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

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Clostridium difficile is a frequent cause of severe, recurrent, post-antibiotic diarrhoea and pseudomembranous colitis. Its pathogenicity is mediated mainly by two toxins, TcdA and TcdB. However, different adhesins have also been described as important colonization factors which are implicated in the first step of the intestinal infection. In this study, we focused our interest on one of these adhesins, fibronectin-binding protein A (FbpA), and on its role in the intestinal colonization process. A mutant of FbpA (CDΔFbpA) was constructed in *C. difficile* strain 630Δerm by using ClosTron technology. This mutant was characterized *in vitro* and *in vivo* and compared to the isogenic wild-type strain. Adhesion of the CDΔFbpA mutant to the human colonic epithelial cell line Caco-2 and to mucus-secreting HT29-MTX cells was examined. Surprisingly, the CDΔFbpA mutant adhered more than the wild-type parental strain. The CDΔFbpA mutant was also analysed in three different mouse models by following the intestinal implantation kinetics (faecal shedding) and caecal colonization (7 days post-challenge). We showed that in monoxenic mice, CDΔFbpA shed *C. difficile* in faeces at the same rate as that of the isogenic wild-type strain but its colonization of the caecal wall was significantly reduced. In dioxenic mice, the shedding rate was slower for the CDΔFbpA mutant than for the isogenic wild-type strain during the first days of infection, but no significant difference was observed in caecal colonization. Similar rates of intestinal implantation and caecal colonization were observed for both strains in assays performed in human microbiota-associated mice. Taken together, our data suggest that FbpA plays a role in intestinal colonization by *C. difficile*.

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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 flagellum and its components (Tasteyre *et al.*, 2001), 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 CDΔFbpA 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 CDΔFbpA 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 via *Hind*III and *Bsr*GI and transformed into *E. coli* TOP10. Sequence confirmation of purified plasmid constructs was performed using 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 electrotransferred 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 1/2500. Antibodies were then detected by alkaline phosphatase-conjugated anti-rabbit IgG (Sigma; 1/2500) 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 Salubini chamber, the bacterial culture was adjusted to 10⁸ 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')
IBS ₆₄₀	AAAAAAGCTTATAATTATCCTTACTGCTCTGTAGCGTGCGCCAGATAGGGTG
EBS1d ₆₄₀	CAGATTGTACAAATGTGGTGATAACAGATAAGTCTGTAGCAATAACTTACCTTTTCGT
EBS2 ₆₄₀	TGAACGCAAGTTTCTAATTTTCGGTTAGCAGTCGATAGAGGAAAGTGCT
IBS ₁₃₇₀	AAAAAAGCTTATAATTATCCTTAGTATCCTTTTATAGTGCGCCAGATAGGGTG
EBS1d ₁₃₇₀	CAGATTGTACAAATGTGGTGATAACAGATAAGTCTTTTACTTAACCTTACCTTTCTGT
EBS2 ₁₃₇₀	TGAACGCAAGTTTCTAATTTTCGATTGATACTCGATAGAGGAAAGTGCT
EBSu	CGAAATTAGAACTTTCGGTTTCAGTAAAC
FbpA ₆₄₀ -F	TTCATTGCCACCAGAACAAA
FbpA ₆₄₀ -R	AGAAATCTTCCAGAATACGAGACA
FbpA ₁₃₇₀ -F	CACCATCTGAAAATGCTCAAAA
FbpA ₁₃₇₀ -R	GGGACTTCCTTACCAGCACA
ErmRAM-F	ACGCGTTATATTGATAAAAAATAATAAGTGGG
ErmRAM-R	ACGCGTGCGACTCATAGCCTTATTTCTCTCCCG

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

Contact angle measurements. Contact angle measurements were carried out using a G1/G40 (Krüss) goniometer coupled to the Drop Shape Analysis DSAv1.80 software. One hundred and twenty microliters of a bacterial suspension (2×10^{10} bacteria ml^{-1} in 0.15 M PBS) was deposited on a microscope slide and then left to dry for 2 h at 37 °C. The homogeneity of the bacterial film was checked by differential interference contrast microscopy using a confocal laser scanning microscope (Zeiss LSM 510) with a Plan-Apochromat $\times 63/1.40$ oil objective. Drops of ultrapure water were deposited onto the surface and the contact angle was measured immediately after their deposition onto the bacterial film. The values obtained were mean contact angles from three different experiments with three independently grown cultures. For each strain and each experiment, at least eight different drops were measured.

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 A_{405} of this suspension (A_0) 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 was subsequently calculated by the following equation: % adherence = $(1 - A/A_0) \times 100$, where A_0 is the A_{405} of the bacterial suspension before mixing and A is the absorbance after mixing. Each experiment was carried out five times with bacteria 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 Salubini 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 0.5 ml bacterial suspension and monitored for up to 7 days. Faecal samples were collected every day from each mouse. They were weighed and diluted in PBS in order to obtain a concentration of 10 mg 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 CDΔFbpA 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 intragastric 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 (CDΔFbpA₆₄₀) and 1370 bp (CDΔFbpA₁₃₇₀). 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 CDΔFbpA₆₄₀ mutant in Western blot analysis. Whole proteins extracted from cultures (10 ml) of CD630Δerm (lane 1) and CDΔFbpA₆₄₀ (lane 2) were subjected to SDS-PAGE, transferred to Hybond-P membrane and then probed with 1/10 000 anti-FbpA polyclonal antibodies. The arrow indicates the location of FbpA, which is present in CD630Δerm but not in CDΔFbpA₆₄₀. The same result was obtained for CDΔFbpA₁₃₇₀.

mutants compared to those of the wild-type strain showed that both mutants were not able to bind fibronectin immobilized on immunoplates (Fig. 2). These results show that the *fbpA* gene was really inactivated and that the FbpA 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-Provencio *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.

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 A₄₀₅ was measured. The same result was obtained for CDΔFbpA₁₃₇₀.

Fig. 3. Hydrophobicity of the wild-type strain CD630Δerm and the CDΔFbpA₆₄₀ mutant. (a) Contact angle measurements. One hundred and twenty microlitres of a bacterial suspension (2×10^{10} bacteria ml^{-1}) was deposited on a microscope slide and then left to dry. Contact angles were measured immediately after the deposition of drops of water onto the bacterial film. The reported values are the mean contact angles from three independent experiments. (b) Differential interference contrast microscopy image of a representative bacterial film showing a homogeneous and continuous covering of the slide. Bar, 30 μm . (c) Bacterial adherence to *n*-hexadecane. Each bacterial suspension (2.4 ml; 10^8 bacteria ml^{-1}) was mixed with 0.4 ml *n*-hexadecane and vortexed for 90 min. The mixture was allowed to stand for 15 min. The percentage of adherence to *n*-hexadecane was calculated by using the initial A_{405} of the bacterial suspension and the absorbance of the water phase after mixing as indicated in Methods. The bars represent standard deviation of five independent experiments.

Monoxenic mice. In this model, mice were challenged with either the *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 *fbpA* mutant was no different from that of the wild-type strain, a significant decrease in caecal colonization was noted [2.51×10^6 c.f.u. (g caeca) $^{-1}$ and 7.88×10^5 c.f.u. (g caeca) $^{-1}$ for the wild-type strain and CDΔFbpA₆₄₀ mutant, respectively; $P < 0.05$]. 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 CDΔFbpA₆₄₀ mutant and the wild-type strain. The CDΔFbpA₆₄₀ mutant and CD630Δerm showed two different kinetics (Fig. 5). In fact, the mutant shed less *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 CDΔFbpA₆₄₀ 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 CDΔFbpA₆₄₀ 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 CDΔFbpA₆₄₀ mutant to colonize the gut and adhere to the caeca of human microbiota-associated mice. Wild-type and CDΔFbpA₆₄₀ 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.

Fig. 4. Role of FbpA in intestinal colonization of monoxenic mice by *C. difficile*. Kinetics of intestinal implantation by wild-type strain CD630Δerm (◆) and the CDΔFbpA₆₄₀ mutant (■). Mice were challenged ($n=6$) with a total of 5×10^6 bacteria per mouse of each of the two strains. Faeces were collected and treated daily for a week to obtain the number of c.f.u. (g faeces)⁻¹.

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

Fig. 5. Role of FbpA in intestinal colonization of dioxenic mice by *C. difficile*. Kinetics of intestinal implantation by wild-type strain CD630Δerm (■) and the CDΔFbpA₆₄₀ mutant (◆). The same protocol as described for monoxenic mice was used but mice were co-infected with the two strains. Statistically significant differences ($P<0.05$) are indicated by *.

Fig. 6. Role of FbpA in colonization of human microbiota-associated mice by *C. difficile*. Kinetics of intestinal implantation by wild-type strain CD630Δerm (◆) and by the CDΔFbpA₆₄₀ mutant (■). Human microbiota-associated mice ($n=6$) were inoculated with each of the two strains and the kinetics of colonization was followed by analysing faecal shedding over time. Results are expressed as number of colonies obtained (g faeces)⁻¹.

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 knockout 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* 630Δerm to epithelial cell lines and that adherence is increased. Both caecal colonization (in monoxenic mice) and intestinal implantation (in dioxenic mice) of the CDΔFbpA 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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