Plasminogen-dependent proteolytic activity in *Bifidobacterium lactis*

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Bifidobacteria represent one of the most important health-promoting bacterial groups of the intestinal microbiota. The binding of plasminogen to species of *Bifidobacterium* has been recently reported. To further explore the interaction between bifidobacteria and plasminogen, we investigated the role of *Bifidobacterium lactis* BI07 plasminogen-dependent proteolytic activity in the degradation of host-specific substrates. Our experimental data demonstrate that the recruitment of plasminogen on the bacterial cell surface and its subsequent conversion into plasmin by host-derived plasminogen activators provide *B. lactis* BI07 with a surface-associated plasmin activity effective in degradation of physiological substrates such as extracellular matrix, fibronectin and fibrinogen. The ability of bifidobacteria to intervene in the host plasminogen/plasmin system may contribute to facilitating colonization of the host gastrointestinal tract.

INTRODUCTION

Bifidobacteria are autochthonous members of the human intestinal microbiota. Even if they represent only a minor component of the human intestinal microbial ecosystem (Palmer et al., 2007), their presence in the human gastrointestinal tract (GIT) has been commonly associated with the concept of a healthy microbiota (Schell et al., 2002; Servin, 2004; Ventura et al., 2004; Klijn et al., 2005). Several health-promoting activities have been directly related to the presence of bifidobacteria in the GIT, such as maintenance of normal microflora, immunostimulation and immunomodulation, improvement of lactose utilization and reduction of serum cholesterol levels (Salminen et al., 1996; Guarner & Malagelada, 2003). Due to these beneficial effects, some *Bifidobacterium* species have become common components in many dairy and pharmaceutical products. However, knowledge of the mechanisms involved in the health-promoting activities of bifidobacteria and in the interaction of these commensals with the host is very limited.

Recently, the interaction between *Bifidobacterium* and the human plasminogen (Plg) system has been reported (Candela et al., 2007). Four strains belonging to the bifidobacterial species *B. lactis*, *B. bifidum* and *B. longum* showed a dose-dependent binding activity to human Plg, and for the model strain *B. lactis* BI07, five putative Plg-binding proteins were identified in the cell wall fraction.

The proteolytic Plg/plasmin system plays a pivotal role in mammalian physiology. Plg is a single-chain glycoprotein with a molecular mass of 92 kDa and comprises an N-terminal pre-activation peptide (~8 kDa), five consecutive disulfide-bonded triple-loop kringle domains (K1–5), and a serine-protease domain containing the catalytic triad (Vassalli et al., 1991). It is produced mainly by hepatocytes; however, other tissue sources for Plg synthesis have been identified and include the intestine (Zhang et al., 2002). Plg circulates at a concentration of 180–200 µg ml⁻¹ (~2 µM) in plasma, but it is also present in several interstitial fluids (Myohanen & Vaheri, 2004). The conversion of the single-chained zymogen to its active form, plasmin, is mediated by proteolytic activation via mammalian Plg activators (PAs), tissue-type Plg activator (tPA) and urokinase (uPA) (Castellino & Powell, 1981). Plasmin is a trypsin-like serine protease with a broad substrate specificity. It is involved in fibrinolysis (Collen & Verstraete, 1975), homeostasis, and degradation of the extracellular matrix (ECM) and basement membrane (Saksela & Rifkin, 1988).
The human Plg/plasmin system is employed by numerous microbial pathogens for migration across host tissue barriers in a process called bacterial metastasis (Bergmann & Hammerschmidt 2007; Bergmann et al., 2001; Lahteenmaki et al., 2005; Pancholi et al., 2003; Parkkinen & Korhonen, 1989; Schaumburg et al., 2004; Sibbrendi et al., 2005; Sun, 2006; Sun et al., 2004). In particular, within the gastrointestinal niche, enteropathogenic bacteria such as Salmonella enterica, Listeria monocytogenes, Helicobacter pylori, Escherichia coli and Bacteroides fragilis also studied. According to our results, in the presence of fibrin, B. lactis BI07 cells were able to transmigrate through a matrix of fibrin. This surface-fibrinogen (Fn) and fibrinogen (Fg), as well as the ability to transmigration of BI07 through a fibrin matrix was independent of the lysine-binding sites in Plg recruitment on the bacterial cell surface, that allow the recruitment of the host Plg. Whereas Yersinia pestis possesses surface proteases that specifically act as endogenous PAs, for the majority of enteric bacteria Plg activation depends on the presence of host PAs (Lahteenmaki et al., 1995). Independently of the strategy of activation, by recruiting human Plg on their cell surface and subsequently converting it to plasmin, microorganisms acquire a host-derived surface-associated proteolytic activity that triggers damage of ECMs, as well as the spread of bacteria and organ invasion during the host infection (Lahteenmaki et al., 2005).

In order to further explore the interaction between bifidobacteria and Plg, we investigated here the role of the B. lactis BI07 Plg-dependent proteolytic activity in the degradation of specific substrates. The plasmin-mediated transmigration of B. lactis BI07 through a fibrin matrix was also studied. According to our results, in the presence of Plg and PAs, B. lactis BI07 acquires the capability to degrade ECM and plasmin-specific substrates such as fibrinectin (Fn) and fibrinogen (Fg), as well as the ability to transmigrate through a matrix of fibrin. This surface-bound Plg-derived plasmin activity may have a role in bifidobacterial colonization of the host GIT.

METHODS

Bacterial strains, media and growth conditions. B. lactis BI07 (Candela et al., 2007) was cultured in MRS medium (Difco) with added l-cysteine 0.05 % (w/v) at 37 °C in anaerobiosis. The anaerobic condition was obtained in jars by using Anaerocult A (Merck). Bifidobacterial cells were grown for 18 h until they reached the stationary phase.

Plasmin activity assay. B. lactis BI07 cells in the stationary phase were washed in phosphate-buffered saline (PBS) at pH 7.4, adjusted to 1 × 10^8 c.f.u. ml^-1 and incubated for 30 min at 37 °C with Plg (Sigma-Aldrich), 20 µg ml^-1 in PBS. Bacteria were washed twice with PBS to remove unbound Plg and resuspended in 50 mM Tris/HCl, pH 7.5 (TBS). A volume of 100 µl of the bacterial cell suspension was added per well of a 96-well microtitre plate. Plg was activated with 0.24 KIU tPA (Calbiochem) or 0.06 KIU uPA (Calbiochem), and 30 µl plasmin-specific chromogenic substrate solution, containing 0.34 mM D-valyl-leucyl-lysine-p-nitroanilide dihydrochloride (S-2251, Sigma-Aldrich), was added (Bergmann et al., 2005). Absorbance at 405 nm was measured immediately after the addition of S-2251 (time point t0) and after 1.5 h of incubation at 37 °C (time point t1) with a Multiskan Ascent V1.24 (Thermo Electron Corporation). The plasmin activity was evaluated by calculating ∆A405 = A405(t1) - A405(t0). Bacterial cells not incubated with Plg were used as a negative control. Controls for spontaneous hydrolysis of S-2251 were carried out with the chromogenic substrate alone and in the presence of PAs. In order to distinguish between bacterial surface-bound plasmin activity and the activity of plasmin released into the supernatant, B. lactis BI07 cells preincubated with Plg were incubated with tPA or uPA. Thereafter, plasmin activity of both bacterial pellet and supernatant was measured as reported above. Finally, to prove the role of the lysine-binding sites in Plg recruitment on the bacterial cell surface, B. lactis BI07 cells were incubated with Plg in the presence of 0.1 M ε-aminoacapric acid (EACA) (Sigma-Aldrich) and the plasmin activity was evaluated.

Preparation of 35S-radiolabelled NCI-H292 ECM. The epithelial cell line NCI-H292 (ATCC CRL-1848), derived from a human lung mucoeoeipidermoid carcinoma, was grown to confluence in RPMI 1640 medium (PAA Laboratories) supplemented with 2 mM l-glutamine and 10 % fetal calf serum at 37 °C under a 5 % CO2 atmosphere. Then 5 × 10^5 cells per well were seeded in a 24-well tissue culture plate (Greiner) and incubated for 3 days at 37 °C under a 5 % CO2 atmosphere. Each well was washed three times with PBS and 1 ml per well methionine-free cell culture medium (DMEM containing 10 % FCS, 2 mM glutamine and 10 % RPMI 1640 medium; Neustadt) was added. After 1 h incubation the medium was replaced with fresh cell culture medium containing 30 µCi (1.1 MBq) per well of [35S]methionine (GE Healthcare) and incubated at 37 °C for 18 h.

Preparation of the NCI-H292 radiolabelled ECM was performed as described by Hedman et al. (1979). Briefly, cells were washed three times with PBS followed by a 30 min incubation at room temperature with 10 mM Tris/HCl (pH 8.0) containing 0.5 % sodium deoxycholate. The cell debris was removed and the remaining ECM was incubated for 5 min with 10 mM Tris/HCl (pH 8.0) containing 10 U DNase I ml^-1. Finally, the ECM was washed three times with PBS, pH 7.4. The absence of epithelial cells and cell debris was confirmed by microscopy.

Degradation of 35S-ECM. B. lactis BI07 cells (1 × 10^9 c.f.u.) were resuspended in 100 µl PBS containing 1 % fetal calf serum and incubated with 20 µg human Plg (Sigma-Aldrich) for 30 min at 37 °C. Degradation of radiolabelled ECM was performed as described by Lahteenmaki et al. (1995). Briefly, 1 × 10^5 c.f.u. of B. lactis BI07, pretreated or untreated with Plg, were washed twice in PBS, suspended in 1 ml PBS, and added to a well containing the prepared radiolabelled ECM. Degradation was carried out in the absence or in the presence of 0.24 KIU tPA or 0.06 KIU uPA at 37 °C for up to 4.5 h. Control experiments were carried out in the presence of 500 KIU aprotinin. Further controls included wells with 2 µg Plg in PBS and no bacterial cells in either the absence or the presence of 0.24 KIU tPA or 0.06 KIU uPA. Degradation was quantified by measuring the released radioactivity. Subsamples of 40 µl were taken from the supernatant at different time intervals for up to 4.5 h and transferred into scintillation tubes (4 ml Pico Pro Vial; Packard Instrument) containing 2 ml scintillation liquid (Optiphase Hisafe). Radioactivity was measured in a Packard 1600TR liquid scintillation counter. At each time point the Acp.m with respect to the time point 0 was calculated. The degradation assays were performed three times in duplicate wells.

Degradation of Fn and Fg. B. lactis BI07 cells (1 × 10^9 c.f.u.) were resuspended in 100 µl PBS containing 1 % fetal calf serum and incubated with 20 µg human Plg (Sigma-Aldrich) for 30 min at 37 °C. The bacterial cells were then washed, suspended in PBS-EDTA, and 1 × 10^8 c.f.u. of the Plg-pretreated B. lactis BI07 cells were incubated at 37 °C with 4 µg human plasma Fn (ICN Immunobiologics) or 4 µg human Fg (Calbiochem) and 0.06 KIU uPA. Bacterial cells were then sedimented at different time points and the reactions were stopped with SDS-containing sample buffer. After a 5 min boiling, supernatants were collected, resolved by SDS-PAGE, and proteins were transferred to PVDF membranes (Immobilon-P,
Millipore). After blocking in 10% fat-free milk in PBS, membranes were incubated with rabbit anti-human Fn antibody or goat anti-human Fg antibody (Dako, Cytomatin) for the detection of Fn or Fg, respectively. After three washing steps in PBS, membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (Eurogentech) and HRP-conjugated anti-goat antibody (Sigma), respectively. The membranes were washed again three times in PBS, then were incubated with a detection solution (1 mg 4-chloro-1-naphthol ml⁻¹ and 0.1% H₂O₂ in PBS) until it was possible to detect the bands. Control experiments were carried out with Fn and Fg alone and in the presence of Plg and uPA.

**Transmigration through a fibrin matrix.** A fibrin matrix was produced on membranes of transwell cell culture inserts (polycarbonate membranes with 6.5 mm diameter and 3 μm pore size; Costar) by incubating 1 mg Plg-depleted human Fg (Calbiochem) with 25 U thrombin from bovine plasma (MP Biomedicals) for 14 h at 37 °C in 100 μl PBS. *B. lactis* BI07 cells (1 × 10⁹ c.f.u.) were resuspended in 100 μl PBS containing 1% fetal calf serum and incubated with 20 μg human Plg (Sigma-Aldrich) for 30 min at 37 °C. After washing in PBS-EDTA, Plg-pretreated bacteria were applied to the fibrin matrix at concentration of 2 × 10⁸ per 100 μl PBS-EDTA and, simultaneously, Plg was activated by adding 0.06 KIU uPA. Aprotinin (500 KIU) was used as a serine protease inhibitor in control experiments. Further control experiments were carried out with bacteria untreated with Plg and with Plg-pretreated bacterial cells in the absence of uPA. Bacterial transmigration from the upper to the lower chamber was quantified by plating serial dilutions of the lower chamber solution on MRS agar. Experiments were carried out for up to 7 h and samples were plated at timed intervals (0.5, 1.5, 2, 3, 5 and 7 h). After each time point the transwell inserts were replaced into a new well containing PBS-EDTA buffer.

**RESULTS**

**Plasmin activity of *B. lactis*-bound plasminogen**

In order to investigate the conversion of *B. lactis*-bound Plg to the proteolytically active form plasmin, *B. lactis* BI07 cells were incubated with Plg and the surface-bound Plg-derived plasmin activity was determined in a plasmin activity assay (Bergmann et al., 2005). Plg bound to the *B. lactis* BI07 outer surface was activated to plasmin by uPA or tPA, as measured by the hydrolysis of the plasmin-specific chromogenic substrate S-2251 (Fig. 1a). In the absence of PAs no hydrolysis was measured, demonstrating that *B. lactis* BI07 does not produce any endogenous PA (Fig. 1a). No plasmin activity was measured in the supernatant of the bacterial cultures (Fig. 1b). Hence, it can be concluded that Plg remained bound to the bacterial cell surface after its activation to plasmin. The lysine analogue EACA inhibited the plasmin formation on *B. lactis* BI07 cell surface, indicating the essential role of the lysine-binding sites of the Plg molecule in binding to the bacterial cell surface (Fig. 1a).

**Plasmin-dependent degradation of ECM by *B. lactis* BI07**

To investigate the effect of the *B. lactis* BI07 surface-bound Plg-derived plasmin activity on degradation of ECM material, *B. lactis* BI07 cells were incubated with Plg and the plasmin-mediated degradation of radiolabelled ³⁵S-ECM was evaluated in kinetic experiments for up to 4.5 h. Degradation was determined by measuring the radioactivity released into the medium at each time point. In the presence of uPA or tPA, Plg-pretreated *B. lactis* BI07 cells prompted a time-dependent degradation of ECM (Fig. 2). In accordance with the data measured in our plasmin activity assay, no ECM degradation was detected in the absence of PAs. In addition, aprotinin, a plasmin inhibitor, was effective in inhibiting the degradation of radiolabelled ³⁵S-ECM by Plg-pretreated *B. lactis* BI07, confirming the role of bacteria-bound plasmin in degradation of ECM by the bacteria (Fig. 2). In control experiments, *B. lactis* BI07 cells without Plg pretreatment did not exhibit any time-dependent degradation of ECM (Fig. 2), demonstrating that *B. lactis* BI07 does not possess any endogenous proteolytic activity effective in ECM digestion.

**Plasmin-dependent degradation of Fn and Fg by *B. lactis* BI07**

Human Fn and Fg represent common molecular targets of plasmin. Fn is a multifunctional glycoprotein found in ECM and body fluids, while Fg is a key protein in blood coagulation. The ability of *B. lactis* BI07 cells to degrade Fn and Fg after recruitment of Plg to the bacterial surface was studied. In the presence of uPA, Plg-pretreated *B. lactis*
BI07 cells induced plasmin-dependent degradation of Fn (Fig. 3a). According to our data, the degradation of Fn into smaller fragments by bacterial surface-bound plasmin was time-dependent. No Fn degradation was observed without Plg pretreatment of B. lactis BI07, indicating that the cell-bound plasmin caused Fn proteolysis. In a further control, degradation of Fn was monitored in kinetic experiments carried out without bacterial cells (Fig. 3a). In an analogous experiment, we showed the plasmin-dependent degradation of Fg by Plg-pretreated B. lactis BI07 cells in the presence of uPA (Fig. 3b). The significance of the cell-bound plasmin in Fg proteolysis was indicated by experiments performed without Plg pretreatment of bacterial cells. Degradation of Fg was further monitored without bacteria but in the presence of activated Plg (Fig. 3b).

**Plasmin-dependent transmigration of B. lactis BI07 through a fibrin matrix**

To investigate the plasmin-mediated transmigration of B. lactis BI07 through a matrix, a fibrin matrix was generated on membranes of transwell cell culture inserts. Plg-pretreated B. lactis BI07 cells showed transmigration through the fibrin matrix in the presence of uPA. The number of transmigrated B. lactis BI07 cells increased exponentially for up to 7 h (Fig. 4). No transmigration of bacteria was detected when the experiments were performed in the absence of uPA. No appreciable bifidobacterial transmigration was measured without Plg pretreatment, confirming the crucial role of cell-bound plasmin in bifidobacterial transmigration through the fibrin matrix.

**DISCUSSION**

In this study plasmin activity assays indicated that the Plg bound to the outer surface of B. lactis BI07 is activated to plasmin by tPA and uPA, allowing the bacteria to acquire a surface-associated plasmin activity. According to our data,
B. lactis BI07 does not possess an endogenous plasminogen activator system. The inhibition of plasmin formation on the B. lactis BI07 cell surface in the presence of the lysine analogue EACA underscores the role of the lysine-binding sites of Plg kringle domains for Plg recruitment to the bacterial cell surface. In order to evaluate the significance of the B. lactis BI07 surface-bound Plg-derived plasmin activity in the degradation of a physiological substrate, the time-dependent degradation of ECM material was assessed. Plg-pretreated B. lactis BI07 cells showed a time-dependent ECM degradation activity when PAs were added. Bacteria untreated with Plg did not show any endogenous proteolytic activity effective in ECM degradation, proving the involvement of cell surface-bound plasmin. The complete inhibition of ECM proteolysis by the plasmin inhibitor aprotinin confirmed the importance of bifidobacterial surface-bound plasmin in ECM degradation. Specifically, Plg-pretreated bacterial cells acquired the capability to degrade plasmin-specific substrates such as Fn and Fg when uPA was added. Finally, Plg-coated B. lactis BI07 cells have been shown to transmigrate through a fibrin matrix in the presence of uPA. As expected, Plg-untreated cells did not exhibit any transmigration capacity.

Taken together our experimental data demonstrate that the immobilization of Plg on the B. lactis BI07 cell surface, and its conversion to plasmin by host PAs, endows the bacteria with a host-derived surface-associated proteolytic activity that the bacteria did not evolve on their own. This mode of interaction with the host Plg/plasmin system resembles that reported for several enteropathogens (Bergmann & Hammerschmidt, 2007; Lahteenmaki et al., 2005). In pathogens, the interaction with the Plg system triggers damage of ECMs, as well as the spread of bacteria and organ invasion during the host infection (Lahteenmaki et al., 2005). However, the commensal nature of bifidobacteria is widely accepted, and there is a remarkable amount of evidence that supports the overall safety of Bifidobacterium when employed in foods as well as in pharmaceutical probiotic products (Boyle et al., 2006; Reid, 2006). Moreover, we are not aware of any report in the literature of Bifidobacterium sepsis or endocarditis related to its use as probiotics. Thus, enteropathogens must possess other actors, in addition to the presence of Plg receptors on the cell surface, to take advantage of the host Plg/plasmin system for organ and tissue invasion. The mere capability to intervene in the host Plg/plasmin system via Plg recruitment on the bacterial cell surface could represent a molecular mechanism for host colonization shared by pathogens and commensal bacteria. Supporting our findings, Lactobacillus crispatus, another member of the human intestinal microbiota, has recently been shown to interact with the host Plg/plasmin system (Antikainen et al., 2007; Hurmalainen et al., 2007).

Within the GIT, epithelial surfaces are covered by a layer of mucus which prevents most micro-organisms reaching and persisting on the mucosal surface (Macfarlane et al., 2005). For a member of the human intestinal microbiota, such as Bifidobacterium, the capability to colonize and digest the intestinal mucus is central for the colonization establishment of the host (Deplancke & Gaskins, 2001; Macfarlane et al., 2005; Leitch et al., 2007). Mucus can serve as initial binding site for GIT colonization, and it represents a readily available source of energy on which bacteria can proliferate. The acquisition of a surface-bound plasminogen-derived plasmin activity in the intestinal mucosa may enhance the capability of Bifidobacterium to degrade the intestinal mucus. In fact, bifidobacteria can employ the concerted action of its arsenal of glycosidases (Klijn et al., 2005) and the acquired protease activity to disassemble the mucin polymeric network. Besides representing a source of nutrients, the digestion of the mucus coat overlying the intestinal epithelium allows bifidobacteria to gain access to the epithelial surface (Deplancke & Gaskins, 2001). The establishment of an intimate contact with the host enterocytes is an essential step for all the Bifidobacterium health-promoting activities which depend on bacteria–host molecular cross-talk, such as modulation of the intestinal immune system, maintenance of intestinal barrier integrity, and increase in mucin secretion (Ismail & Hooper, 2005; Otte & Podolsky, 2004).

Even if the intervention in the host Plg/plasmin system may represent a novel component in the molecular cross-talk between bifidobacteria and host enterocytes, further studies are necessary for the understanding of the role of this system in bifidobacterial ecology either in a healthy
gastrointestinal microbial ecosystem or in inflammatory bowel diseases.

REFERENCES


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