Inactivation of a fibronectin-binding TonB-dependent protein increases adhesion properties of Bacteroides fragilis

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Bacteroides fragilis is the Gram-negative strictly anaerobic bacterium most frequently isolated from clinical infections, including intra-abdominal abscess and bacteremia. A number of factors can contribute to its virulence, including the expression of adhesins. Some of them are already characterized and can recognize and bind to extracellular matrix components, such as fibronectin. One of the molecules responsible for fibronectin-binding is an outer-membrane protein previously described by our group, which belongs to the TonB-dependent family. The aim of the present work was to characterize this protein. Initially, it was confirmed by fluorescence and electron microscopy that the fibronectin-binding molecules were located in the bacterial surface, but the distribution of these molecules on the surface was not uniform. To further evaluate the role of this protein, the gene bf1991, responsible for encoding this protein, was inactivated by a suicide vector and the mutant strains generated were used in several experiments to verify possible phenotypical alterations. In adherence assays with fibronectin immobilized on latex beads an increased adhesion was observed with the mutant strains compared with the wild-type strain. Western blot analysis in the mutant strain revealed the absence of the 120 kDa TonB-dependent outer-membrane protein and an alteration in the expression of an unknown 30 kDa protein. Killing assays using peritoneal macrophages were performed to evaluate the role of this protein as a virulence attribute and it was observed that the mutant strains were more efficiently internalized than the wild-type strains, with more internalization in the samples covered with fibronectin than in the samples not covered with it.

INTRODUCTION

Bacteroides fragilis is a bacterium that resides in the human gastro-intestinal tract; however, it is also the most commonly isolated Gram-negative obligate anaerobe from human clinical infections, such as intra-abdominal abscesses, and the most common cause of anaerobic bloodstream infections (Odamaki et al., 2012). In the genus Bacteroides, B. fragilis is considered the most virulent species, and its capsular polysaccharides are especially linked to its pathogenesis (Wexler, 2007). Adhesion and invasion are recurring themes in bacterial pathogenesis. Firm attachment of microbes to molecules in host tissues is often a pre-requisite to colonizing host surfaces or to injecting bacterial effectors into eukaryotic cells. Furthermore, invasion of host cells allows pathogens to hide and persist within host tissues and to escape immune responses (Finlay & Falkow, 1997). The extracellular matrix (ECM), an important structural component underlying epithelial and endothelial cells and surrounding connective tissue cells, is involved in the metabolism and differentiation of animal cells (Westerlund & Korhonen, 1993).

Abbreviations: ECM, extracellular matrix; FnBP, fibronectin-binding protein; OMP, outer-membrane protein.
Certain adhesins present on the bacterial surface can recognize and bind components of ECM including collagens, elastin, laminin and fibronectin (Joh et al., 1999). Binding to ECM components has a decisive role in the pathogenic process of several bacterial species.

Fibronectin is a glycoprotein dimer of 250 kDa subunits that is present in a soluble form in plasma and other body fluids and in an insoluble form in tissues (Cho & Mosher, 2006). In the last few decades, it has become clear that many bacteria possess fibronectin-binding proteins (FnBPs) and that such proteins can bind to a growing number of sites on fibronectin (Henderson et al., 2011). In 1994, Nagy et al. (1994) showed the ability of different species of the Bacteroides genus to adhere to fibronectin. The adherence ability of B. fragilis has already been proposed as a major virulence factor of the species (Pruzzo et al., 1989; Patrick & Lutton, 1990). The number of bacteria known to express FnBP is high (Hasty et al., 2002). However, the presence of FnBP does not indicate that this protein functions as an adhesin. In fact, only a few studies have clearly documented the interaction of the FnBPs as a direct mediator of adhesion (Ofek et al., 2003). In a recent study, our group described an FnBP in B. fragilis as a putative TonB-dependent outer-membrane protein (OMP) of 102 kDa, encoded by the bfpA gene, identified by MALDI-TOF MS (MS/MS) analysis (Pauer et al., 2009). In the present work, we demonstrate that this protein is functionally expressed in the cell outer layer and study the involvement of this FnBP in adherence properties of this bacterium to fibronectin.

**METHODS**

**Culture conditions.** Strains were routinely grown anaerobically (80 % N2, 10 % CO2 and 10 % H2) in brain heart infusion broth (BHI) previously reduced and sterilized anaerobically, supplemented with haemin (5 mg ml–1) and menadione (0.1 mg ml–1). Rifampicin (20 mg ml–1), erythromycin (10 mg ml–1) and gentamicin (100 mg ml–1) were added when required. Escherichia coli strains were grown aerobically at 37 °C in lysogeny broth. Ampicillin (100 μg ml–1), tetracycline (10 μg ml–1), kanamycin (50 μg ml–1) and spectinomycin (50 μg ml–1) were added when required. All these supplements were from Sigma.

**Immunostaining fluorescence microscopy.** Bacteria grown in BHI broth to late-exponential phase were incubated with fibronectin (50 μg ml–1; Sigma) for 1 h at room temperature and later with 2 % BSA. Afterwards, the bacteria were washed twice with PBS (0.01 M, pH 7.2) and fixed in 3.7 % formaldehyde for 1 h. Then, the bacteria were allowed to adhere to coverslips, previously prepared with poly-L-lysine (Sigma), for 30 min at room temperature. After washing twice with PBS, the coverslips were incubated with mouse anti-fibronectin IgG (1 : 10) (Sigma) in PBS for 60 min. After washing twice more with PBS, the bacteria were incubated for 60 min with Alexa Fluor 488 goat anti-mouse IgG (Invitrogen). Then, the coverslips were washed three times with PBS and placed on slides with n-propyl gallate (Sigma). The negative control was performed with the bacteria without fibronectin. Bacterial adhesion was observed in an Axiovert Zeiss microscope.

**Transmission electron microscopy.** Bacteria grown in BHI broth to late-exponential phase were incubated with fibronectin (50 μg ml–1 in PBS) for 1 h at room temperature, followed by incubation with 2 % BSA for 1 h. Then, the bacterial cells were washed twice with PBS (0.01 M, pH 7.2), fixed in 4 % paraformaldehyde/0.1 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 2 h at 4 °C. After washing in PBS twice, the cells were incubated with mouse anti-fibronectin IgG (1 : 1) (Sigma) in PBS for 60 min. The bacterial cells were washed twice with PBS and incubated for 60 min with 10 nm colloidal gold-conjugated goat anti-mouse IgG (1 : 100 in PBS; Invitrogen). The cells were post-fixed in 1 % OsO4 and 1.25 % potassium ferrocyanide for 40 min, dehydrated in graded concentrations of acetone (50–100 %), and embedded in epoxy resin. Thin sections were stained with uranyl acetate and lead nitrate. Bacteria without fibronectin were used as a negative control. The observation was carried out using a Zeiss 900 transmission electron microscope.

**Construction of B. fragilis mutants.** B. fragilis 638R mutants were generated by insertional inactivation using the suicide plasmid pFD516 (Smith et al., 1995). Briefly, an 800 bp fragment of the bfpA gene was amplified by PCR and cloned into the cloning vector pGEM-T Easy (Promega). The primers used were BfompF (5’-AGACCGGAGAATCTGCTGATG-3’) and BfompR (5’-ACATT-GCCATAACGGAAC-3’). The PstI/SphI fragment generated by digestion of the cloning vector was cloned into the PstI/SphI sites of the suicide plasmid pFD516. The resulting plasmid was mobilized into B. fragilis 638R by triparental mating (Guiney et al., 1984). Transconjugants were selected on BHI containing rifampicin (20 μg ml–1), erythromycin (2 μg ml–1) and gentamicin (100 μg ml–1). The mutation was verified by Southern hybridization. The blots were probed with digoxigenin-labelled pFD516 DNA according to the manufacturer’s protocol (Roche Molecular Biochemicals).

**Particle agglutination assay.** Latex particles (0.83 μm diameter; Sigma) coated with plasma fibronectin (Sigma) were prepared according to Pauer et al. (2009). All the B. fragilis strains were grown for 18 h at 37 °C in BHI broth, centrifuged (3000 g for 10 min) and washed once in 0.1 M PBS (pH 7.2). A bacterial suspension of 109 c.f.u. ml–1 (10 μl) was gently mixed on a glass slide with an equal volume of plasma fibronectin-coated latex beads. The agglutination reaction was scored after 2 min and recorded as a particle agglutination assay value that varied from strongly positive (3 +), to weakly positive (1 +), to negative (0), as previously described (Naidu et al., 1988). The strains were also tested for auto-agglutination by suspending them in 10 μl of glycerol-NaOH buffer. Latex beads coated with BSA (2 mg ml–1) were used as a negative control.

**Fibronectin-binding assays.** Ninety-six well plates were coated with fibronectin as previously described (RebiereHuet et al., 2002). Briefly, the wells were coated with plasma fibronectin in PBS (100 μl fibronectin at 25 or 10 μg ml–1 well) or with 20 μg ml–1 BSA overnight at 4 °C. Then, wells were washed four times with 100 μl PBS, and blocked for 1 h at room temperature with 100 μl 1 % BSA in PBS. The wells were washed four times with PBS just before the addition of the bacteria. Fibronectin-binding assays were performed as previously described (RebiereHuet et al., 2002), with some modifications. Briefly, the wild-type and mutant strains were suspended in PBS and collected by centrifugation at 5000 g for 10 min. The cells were washed twice in sterile PBS and suspended in the same sterile buffer (OD550 0.4). Fifty microlitres of bacterial suspension were mixed with 50 μl PBS, added to BSA or fibronectin-coated wells and incubated for 2 h at room temperature. Non-adhered bacteria were discarded and the wells were washed six times with sterile PBS to remove unbound bacteria and fixed at 55 °C for 45 min. Adhered bacteria were detected by staining with crystal violet, and OD570 was determined with an ELISA plate reader (Yhiber et al., 2010).
Extraction of total proteins. Analysis of whole-cell protein extracts was performed as previously described (Taylor et al., 1986). Briefly, after cultivation, bacterial cells were washed twice with 0.1 M PBS (pH 7.2) and centrifuged (4000 g for 10 min) and the pellet was resuspended in Tris/HCl buffer 0.25 M (pH 8.5) containing 0.192 M glycine and 0.1% SDS. The suspension was kept at $-20^\circ C$ until the moment of use.

SDS-PAGE. Standard SDS-PAGE was carried out using discontinuous bis-acrylamide gels (4% stacking gel; 12% separating gel) in Tris-glycine running buffer (Tris/HCl 3 g l$^{-1}$, glycine 72 g l$^{-1}$ and SDS 5 g l$^{-1}$) (Laemmli, 1970). The protein concentration of the enriched OMP extracts was determined by the Quick Start Bradford Protein Assay (Bio-Rad). Protein samples (15 µl) were mixed with sample buffer (Tris/HCl pH 6.8, 10% SDS, 10% v/v glycerol and bromophenol blue, 0.05% w/v), boiled for 5 min and applied to the gel. Electrophoresis was carried out in a vertical slab gel apparatus (20 mA, 100 V). All gels were stained with Coomassie brilliant blue R-250.

Western blotting. Western blotting analyses were performed following SDS-PAGE. The total proteins were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes. After blocking overnight in TBST buffer (10 mM Tris, 150 mM NaCl, 0.1% Tween 20; pH 7.4) supplemented with 5% skimmed milk (Sigma), membranes were incubated with plasma fibronectin (Sigma) (50 µg ml$^{-1}$ in TBST; 1 h), washed with TBST (twice, 20 min each time) and incubated with mouse anti-fibronectin IgG (1:10000) (Sigma) in TBST for 60 min. After washing twice with TBST, membranes were incubated at room temperature for 60 min with anti-mouse polyvalent immunoglobulin peroxidase conjugate (1:5000) (Sigma), membranes were incubated at room temperature for 60 min with anti-mouse polyvalent immunoglobulin peroxidase conjugate (1:5000) (Sigma). The membrane was developed after a final wash.

Peritoneal macrophages. Macrophages were stimulated by intraperitoneal inoculation of 30 mg ml$^{-1}$ thiglycolate in male mice. After 72 h, peritoneal cells were collected aseptically by peritoneal lavage with ice-cold Dulbecco’s modified Eagle’s medium (DMEM). Harvested cells were placed on ice, pelleted by centrifugation at 6000 g for 10 min at 4°C, resuspended in fresh medium, counted, and adjusted to 2.0 x 10$^6$ cells ml$^{-1}$ in fresh medium. To obtain adherent cells, 150 µl of the cell suspension was seeded in 24-well plates. After 1 h of adhesion at 37°C in a 5% CO$_2$ atmosphere, cells were washed to remove non-adherent cells and DMEM (supplemented with 10% heat-inactivated fetal bovine serum, penicillin and streptomycin) was added. On the next day, strains 1405 and 638R and the mutant grown in BHI broth were incubated, or not, with plasma fibronectin (20 µg ml$^{-1}$) for 1 h at 4°C. Three hundred microlitres of the bacterial suspension (10² bacteria ml$^{-1}$) was inoculated into the well. Bacteria–macrophage incubations were performed for 1 h at 37°C in a 5% CO$_2$ atmosphere using a 100:1 m.o.i. After incubation, the wells were washed with PBS and re-incubated with DMEM (supplemented with 10% fetal bovine serum and metronidazole, 200 µg ml$^{-1}$) for 2 h. Then, the macrophages were lysed with 2 µM digitonin (Sigma) for 2 min. The bacteria were recovered, diluted in Blanks buffer (Holdeman et al., 1976), plated onto blood agar and incubated at 37°C for 24 or 48 h for enumeration of c.f.u.

RESULTS

Localization of FnBP in the bacterial surface

Because the TonB-dependent protein is present in the outer membrane, we decided to perform immunostaining for fluorescence microscopy to confirm if this protein was exposed on the surface of the cell. As we observed in Fig. 1(c, d), the localization of the protein was not uniform on the cell surface. Some cells were totally fluorescent while others presented few fluorescent spots. In transmission electron microscopy, strains 1405 and 638R showed weak labelling with gold particle-conjugated antibody (Fig. 1g, h). As positive control, we used a Staphylococcus aureus

![Fig. 1. Binding of fibronectin to cellular surface of B. fragilis by immunostaining fluorescence microscopy and transmission electron microscopy. (a, e) Positive control, S. aureus with fibronectin; (b, f) negative control, B. fragilis and S. aureus, respectively, without fibronectin; (c, g) strain 638R; (d, h) strain 1405. The red arrow indicates the full coverage of the bacterium, and the yellow arrow the marking point. The black arrows indicate weak labelling with gold. Bars: (a–d) 10 µm, (e, f) 0.5 µm, (g, h) 0.1 µm.](image)
strain that possesses several FnBPs (Fig. 1a, e) and as negative control we used the strain without fibronectin (Fig. 1b, f).

**Construction of FnBP mutants**

To determine if expression of the TonB-dependent protein contributes to the binding of *B. fragilis* cells to fibronectin and to other adherence properties, a mutant of strain 638R that expresses FnBP was constructed by insertion inactivation. To verify that the recombination event had occurred in the mutated strain, chromosomal DNA from the wild-type and mutated strains were isolated and used as template in Southern blot analysis. The results demonstrated that the suicide vector pFD516 was inserted into the gene *bf1991* of the mutated 638R strain (data not shown). SDS-PAGE analysis with total protein lysates of the wild-type 638R and mutant strains was used to determine if the modification in the genome interrupted the expression of the 102 kDa TonB-dependent protein. The results showed that, in the electrophoresis profile of the total protein extract of the mutated strain, a protein of about 102 kDa could not be detected (Fig. 2).

**Bacterial binding to fibronectin-coated latex beads**

The particle agglutination assay was used as a preliminary experiment to determine if the absence of protein expression in the mutant strain is associated with a decrease in the capacity to bind to fibronectin. The results from this assay showed that the binding to immobilized fibronectin by the mutant strain was stronger than that by the wild-type strain (Fig. 3). The tests were performed in triplicate.

**Evaluation of change in biofilm production**

Evaluation of biofilm formation was another method used to verify if the absence of the protein under study led to increased adhesion properties in *B. fragilis*. As we can observe in Fig. 4(a, b), the mutant strains were more adherent than the wild-type strain. At all the concentrations tested, the mutant strains showed a significant increase in their ability to form biofilm, proving again the increase in adhesive properties of these bacteria. The tests were performed in triplicate and the results were analysed with Student’s *t*-test. The results that had greater significance (*P*<0.05) are marked with an asterisk.

**Analysis of the protein profile by Western blot**

To understand why the mutants were adhering more to fibronectin, an analysis was carried out by Western blot. Total protein extracts were separated by SDS-PAGE, transferred to a nitrocellulose membrane and allowed to interact with fibronectin. The specificity of the binding assay was demonstrated using anti-fibronectin antibodies. In this experiment, it was observed (Fig. 2) that the strain MBA8, carrying the inactivated *bf1991* gene and consequent loss in the expression of the 102 kDa TonB protein, had a slight increase in the expression of another FnBP of about 30 kDa (arrow) compared with the wild-type strain (asterisk).

**Evaluation of bacterial internalization by peritoneal macrophages**

As shown in Fig. 5, strain MBA8, coated or not with plasma fibronectin, was more efficiently recognized and internalized by macrophages independent of the fibronectin coating. This result also seems to be an indication that the expression of surface proteins may have been changed due to mutation, altering, therefore, their capacity to recognize cellular components.
DISCUSSION

Little is known about the possible involvement of surface molecules, especially OMPs, in the pathogenicity of *B. fragilis*. These surface proteins have an important role in events such as adhesion, invasion and evasion of host defences and antimicrobial agents (Wexler, 2002). Furthermore, OMPs have already been considered as adhesins to other bacterial species (Lantz et al., 1991; Patti et al., 1994; Marques et al., 2001). Hence, we decided to evaluate the involvement of these surface components in the attachment to fibronectin.

The first step was to establish the location of the FnBP on the surface of *B. fragilis*. By fluorescence and transmission electron microscopy, it was confirmed that the proteins were located on the bacterial surface in a stochastic form, without a distribution pattern for the species or differences within the same strain. For some bacteria the whole surface was stained and for others only punctate labelling was found. The fact that the detection of this protein was not uniform on the cell surface may be an indication that the FnBP is not constantly expressed, but is induced, for example, by an environmental trigger, such as the presence of oxygen. In fact, oxidative stress was shown to affect up to 45% of the genomic expression in *B. fragilis* (Sund et al., 2008). In addition to that, differences in the sequence and conformation of the protein between the cellular and plasma fibronectins are known to occur due to alternative splicing (Henderson et al., 2011), which may lead to variation in the capacity of bacteria to adhere to these two molecules. Differences in adherence to plasma fibronectin may also occur depending on the form in which it is found, soluble or immobilized, as seen with *Yersinia enterocolitica*, where the protein YadA can adhere to the immobilized form but not to the soluble form due to a conformational change that exposes the bacterial binding epitopes when fibronectin is immobilized (Schulze-Koops et al., 1993; Tertti et al., 1992). Moreover, the bacteria could be expressing simultaneously more than one FnBP, which would not be discriminated by the methodology used.

Several studies have revealed that, after inactivation of the proteins responsible for the adhesion, there is a decrease in the adherence of bacteria to ECM components. Miller-Torbert et al. (2008) demonstrated that when the protein SmFnB, expressed by *Streptococcus mutans*, is inactivated, there is a 30% reduction in adhesion to fibronectin. Similar results also occur when the FBPA protein in *Streptococcus gordonii* is inactivated (Christie et al., 2002). However, contrary to expectations, on testing adhesion to fibronectin in *B. fragilis* insertional mutants, it was found that a *bf1991* mutation enhanced its adherence. These results demonstrate that the TonB-dependent OMP is not the major FnBP of *B. fragilis* and that, when the gene responsible for its transcription is inactivated, other mechanisms of adherence are in place. To prove this hypothesis, we performed an analysis of the protein profile of wild-type and mutant strains by Western blot. In these assays, increased expression of another FnBP (30 kDa) was found. This result explains the fact that the mutant strains show a greater adherence to fibronectin than does the wild-type strain. However, further analysis must be performed to identify these proteins and understand their connection with the TonB-dependent OMP.
Phagocytosis is an essential step in protecting the host against pathogenic micro-organisms and to removing dead cells from the circulation (Aderem & Underhill, 1999). When \textit{B. fragilis} reaches the bloodstream, it encounters an environment with an abundance of plasma fibronectin, which some studies have reported to increase the recognition and phagocytosis by macrophages (Rodrigues et al., 2003; Romberger, 1997). This result was confirmed in our experiments, where the strains coated with fibronectin were phagocytosed more efficiently. Similarly to our previous results, the mutant strain showed enhanced adhesion properties compared with the wild-type strain 638R, and was more recognized and phagocytosed both with and without fibronectin.

It is known that microbial adhesion to surfaces is the first stage in the formation of biofilms, which allow the bacteria to survive in harsh environments and resist the effect of antimicrobials (Pumbwe et al., 2008). Studies have shown that FnBPs seem to be important mediators of biofilm formation. In \textit{S. aureus}, for example, mutations in the loci responsible for encoding these proteins led to dramatic decrease in the ability to form this structure (Vergara-Irigaray et al., 2009). In the present study, however, it was noted that the inactivation of an FnBP led to a greater capacity for biofilm formation, possibly due to increased expression of another FnBP after the mutation.

The influence of adhesion as a virulence factor for \textit{B. fragilis} still remains an open question. Our results clearly show that the TonB protein coded by the \textit{bf1991} gene binds to fibronectin on the surface of \textit{B. fragilis}. It seems evident however that, for some strains, certain proteins have their expression altered in response to this OMP mutation. Additional studies will be necessary for us to better evaluate the role of these new adhesive properties of the TonB mutant strains and understand the factors that led to these modifications.

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