mutants showed the same colony aspect and growth rate as the isogenic wild-type strain (data not shown).

**Absence of FbpA affects adhesion of *C. difficile* to intestinal epithelial cells**

In order to elucidate whether FbpA plays a role in the colonization process, which is the first step in the course of infection, CDΔFbpA640 and CDΔFbpA1370 mutants were analysed for their ability to adhere to cultured intestinal epithelial cells. Only the results obtained with the CDΔFbpA640 mutant are presented. First, we measured adhesion of the CDΔFbpA mutants to Caco-2 cells. Surprisingly, a small but significant increase in adhesion was observed for the mutants (1.17 c.f.u. per cell for CDΔFbpA640) as compared to the wild-type strain (5.47 × 10⁻² c.f.u. per cell) (P<0.05). The CDΔFbpA640 mutant also adhered slightly more (2.6 c.f.u. per cell) than the wild-type strain (3.15 × 10⁻² c.f.u. per cell) onto HT29-MTX cells (P<0.05). The same results were obtained with the two CDΔFbpA mutants (data not shown for the CDΔFbpA1370 mutant). These striking results suggest that the absence of FbpA leads to some changes in the adhesion properties of *C. difficile*. One hypothesis is that there could be changes in the hydrophobic properties of the bacterial surface. In order to test this hypothesis, we performed measurements of the contact angle between water drops and bacterial films as well as tested adherence to hexadecane for the two CDΔFbpA mutants and the wild-type strain. Using these two techniques we did not observe any significant difference between strain 630Δerm, CDΔFbpA640 and CDΔFbpA1370 (Fig. 3a, c; data not shown for the CDΔFbpA1370 mutant). Microscopic analysis (differential interference contrast microscopy) of the bacterial films used for contact angle measurements showed a flat, regular and homogeneous surface for all the tested biofilms (Fig. 3b). These results suggest that hydrophobic properties are not involved in the increased adhesion of CDΔFbpA mutants to intestinal epithelial cells. The observed increased adherence of these mutants could also be explained by changes in the bacterial surface other than hydrophobicity which lead to an overexposure of other adhesins. Another hypothesis could be changes in the regulation of one or more of the cell wall proteins. Further experiments should be performed in order to confirm these hypotheses. In a previous study, it has already been reported that a ΔFbpA mutant in *Lactobacillus casei* adhered more than the wild-type strain to the HT29-MTX cell line whereas no significant changes were detected in the ability to bind to Caco-2 (Muñoz-Navarro et al., 2010). It was speculated that *L. casei* probably utilizes other FbpA-independent mechanisms for attachment to the HT29 and Caco-2 cell lines or that the contribution of FbpA to binding in these models is small. In another recent report, a CDΔFbp68 mutant was constructed in *C. difficile* 630 (insertion site at 102 bp) by using the same Clostron gene knockout system (Lin et al., 2011). The authors assayed adherence to Caco-2 cells and showed that their mutant had similar fibronectin- and cell-binding activities to the wild-type strain. Contrary to their results, our CDΔFbpA mutants did not adhere to human fibronectin by comparison with the wild-type CD630Δerm strain. Moreover, adherence of the mutants to cultured cells was tested on two different cell lines, which showed that the CDΔFbpA mutants adhere slightly more to both Caco-2 cells and mucus-secreting HT29-MTX cells. It is possible that the different genetic background of the mutated bacteria plays a role in the differences observed in the adherence properties.

Still in order to determine the role of FbpA and the implication of its adhesive properties in the colonization of *C. difficile*, we tested the capacity of the CDΔFbpA640 mutant to colonize the intestine of mice.

**FbpA plays a role in intestinal colonization of mice**

In order to assess the capacity of the CDΔFbpA640 mutant to colonize mice, the kinetics of faecal shedding and caecal colonization were studied in three different mouse models. Kinetics of faecal shedding allows measurement of the intestinal implantation of a strain, while caecal colonization allows evaluation of the capacity of one strain to interact with the intestinal epithelium. For each experiment, faeces were collected at specific times during a week and caeca were obtained by sacrificing mice 7 days post-challenge.

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**Fig. 2.** Binding of the wild-type strain CD630Δerm (○) and the CDΔFbpA640 mutant (▲) to immobilized human fibronectin. Various concentrations of CD630Δerm or CDΔFbpA640 were added to fibronectin (10 μg ml⁻¹)-coated wells. Binding was measured by ELISA using an anti-*C. difficile* polyclonal antibody and an alkaline phosphatase-conjugated anti-rabbit secondary antibody. The A₄₅₀ was measured. The same result was obtained for CDΔFbpA1370.
Monoxenic mice. In this model, mice were challenged with either the \textit{fbpA} mutant or the wild-type strain. The same faecal shedding kinetics was observed for the two strains (Fig. 4). The rate of faecal shedding increased during the first hours of infection, reached a plateau after about 8 h and then remained constant. Even though the faecal shedding kinetics of the \textit{fbpA} mutant was no different from that of the wild-type strain, a significant decrease in caecal colonization was noted \cite{2.51 \times 10^6 \text{ c.f.u. (g caeca)^{-1}}} and \text{7.88 \times 10^5 \text{ c.f.u. (g caeca)^{-1}}} for the wild-type strain and \text{CD\Delta FbpA_{640}} mutant, respectively; \text{P<0.05}. This reduced caecal colonization suggests a role for FbpA in adherence and in intestinal colonization.

Dixenic mice. We carried out a competition assay in which mice were simultaneously challenged with both the \text{CD\Delta FbpA_{640}} mutant and the wild-type strain. The \text{CD\Delta FbpA_{640}} mutant and \text{CD630\Delta erm} showed two different kinetics (Fig. 5). In fact, the mutant shed less \textit{C. difficile} than the wild-type strain during the first 5 days after challenge. We particularly observed a significant and important reduction of the shedding rate at 48 h, 72 h and 96 h post-challenge for the \text{CD\Delta FbpA_{640}} mutant. After 120 h post-challenge, the shedding rate became similar for the two strains. Even though a lower rate of caecal colonization was observed for the \text{CD\Delta FbpA_{640}} mutant in this model, this difference was not statistically significant. These results as well as those observed in monoxenic mice support the fact that FbpA plays a role in intestinal colonization of mice.

Human microbiota-associated mice. We also tested the capacity of the \text{CD\Delta FbpA_{640}} mutant to colonize the gut and adhere to the caeca of human microbiota-associated mice. Wild-type and \text{CD\Delta FbpA_{640}} mutant strains colonized at exactly the same rate (Fig. 6). The absence of difference in this model is not so surprising and could be explained by the barrier effect of the intestinal microbiota. Moreover, the colonization process is multifactorial and probably involves several adhesins. Consequently, the effect of only one adhesin is difficult to observe in such a model.
C. difficile pathogenicity is mainly mediated by its two toxins, TcdA and TcdB, but other factors undoubtedly contribute to its virulence, particularly in the initial colonization process. This first step occurs before the production of toxins and involves several adhesins. Thus, it is very important to study the precise role of these adhesins in the colonization process. FbpA represents one of these adhesins. It has already been reported that FbpA is able to bind to soluble and to immobilized fibronectin and that it is probably implicated in cell adherence. These conclusions are based on a competitive inhibition assay using anti-Fbp antibodies and on an adherence assay of Fbp to radiolabelled Vero cells which carry fibronectin on their surface (Hennequin et al., 2003). In our study, we tried to confirm the role of FbpA in cell adherence and to elucidate the role of FbpA in the initial C. difficile colonization process in particular. For this purpose, we constructed a CDΔFbpA mutant and studied the effect of this mutation in vitro on cultured epithelial cells and also in vivo using different mouse models. We demonstrated that mutation in the fbpA gene affects the adherence of C. difficile 630 Derm to epithelial cell lines and that adherence is increased. Both caecal colonization (in monoxenic mice) and intestinal implantation (in dixenic mice) of the CD ΔFbpA mutant are slower than those of the wild-type strain. Taken together, these data suggest a role for FbpA in intestinal colonization by C. difficile. This role is minor but not insignificant and is probably shared with other adhesins. This strengthens the idea that intestinal colonization is a complicated process which involves several colonization factors. The observed effect might also be different between strains. Thus, it could be interesting to test the effect of FbpA mutation on other strains, especially hypervirulent strains such as the NAP1/027 strain.

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