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The Bfp60 surface adhesin is an extracellular matrix and plasminogen protein interacting in *Bacteroides fragilis*

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ABSTRACT

Plasminogen (Plg) is a highly abundant protein found in the plasma component of blood and is necessary for the degradation of fibrin, collagen, and other structural components of tissues. This fibrinolytic system is utilized by several pathogenic species of bacteria to manipulate the host plasminogen system and facilitate invasion of tissues during infection by modifying the activation of this process through the binding of Plg at their surface. *Bacteroides fragilis* is the most commonly isolated Gram-negative obligate anaerobe from human clinical infections, such as intra-abdominal abscesses and anaerobic bacteraemia. The ability of *B. fragilis* to convert plasminogen (Plg) into plasmin has been associated with an outer membrane protein named Bfp60. In this study, we characterized the function of Bfp60 protein in *B. fragilis* 638R by constructing the bfp60 defective strain and comparing its with that of the wild type regarding binding to laminin-1 (LMN-1) and activation of Plg into plasmin. Although the results showed in this study indicate that Bfp60 surface protein of *B. fragilis* is important for the recognition of LMN-1 and Plg activation, a significant slow activation of Plg into plasmin was observed in the mutant strain. For that reason, the possibility of another unidentified mechanism activating Plg is also present in *B. fragilis* cannot be discarded. The results demonstrate that Bfp60 protein is responsible for the recognition of laminin and Plg-plasmin activation. Although the importance of this protein is still unclear in the pathogenicity of the species, it is accepted that since other pathogenic bacteria use this mechanism to disseminate through the extracellular matrix during the infection, it should also contribute to the virulence of *B. fragilis*.

Introduction

Plasminogen (Plg), an abundant glycoprotein found in the blood plasma, is a central component of the fibrinolytic system. It is converted to plasmin, a broad-spectrum serine protease, by the action of urokinase (uPA) or the tissue-type Plg activator (tPA). Since plasmin can degrade structural proteins and activate other proteolytic enzymes, it is highly important for the degradation of the tissue barriers and cell migration. The recruitment and activation of Plg must be well controlled. However, several pathogens can interfere with the host Plg-plasmin system by expressing Plg receptors or activators (Lähteenmäki et al., 1998, 2001a, 2005; Stie et al., 2009). The surface molecules bind to Plg and enhance its activation by tPA on the bacterial surface, providing proteolytic activity to a nonproteolytic bacterium with the help of a host-derived proteolytic system. Since metastatic tumor cells utilize Plg activation to penetrate the basal membrane (BM), the term bacterial metastasis was coined to describe the analogous mechanism that bacteria employ to cross this barrier (Lähteenmäki et al., 2005). Furthermore, Plg activators efficiently enhance bacterial adherence to human extracellular matrix (ECM) and mouse BM (Lähteenmäki et al., 1998). Several human invasive pathogenic bacteria, such as, *Neisseria meningitidis* (Ullberg et al., 1992), *Salmonella typhimurium* (Lähteenmäki et al., 1995), *Yersinia pestis* (Kukkonen et al., 2001), *Staphylococcus aureus* (Mökänen et al., 2002) and *Helicobacter pylori* (Jönsson et al., 2004) are examples of human pathogens that interact with the host Plg system to enhance their pathogenicity.

The phylum *Bacteroidetes* is a predominant component of the gastrointestinal tract microbiota and by far the most-well studied is the *Bacteroides fragilis* species which have shown to prime T cells responses in animals models via the capsular polysaccharide PSA.

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Table 1
Strains and plasmids used in this study.

Strain or plasmid	Phenotype and/or genotype[a]	Reference or source
B. fragilis strains		
638R	Clinical isolate Rif^r	Privitera et al. (1979)
Bf6m	638R Δ*bfp60*R:: pFD516 *erm*^{r}	This study
Bfp6c	638R *bfp60* (Con) – constitutively activating the outer membrane protein Bfp60	This study
E. coli DH10B	Cloning host strain	Invitrogen
Plasmids		
pGem T easy	Cloning vector, (Amp^r)	Promega
pFD340	*Bacteroides* – *E. coli* expression shuttle vector, (Sp^r) Erm^r	Smith et al. (1992)
pFD540	Suicide vector, derivated from deletion of pBI143 in pFD288 (Sp^R) Erm^r	Smith et al. (1995)
ATCC 968 *S. aureus*	Positive for the plasminogen activation	Ferreira et al. (2009)

[a] Erm^r, Erythromycin resistance; Rif^r, Rifamycin resistance; Sp^r, spectinomycin resistance; Amp^r, ampicillin resistance. Parentheses indicate antibiotic resistance expression in *E. coli*.

In human stool samples this taxon represents a level of at least 0.1% in 16% of samples (over 1% abundance in 3%) (Hunttenhower et al., 2013; Patrick et al., 2011) and is the anaerobic pathogen most frequently isolated from endogenous infections (Finegold and Wexler, 1996), especially from patients with intra-abdominal infections (Gibson et al., 1998) and bacteraemia (Brook and Frasier, 2000). Several virulence factors have been described for this microorganism, such as, proteases (Patrick et al., 1996), enterotoxin (Wu et al., 1998) and lipopolysaccharide (Pumbwe et al., 2006), but their roles in pathogenicity are still not well elucidated. Without a doubt, the most studied virulence factor in the species is the capsular polysaccharide complex (CPC), which can modulate its antigenicity by expressing at least eight distinct polysaccharides (Gibson et al., 1998; Krinos et al., 2001).

The ability of *B. fragilis* to strongly adhere to laminin-1 (LMN-1) (Ferreira et al., 2006) and to convert Plg into plasmin (Ferreira et al., 2009) has been associated with an outer membrane protein (OMP) of approximately 60 kDa, Bfp60. Bfp60 was previously identified by Sijbrandi and colleagues (2005, 2008) as cell-surface Plg binding protein. However, its involvement in the pathogenicity of *B. fragilis* remains unclear. The purpose of this study was, therefore, to characterize the function of Bfp60 protein as a cell surface in *B. fragilis* 638R by constructing the bfp60 defective strain and comparing the properties of the wild type and mutant strains to bind LMN-1 and activate Plg into plasmin.

Materials and methods

Bacterial strains and growth conditions

B. fragilis strains used in this study are listed in Table 1. Strains were routinely grown anaerobically in brain heart infusion broth supplemented (BHIS) with hemin (5 mg/mL) and L-cystein 0.5 g/L) (Jousiemies-Somier et al., 2002). *Escherichia coli* strains were cultured in Luria-Bertani Broth (LB) or agar. Rifampicin (20 μg/mL), gentamicin (100 μg/mL) and erythromycin (10 μg/mL) were added to the media when required.

Construction of bfp60 insertional mutant and constitutive expressing strains

All DNA modifications and manipulations were carried out according to standard protocols (Sambrook et al., 1989). To construct of a *bfp*60 insertion mutant a DNA fragment of 600 bp, corresponding to an internal fragment of the *bfp*60 gene of *B. fragilis*, was used. The following two oligonucleotide primers were designed based on the whole nucleotide sequence of the *bfp*60 gene (1700 bp) available in the GenBank (EMBL/GenBack nucleotide database accessing number: AJ786264.1; Sijbrandi et al., 2005). The sense and antisense oligonucleotide sequences are as follows: Bfp60F 5′ CTTTACTTATGGCATTGG 3′ (forward) and Bfp60R 5′ GTTGTCCGTTGTAGGC 3′ (reverse). The DNA fragment was amplified by PCR using Taq polymerase (Invitrogen) in 50 μL containing 1× Taq polymerase buffer, 1 mM $MgCl_2$, 0.3 mM dNTP, 0.5 pmol of each primer and 2 μL of DNA (20 μg/μL). The following cycle program was used: 95 °C for 1 min, 35 cycles at 94 °C for 30 s, 40 °C for 30 s and 72 °C for 30 s; and a final extension at 72 °C for 10 min. The amplified fragment was cloned into the pGEM-T easy vector (Promega) according to manufacturer's instructions. The plasmid was sequenced to confirm the cloned DNA fragment. The *bfp60* fragment was excised from the pGEM-T easy vector with *Sph*I and *Pst*I enzymes and ligated into the suicide vector pFD516, previously digested with the same enzymes. The new construct was transformed by electroporation into *E. coli* DH10B. The suicide vector containing the 600 bp *bfp*60 fragment was mobilized into *B. fragilis* by the triparental filter mating protocol (Shoemaker et al., 1986). The transconjugants with insertional mutations were selected on BHIS agar plates containing 20 μg/mL rifampicin, 100 μg/mL gentamicin and 10 μg/mL erythromycin. The *B. fragilis bfp60*::pFD516 construct, Bfp6 m, was confirmed by PCR using the following oligonucleotides: Bfp60Fmut 5′CCAAAGCGATCCAGGAAATA3′ and Bfp60mut TGCACCTGTCATAGCCTCAG 3′.

A plasmid constitutively expressing *bfp60* was constructed by PCR amplification of a 1692 bp promoterless *bfp*60 gene containing 53 bp upstream of the ATG start codon using the following primers Bfp60XmaI 5′ATGCCCGGGGAATAGACAAAATTTCTC 3′and Bfp60SacI 5′GAGCTCTCGCTTATTTTACGATGC3′ containing recognition sites for *Xma*I and *Sac*I enzymes, respectively. The promoterless *bfp*60 gene fragment was cloned into the expression vector pFD340 in the same orientation as the IS4351 promoter. The new construct pFD340*bfp60* was mobilized in the *B. fragilis* 638R strain as previously described. Transconjugants were selected on BHIS containing 20 μg/mL rifampicin, 100 μg/mL gentamicin and 10 μg/mL erythromycin and the resulting strain was designated Bf6c.

Adhesion and inhibition assays

Laminin from Engelbreth-Holm Swarm tumor (LMN-1; Sigma) or human plasminogen (Plg; Sigma) were used throughout this study. LMN-1 and Plg were immobilized onto glass coverslips as described previously (Ferreira et al., 2009) and placed into 24-well culture plates. Briefly, to prepare the plates, 15 μg/mL or 10 μg/mL of Plg or LMN-1 were suspended in 0.01 M PBS and immobilized onto glass coverslips for 1 h at room temperature. Immediately, LMN-1 or Plg-coated coverslips were carefully washed with PBS containing 0.1% bovine serum albumin (BSA) (w/v) to remove unbound LMN-1, avoiding non-specific association, and blocked with PBS containing 2% BSA (PBSB) for 1 h at room temperature. Coverslips incubated with 2% BSA alone were used as the control.

For the adhesion assay, 200 μL of a suspension of cells of *B. fragilis* 638R wild type, *bfp60* mutant or constitutively expressing bfp60 strains (10^9 CFU/mL in 0.1% PBSB), were placed into 24-well culture plates containing the coverslips covered with either LMN-1 or Plg.

For the inhibition assay, *B. fragilis* 638R was mixed with 10 μg/mL Bfp60r protein (Ferreira et al., 2009) or with 1:100 dilution of anti-Bfp60 polyclonal antibody 0.1 M PBSB for 1 h at room temperature before adding the suspension to respective coverslips placed in the microplate wells. After the 1 h incubation at room temperature, the coverslips were washed twice with 0.1 M PBS, and allowed to interact with the coverslips covered with LMN-1, for 1 h

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