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DnaK from *Bifidobacterium animalis* subsp. *lactis* is a surface-exposed human plasminogen receptor upregulated in response to bile salts

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Bifidobacterium animalis subsp. *lactis* lives in the gastrointestinal tract of most mammals, including humans. Recently, for the probiotic strain *B. animalis* subsp. *lactis* BI07, a dose-dependent plasminogen-binding activity was demonstrated and five putative plasminogen-binding proteins were identified. Here we investigated the role of surface DnaK as a *B. animalis* subsp. *lactis* BI07 plasminogen receptor. DnaK was visualized on the bacterial cell surface by transmission electron microscopy. The His-tagged recombinant DnaK protein showed a high affinity for human plasminogen, with an equilibrium dissociation constant in the nanomolar range. The capability to tolerate physiological concentrations of bile salts is a crucial feature for an intestinal symbiont micro-organism. By proteome analysis we demonstrated that the long-term exposure of *B. animalis* subsp. *lactis* BI07 to bile salts results in the upregulation of important surface plasminogen receptors such as DnaK and enolase. Moreover, adaptation of *B. animalis* subsp. *lactis* BI07 to physiological concentrations of bile salts significantly increased its capacity to interact with the host plasminogen system. By enhancing the bacterial capacity to interact with the host plasminogen, the gut bile environment may facilitate the colonization of the human host by *B. animalis* subsp. *lactis* BI07.

INTRODUCTION

Human beings are in coevolution with a complex microbial community living in their gastrointestinal tract (GIT), the human intestinal microbiota (Gill *et al.*, 2006; Ley *et al.*, 2008). Active and metabolizing, the intestinal microbiota affects several host physiological features and has recently been considered as an integral component of the human physiology (Neish, 2009; Round & Mazmanian, 2009). Bifidobacteria are obligate anaerobes in the *Actinomycetales* branch of the high-G+C Gram-positive bacteria and can be found in the human GIT as both autochthonous and allochtonous residents (Klijn *et al.*, 2005). As inhabitants of the human gut they are only a minor component of the adult intestinal microbiota, but they largely predominate in

the gastrointestinal ecosystem of breast-fed infants until weaning (Ventura *et al.*, 2009). The presence of bifidobacteria in the human GIT has been correlated with several health-promoting effects (Guarner & Malagelada, 2003); however, there is only fragmentary information about their ecology, physiology and interaction with the host. Originally isolated from fermented milk (Meile *et al.*, 1997), the species *Bifidobacterium animalis* subsp. *lactis* is commonly found in the guts of healthy adults and infants (Turroni *et al.*, 2009). The complete genome sequence of three *B. animalis* subsp. *lactis* strains has been recently determined, and important insights into its genetic basis for the interaction with the host and the adaptation to the human gut environment have been provided (Barrangou *et al.*, 2009). In particular, four genes with a possible role in the interaction with human epithelial cells have been identified, including two putative collagen-adhesion proteins, an elastin-binding protein and a fibronectin-binding protein. Another aspect of the *B. animalis* subsp. *lactis*–

Abbreviations: Anti-Eno, anti-pneumococcal enolase antiserum; BSH, bile salt hydrolase; EACA, ε-aminocaproic acid; GIT, gastrointestinal tract; PA, plasminogen activator(s); Plg, plasminogen; uPA, urokinase plasminogen activator.

host interaction was revealed by the dose-dependent human plasminogen (Plg)-binding activity demonstrated for the BI07 and DSM 10140 strains (Candela *et al.*, 2007).

Plg is the zymogen of plasmin, a trypsin-like serine protease with a broad substrate specificity. 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 lysine-binding 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, including the intestine (Zhang *et al.*, 2002). The active form, plasmin, is involved in fibrinolysis (Collen & Verstraete, 1975), homeostasis and degradation of the extracellular matrix and basement membrane (Saksela & Rifkin, 1988).

The capability to intervene with the Plg/plasmin system is a strategy for host colonization shared by several pathogens and commensals of the human GIT (Parkkinen & Korhonen, 1989; Schaumburg *et al.*, 2004; Lähteenmäki *et al.*, 2005; Sijbrandi *et al.*, 2005; Bergmann & Hammerschmidt, 2007; Hurmalainen *et al.*, 2007; Candela *et al.*, 2008b). The binding of Plg to the bacterial cell surface depends on the presence of surface Plg-binding proteins which can interact with Plg by means of a C-terminal lysine residue(s) or/and lysine-enriched internal Plg-binding motive(s) (Bergmann *et al.*, 2003, 2005; Bergmann & Hammerschmidt, 2007; Candela *et al.*, 2009; Sha *et al.*, 2009). With the recruitment of human Plg on the cell surface, and its subsequent conversion to plasmin, the micro-organism acquires a surface-associated and host-derived proteolytic activity useful for facilitating migration across physical and molecular barriers and for responding to the nutritional demands during the colonization process (Lähteenmäki *et al.*, 2005).

Similarly to the bifidobacterial species of human origin *Bifidobacterium bifidum* and *Bifidobacterium longum*, the probiotic species *B. animalis* subsp. *lactis* showed a dose-dependent binding activity to human Plg (Candela *et al.*, 2007). In the presence of host-derived Plg activators (PA), Plg captured on the *B. animalis* subsp. *lactis* cell surface was converted to plasmin, endowing the bacteria with a Plg-dependent surface-associated plasmin activity effective in the degradation of physiological host substrates (Candela *et al.*, 2008b). The complete inhibition of Plg binding to *B. animalis* subsp. *lactis* BI07 in the presence of the lysine analogue ε-aminocaproic acid (EACA) suggested that Plg binding is strongly dependent on lysine residues of surface Plg receptors. Five putative human Plg receptors were identified in the cell-wall fraction of *B. animalis* subsp. *lactis* strain BI07: DnaK, glutamine synthetase, enolase, bile salt hydrolase (BSH) and phosphoglycerate mutase (Candela *et al.*, 2007). Analogously to other Gram-positive bacteria (Lähteenmäki *et al.*, 2001), the *B. animalis* subsp. *lactis* Plg-binding proteins are highly conserved cytoplas-

mic proteins that, expressed on the bacterial cell wall, acquire a secondary 'moonlighting' function different from the one exerted in the cytoplasm. The glycolytic enzyme enolase of *B. animalis* subsp. *lactis* BI07 was characterized with respect to surface localization and Plg-binding activity. Proving its role as a bifidobacterial Plg receptor, *B. animalis* subsp. *lactis* BI07 enolase showed a high affinity for human Plg, with an equilibrium dissociation constant in the nanomolar range (Candela *et al.*, 2009).

Adaptation and tolerance to physiological concentrations of bile salts (usually below 5 mM) is a vital factor for bacterial colonization of the human gut ecosystem (Ridlon *et al.*, 2006; Sanchez *et al.*, 2007). Bile salts are detergent-like compounds that are secreted into the intestine during digestion. Besides their primary role in the emulsification and absorption of fats, bile salts also exert a strong antimicrobial activity by inducing membrane damage and causing oxidative stress to DNA (Bernstein *et al.*, 1999). The adaptation of *B. animalis* subsp. *lactis* to bile salts involves a global change in its protein expression profile (Sanchez *et al.*, 2007), and the issue of whether these changes could affect the manner of interaction with the host has been addressed. Very recently, with the attempt to specifically investigate if bile adaptation could influence the *Bifidobacterium*–host interaction, Ruiz *et al.* (2009) studied the effect of bile salts on the *B. longum* cell-wall proteome. The authors reported that the surface Plg receptors enolase and glutamine synthetase were upregulated in response to bile salts, suggesting that the adaptation of *B. longum* to the bile environment triggers the production of bacterial factors for intestinal colonization.

Here we assessed the role of DnaK as a surface Plg receptor in *B. animalis* subsp. *lactis* BI07 and also analysed the effect of the adaptation to the bile salts environment on the Plg-binding capacity of this bacterium. Demonstrating the influence of bile adaptation on the *B. animalis* subsp. *lactis*–host interaction process, the bile salts treatment induced the upregulation of surface Plg receptors and significantly increased the ability of the bacteria to interact with the host Plg system.

METHODS

Bacterial strains, media and growth conditions. *B. animalis* subsp. *lactis* BI07 was cultured in MRS medium (Difco) supplemented with 0.05 % (w/v) L-cysteine at 37 °C in anaerobic conditions, obtained by using Anaerocult A (Merck) in a jar. The bifidobacterial cells were grown for 18 h until they reached the stationary phase. Liquid MRS cultures were also performed with bile salts (oxgall; Difco) at the final concentrations of 1.2 and 3 g l^{-1}. Since human bile salts are difficult to retrieve, oxgall was used in this study. Oxgall has higher concentrations of bile salts and phospholipids and lower phospholipid/bile salts molar ratios than human bile (Coleman *et al.*, 1979), whereas the ratio of glycine- to taurine-conjugated bile acids is similar (Hafkenscheid & Hectors, 1975). *Escherichia coli* OneShot TOP10, BL21Star(DE3) and XL10-Gold strains were cultured at 37 °C in Luria–Bertani (LB) medium with shaking. MagicMedia (Invitrogen) was used for recombinant protein expression.

Kanamycin (50 µg ml^{-1}) was added as selective agent when appropriate.

Extraction of bifidobacterial cytoplasmic and cell-wall fractions. Cytoplasmic proteins were extracted as reported by Candela *et al.* (2009). Briefly, stationary-phase cells of *B. animalis* subsp. *lactis* BI07 were collected, washed in 50 mM Tris/HCl (pH 7.6), resuspended in 500 µl TE buffer [50 mM Tris/HCl (pH 7.6), 5 mM EDTA], and 50 µl Complete Protease Inhibitors Solution (Roche) was added. The suspension was sonicated and centrifuged for 10 min at 14 000 r.p.m. (22 000 *g*) at 4 °C. The supernatant was collected and centrifuged for 2 h at 45 000 r.p.m. (176 000 *g*) at 4 °C by using a Beckman Ultracentrifuge L7-55. The supernatant, containing cytoplasmic proteins, was stored at −20 °C. Cell-wall proteins were extracted as reported by Hardie & Williams (1998). Briefly, after washing in 50 mM Tris/HCl (pH 7.6), stationary-phase *B. animalis* subsp. *lactis* BI07 cells were resuspended in 2 ml protoplast buffer [50 mM Tris/HCl (pH 7.6), 1 M sucrose, 1.4 mM phenylmethylsulfonyl fluoride, 15 mg lysozyme ml^{-1}]. The suspension was incubated for 90 min at 37 °C and centrifuged for 3 min at 4000 r.p.m. (1667 *g*) at 4 °C. The supernatant, containing the cell-wall proteins, was collected and stored at −20 °C.

Electron microscopy and immunoblot analysis. In order to visualize bifidobacterial DnaK on the *B. animalis* subsp. *lactis* BI07 cell surface, we performed pre-embedding immunogold experiments using whole bacterial cells. PBS-washed stationary-phase cells were adjusted to a concentration of 1×10^9 c.f.u. ml^{-1}, resuspended in 100 µl rabbit polyclonal anti-meningococcal DnaK antiserum (Knaust *et al.*, 2007) diluted 1 : 100 in PBS-1 % BSAe (BSA), and incubated for 1 h at 25 °C under constant agitation. After two washings with 1 ml PBS-1 % BSA, bacteria were resuspended in 25 µl anti-rabbit IgG coupled to 10 nm gold particles (Auro Probe, GE Healthcare) diluted 1 : 5 in PBS-1 % BSA and incubated for 30 min at 25 °C with constant agitation. Bacteria were then collected and washed twice in PBS-1 % BSA and fixed with 2.5 % glutaraldehyde in 0.1 M cacodylate buffer for 4 h at 4 °C. Transmission electron microscopy (TEM) processing was carried out as reported by Candela *et al.* (2007). Electron microscopic experiments were repeated four times. For immunoblot analysis, *B. animalis* subsp. *lactis* BI07 cytoplasmic or cell-wall proteins (10 µg) were subjected to SDS-PAGE with 12 % polyacrylamide and blotted onto a nitrocellulose membrane (Bio-Rad) by using the Minitrans-Blot Electrophoretic Cell (Bio-Rad). Post-transfer, the membrane was blocked in a solution of 4 % skim milk (Biolife) in TBS-T (0.15 % Tween 20 in TBS) and then incubated with anti-meningococcal DnaK antiserum (Knaust *et al.*, 2007). Subsequently, the membrane was washed in TBS-T and incubated with the peroxidase-conjugated anti-rabbit IgG (GE Healthcare). After TBS-T washing, the membrane was incubated with ECL Plus (GE Healthcare), and the signal was detected by using the PhosphorImager Storm system (GE Healthcare).

Construction of a *B. animalis* subsp. *lactis* BI07 genomic library and screening for the *dnaK* gene. Three hundred micrograms of *B. animalis* subsp. *lactis* BI07 chromosomal DNA was partially digested with the restriction enzyme *Mbo*I. DNA fragments of about 20 kb were isolated by ultracentrifugation at 26 000 r.p.m. for 24 h at 18 °C in a sucrose gradient (10–40 %), purified by precipitation with ethanol and ligated to *Bam*HI-digested Lambda EMBL3 vector (Stratagene). Recombinant lambda phages were packaged by using the Lambda EMBL3/*Bam*HI Gigapack III Gold Cloning kit (Stratagene). The library was amplified and titrated as described in the kit manual. A digoxigenin-labelled DNA probe targeting the *dnaK* gene was obtained by amplifying the chromosomal DNA of *B. longum* NCC 2705 with the primer set L-DnaK (5′-TTGGCACGTGCAGTTG-3′) and R-DnaKwt (5′-TCACTTGTTG-TCCTTGTCG-3′) (designed based on the genome sequence of

B. longum NCC 2705) by using the DIG DNA Labelling and Detection kit (Roche). For screening, the phage library was plated on 120 mm NZY agar plates using *E. coli* XL1-Blue MRA P2 as host strain. Plates were blotted onto nylon membrane (Hybond-N+, GE Healthcare). Neutralization, denaturation, cross-linking, membrane hybridization with the DIG-labelled DNA probe, and detection were carried out as described by the DIG-DNA Labelling and Detection kit manual (Roche). Plaques corresponding to the positive signals in the original plates were cut out and resuspended in SM buffer to isolate the recombinant phage population. Phage DNA was extracted by using the Qiagen Lambda mini kit (Qiagen). Positive clones were sequenced and the sequence of the *B. animalis* subsp. *lactis* BI07 *dnaK* gene obtained.

Cloning, expression, purification of recombinant *B. animalis* subsp. *lactis* BI07 His$_6$-DnaK. *B. animalis* subsp. *lactis* BI07 chromosomal DNA was used as template in a PCR for the amplification of the *dnaK* gene using the primer set DnaK-TOPO-L (5′-CACCATGGGACGCGCAGTTGGT-3′) and DnaK-TOPO-R (5′-TTACTTGTTGTCCTTGTCGTCGTC-3′). The PCR product was cloned in the expression vector pET200/D-TOPO (Invitrogen) to obtain the construct pDKwt. Cloning and expression of *B. animalis* subsp. *lactis* BI07 *dnaK* gene were carried out in *E. coli* TOP-10 and *E. coli* BL21Star(DE3) (Invitrogen), respectively. The His-tagged fusion *B. animalis* subsp. *lactis* BI07 DnaK was purified by affinity chromatography under native conditions on Ni-nitrilotriacetic acid resin according to the manufacturer's protocol (Invitrogen). The purified protein was dialysed at 4 °C using Spectra/Por membranes 6000–8000 Da (Spectrum Laboratories) and 20 mM Tris, 120 mM NaCl as dialysis buffer. The expression of the His-tagged recombinant protein His$_6$-DnaK was verified by Western blot analysis with polyclonal anti-meningococcal DnaK antiserum (Knaust *et al.*, 2007) and anti-HisTag antibody (Sigma).

Plg-binding analysis by solid-phase binding assay. Human Plg (Sigma-Aldrich) was biotinylated by using the EZ-Link Micro Sulfo-NHS-SS Biotinylation kit (Pierce). The solid-phase Plg-binding assay was performed as previously described by Sanderson-Smith *et al.* (2006, 2007). Microtitre plates (96-well; OptiPlate-96, Perkin Elmer) were coated with 150 nM recombinant DnaK (50 µl in 0.1 M NaHCO$_3$) and incubated at 4 °C. After washing with PBS, plates were blocked with 200 µl BSA 2 % in PBS for 1 h at 37 °C. After three PBS washings, increasing concentrations of biotinylated Plg (2, 6, 18, 36, 55, 110, 166, 250 nM) in PBS were added to the plates, in the presence or absence of 50-fold molar excess of unlabelled Plg. Plg was allowed to bind to immobilized proteins for 2 h at 25 °C. Plates were washed three times in PBS and 50 µl ExtrAvidin HRP conjugated (Sigma-Aldrich) diluted 1 : 5000 in PBS-1 % BSA was added to each well. Plates were incubated for 2 h at 25 °C and washed four times with PBS and 0.05 % Tween 20. One hundred microlitres of Chemiluminescent Peroxidase Substrate for ELISA (Sigma-Aldrich) was added to each well and luminescence was read by using the Victor^{3}V 1420 Multilabel Counter scanner (Perkin Elmer) and the software Wallac 1420 WorkStation. As negative control for Plg binding, BSA-coated wells were utilized. Plg-binding experiments were repeated in three biological replicates where, for each Plg concentration, three technical replicates were performed. To assess the involvement of lysine residues in the DnaK–Plg interaction, experiments were repeated in the presence of the lysine analogue ε-aminocaproic acid (EACA) (0.5 M). Data were normalized against the highest and lowest luminescence value, and non-linear regression analysis was carried out by using GraphPad Prism (version 5.0). For calculation of the equilibrium dissociation constant (K_D), one- and two-site binding analyses were performed and the best-fit curve was chosen.

Two-dimensional gel electrophoresis and comparative analysis of protein expression. Experiments were carried out as

reported by Candela *et al.* (2007). Briefly, 40 µg samples of *B. animalis* subsp. *lactis* BI07 cell wall proteins were resolved by two-dimensional electrophoresis. Isoelectric focusing was carried out using Immobiline DryStrips with a linear pH gradient between 4 and 7 (7 cm) on an IPGphor system (GE Healthcare) and proteins were separated by SDS-PAGE at 160 V for 2.5 h. Spots were visualized by silver staining. Protein patterns in the gels were recorded as digitized images using a GS-800 imaging densitometer (Bio-Rad). Spot detection, matching and the examination of differentially expressed proteins were performed by PDQuest v. 8.0.1 software (Bio-Rad). Two biological and three technical replicates were made per condition and formed one replicate group with average normalized spot intensities. Comparison was carried out between cell-wall fractions from stationary-phase cells grown in MRS medium and in MRS in the presence of 1.2 g bile salts l^{-1} (oxgall; Difco). A threshold of at least two times enhanced or decreased expression level was chosen to consider the differentially expressed proteins. The Plg-binding proteins were identified by comparison of the two-dimensional gels with the reference map reported by Candela *et al.* (2007).

MALDI-TOF-MS analysis of Plg binding to *B. animalis* subsp. *lactis* BI07. Plg binding to *B. animalis* subsp. *lactis* BI07 cells was evaluated by using a MALDI-TOF-MS-based approach, as reported by Candela *et al.* (2008a). Stationary-phase *B. animalis* subsp. *lactis* BI07 cells (1×10^9 c.f.u.) grown in MRS with and without 1.2 g bile salts l^{-1} (oxgall; Difco) were incubated with 0, 10, 20, 50, 100 and 200 µg human Plg (Sigma-Aldrich). Subsequently, bifidobacteria were washed three times with PBS to remove unbound Plg. After washing, bacterial cells were resuspended in 20 µl 2 % NH$_4$Cl for MALDI-TOF analysis. In order to detect Plg recruited on the bacterial cell surface, 1.0 µl of each bacterial suspension was spotted, airdried, and then overlaid with 1.0 µl matrix solution (12.5 mg ferulic acid ml^{-1} in a mixture of formic acid/acetonitrile/water 17 : 33 : 50). The resulting droplet was left to crystallize by airdrying and analysed in a Voyager DE Pro MALDI-TOF mass spectrometer (Applied Biosystems) operating in linear positive ion mode in the range of 20 000–100 000 Da and equipped with a pulsed N$_2$ laser at 337 nm. Default operating conditions were as follows: accelerating voltage 25 000 V, grid voltage 22 500 V, extraction delay time 750 ns. All acquisitions were generated automatically on the instrument software (Voyager, Biospectrometry Workstation) using a random search pattern mode and based on overhanging 1600 shots from 20 non-overlapping positions (80 shots per position). Data were externally calibrated with a standard BSAe (BSA) solution (67 kDa). As analytical standard, 200 ng human Plg were spotted. For each Plg concentration three biological and three technical replicates were carried out.

In order to evaluate the impact of DnaK and enolase on *B. animalis* subsp. *lactis* BI07 Plg-binding activity, Plg-binding assay of whole *B. animalis* subsp. *lactis* BI07 cells was performed after pre-treatment of cells with cross-reactive anti-meningococcal DnaK antiserum (Knaust *et al.*, 2007) or anti-pneumococcal enolase antiserum (anti-Eno) (Bergmann *et al.*, 2003), respectively. To this aim, 1×10^9 c.f.u. stationary-phase *B. animalis* subsp. *lactis* BI07 cells were washed in PBS and incubated with anti-meningococcal DnaK antiserum diluted 1/250 or anti-Eno diluted 1/100 in PBS-1 % BSA, for 1 h at 25 °C under constant agitation. After two washings with PBS-1 % BSA, cells were resuspended in PBS and incubated with 100 µg human Plg (Sigma-Aldrich) for 30 min at 37 °C with constant agitation. Subsequently, bifidobacteria were washed three times with PBS to remove unbound Plg. After washing, bacterial cells were resuspended in 20 µl NH$_4$Cl 2 % for MALDI-TOF analysis of Plg binding, as previously described. For each experimental condition three biological and three technical replicates were carried out.

Plasmin activity assay. This was carried out as reported by Candela *et al.* (2008b). Briefly, stationary-phase *B. animalis* subsp. *lactis* BI07 cells grown in MRS with and without 1.2 g bile salts l^{-1} (oxgall; Difco) were washed with PBS, adjusted to a concentration of 1×10^9 c.f.u. ml^{-1} and incubated for 30 min at 37 °C with 20 µg Plg ml^{-1} (Sigma-Aldrich) in PBS. After two PBS washing steps, cells were 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 0.06 KIU urokinase PA (uPA) (Calbiochem), and 30 µl of plasmin-specific chromogenic substrate solution, containing 0.54 mM D-valyl-leucyl-lysine-*p*-nitroanilide dihydrochloride (S-2251) (Sigma-Aldrich), was added. Absorbance at 405 nm was measured by using a Multiskan Ascent V1.24 (Thermo Electron Corporation) immediately after the addition of S-2251 and at intervals of 7 min during 1 h incubation at 37 °C. As negative control the kinetic experiment was carried out with *B. animalis* subsp. *lactis* BI07 cells not incubated with Plg. Further controls were performed with *B. animalis* subsp. *lactis* BI07 cells incubated with Plg in the presence of 0.1 M EACA (Sigma-Aldrich) and in the absence of uPA. Controls for spontaneous hydrolysis of S-2251 were carried out with the chromogenic substrate alone and in the presence of PA. Experiments were repeated twice and 10 technical replicates were carried out for each condition

RESULTS

Localization of DnaK protein on the cell surface of *B. animalis* subsp. *lactis* BI07

The presence of the DnaK protein in the cytoplasmic and cell-wall fractions of *B. animalis* subsp. *lactis* BI07 was demonstrated by immunoblot analysis using a cross-reactive anti-meningococcal DnaK antiserum (anti-DnaK) (Knaust *et al.*, 2007) (Fig. 1). Immunoelectron microscopy was conducted to confirm the presence of DnaK on the surface of the bacteria. *B. animalis* subsp. *lactis* BI07 cells were incubated under pre-embedding labelling conditions with the anti-DnaK antiserum followed by the secondary antibody labelled with 10 nm gold particles. Analysis of ultrathin sections at a magnification of ×22 000 showed the presence of DnaK in the bacterial cell-wall region (black dots, arrowed in Fig. 2). No non-specific binding of the secondary antibody was detected (data not shown).

Analysis of Plg binding to *B. animalis* subsp. *lactis* BI07 DnaK

A genomic library of *B. animalis* subsp. *lactis* BI07 was constructed and screened for the *dnaK* gene by using a digoxigenin-labelled DNA probe targeting the *B. longum* NCC 2705 *dnaK* gene. The nucleotide sequence of *B. animalis* subsp. *lactis* BI07 *dnaK* (GenBank accession no. AB514431) showed 100 % identity with the *dnaK* gene of *B. animalis* subsp. *lactis* DSM 10140 (GenBank accession no. CP001606) and 87 % with that of *B. longum* NCC 2705 (GenBank accession no. AE014295). In order to study the specific Plg-binding activity of *B. animalis* subsp. *lactis* BI07 DnaK, a His$_6$-tagged DnaK protein was expressed and, after purification, a solid-phase Plg-binding assay was carried out (Sanderson-Smith *et al.*, 2007). The binding
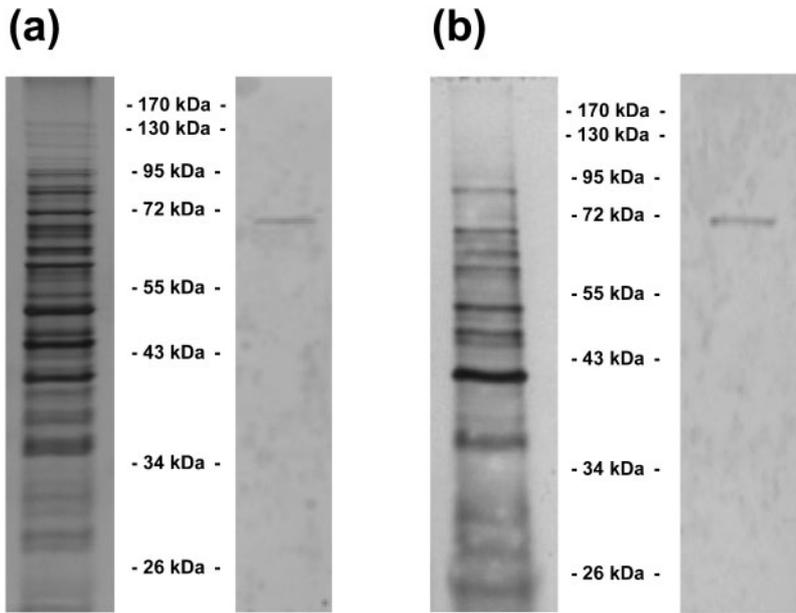
Fig. 1. Immunoblot analysis (right panels) using anti-DnaK antiserum of cytoplasmic (a) and cell wall (b) protein fractions of *B. animalis* subsp. *lactis* Bl07 resolved by SDS-PAGE. (right panels).
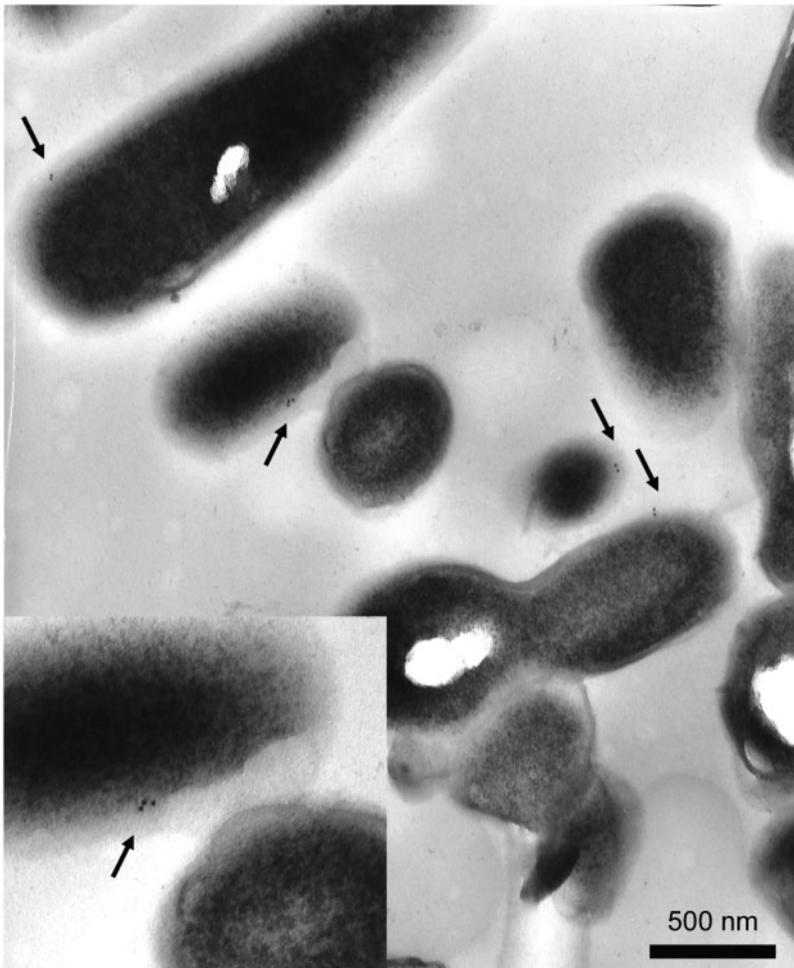

Fig. 2. Immunoelectron microscopic localization of DnaK on the cell surface of *B. animalis* subsp. *lactis* Bl07. DnaK was detected on the bacterial surface by anti-DnaK antiserum and secondary antibody coupled to 10 nm gold particles in pre-embedding experiments. An ultrathin section shows DnaK (black dots, arrowed) localized directly in the cell-wall region.

assay demonstrated a dose-dependent interaction between biotinylated Plg and immobilized recombinant *B. animalis* subsp. *lactis* BI07 DnaK (Fig. 3). In the presence of 150 nM Plg a saturation binding was achieved. Non-specific binding was determined in the presence of 50-fold molar excess of unlabelled Plg, and specific binding was calculated by subtracting non-specific binding from the total binding obtained at each concentration of biotinylated Plg. As negative control BSA-coated wells were utilized. The best-fit non-linear regression analysis allowed calculation of an equilibrium dissociation constant (K_D) for the interaction with Plg of about 11 nM. The pivotal role of DnaK lysine residues in Plg binding was demonstrated with 0.5 M EACA as competitor, which completely inhibited Plg binding (Fig. 3).

Role of surface DnaK and enolase in Plg binding to *B. animalis* subsp. *lactis* BI07

In order to provide experimental evidence of the role of surface DnaK and enolase as Plg receptors, we evaluated the impact of pre-treatment with anti-DnaK or anti-enolase antibodies on *B. animalis* subsp. *lactis* BI07 Plg-binding activity. Bacterial cells were pre-treated with anti-DnaK or anti-Eno (Candela *et al.*, 2009) antisera and their Plg-binding activity was compared with that of untreated bacteria using MALDI-TOF analysis. As a control, *B. animalis* subsp. *lactis* BI07 cells not treated with Plg were utilized. The results showed that treatment with the anti-Eno antiserum decreased the Plg-binding activity by about 30 %, and treatment with the meningococcal anti-DnaK
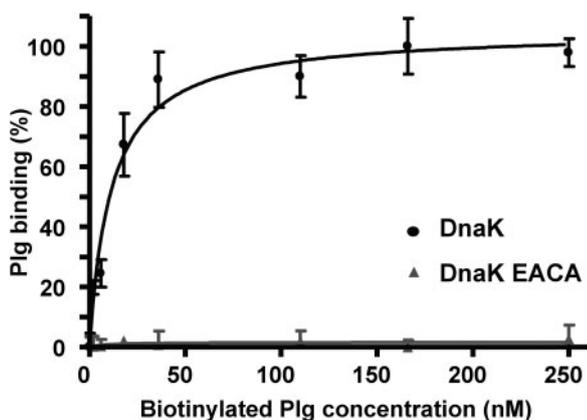

Fig. 3. Saturation binding analysis of biotinylated Plg to immobilized recombinant *B. animalis* subsp. *lactis* BI07 His-tagged DnaK. The black dots represent the specific binding. Specific Plg binding was calculated at each concentration of biotinylated Plg by subtracting non-specific binding obtained in the presence of 50-fold molar excess of unlabelled Plg from total binding. A one-site hyperbolic binding function was fitted to the data and the K_D determined. The experiment was repeated in the presence of the lysine analogue, EACA (0.5 M, grey triangles). Values represent the mean ± SD of 9 measurements.

antiserum decreased it by about 80 % (Fig. 4). In contrast, no reduction in Plg binding was measured when using anti-rabbit IgG as blocking antibodies. These results suggest that both enolase and especially DnaK are of importance for the sequestration of plasminogen by *B. animalis* subsp. *lactis* B107.

Impact of bile salts exposure on the *B. animalis* subsp. *lactis* BI07–plasmin(ogen) interaction

First, the capability of *B. animalis* subsp. *lactis* BI07 to grow in the presence of bile salts was tested. To this end, *B. animalis* subsp. *lactis* BI07 was grown in batch cultures in the absence and in the presence of bile salts at concentrations of 1.2 and 3 g l^{-1} (Sanchez *et al.*, 2005). As reported for *B. longum* NCIMB 8809, growth rates were very similar during the exponential phase in each of the three different conditions (Fig. 5). In contrast, at the end of the stationary-phase the c.f.u. ml^{-1} were significantly lower in the presence of bile salts (1.2×10^9, 5×10^8 and 1×10^8 c.f.u. ml^{-1} for bile salts concentrations of 0, 1.2 and 3 g l^{-1}, respectively). In order to assess whether the physiological changes that are a consequence of the long-term exposure of *B. animalis* subsp. *lactis* BI07 to bile salts, can affect the expression of surface Plg receptors, a comparative proteomic approach was performed. The cell-wall proteomes of *B. animalis* subsp. *lactis* BI07 grown in media supplemented or not supplemented with bile salts at the concentration of 1.2 g l^{-1} were compared by two-dimensional gel electrophoresis (Fig. 6). Plg-binding proteins are numbered as reported by Candela *et al.* (2007). The expression profiles showed that exposure to bile salts induced a 2.8-, 2.3- and 10-fold upregulation of three Plg-binding proteins: DnaK, enolase and phosphoglycerate mutase, respectively. Surprisingly, the putative Plg-binding protein BSH was slightly downregulated (by about 1.7-fold) after bile exposure. A downregulation was also observed for the as yet unidentified Plg-binding proteins no. 2 and no. 8.

To investigate whether bile salts adaptation affects the Plg-binding capacity of *B. animalis* subsp. *lactis* BI07, we compared the Plg-binding activity of stationary-phase cells grown in the presence or in the absence of 1.2 g bile salts l^{-1}. *B. animalis* subsp. *lactis* BI07 cells were incubated with different concentrations of Plg and the bacterial-bound Plg was measured by MALDI-TOF (Fig. 7). Although *B. animalis* subsp. *lactis* BI07 showed a dose-dependent Plg-binding activity independently of bile salts supplementation, bacteria grown in the presence of bile salts were about 10 times more efficient in Plg binding. In a plasmin activity assay we compared the capability of *B. animalis* subsp. *lactis* BI07 cells grown with and without 1.2 g bile salts l^{-1} to acquire a Plg-dependent surface-associated plasmin activity after the addition of host PA. To this end, *B. animalis* subsp. *lactis* BI07 stationary-phase cells were incubated with 20 μg Plg ml^{-1} and the cell-captured Plg was then activated to plasmin by the addition of uPA. The
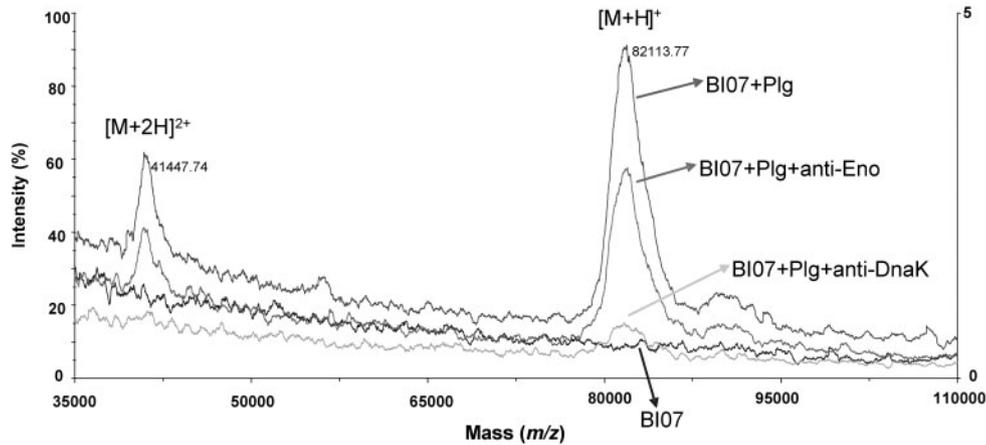
Fig. 4. MALDI-TOF analysis of Plg binding to *B. animalis* subsp. *lactis* BI07 cells untreated (BI07 + Plg), and pre-treated with anti-DnaK (BI07 + Plg + anti-DnaK) or anti-Eno (BI07 + Plg + anti-Eno). As negative control, *B. animalis* subsp. *lactis* BI07 cells not incubated with Plg (BI07) are shown. Plg [M+H]$^+$ signal at 81 kDa, [M+2H]$^{2+}$ at 40 kDa.

acquired bacterial surface-associated plasmin activity was evaluated in kinetic experiments by measuring the hydrolysis of the plasmin-specific chromogenic substrate S-2251 at intervals of 7 min over a period of 1 h (Fig. 8). The *B. animalis* subsp. *lactis* BI07 cells grown in the presence of bile salts showed a significant increase in the rate of S-2251 hydrolysis, demonstrating a higher rate of surface-associated Plg-derived plasmin activity compared with cells grown in standard conditions. As expected, the lysine analogue EACA was effective in inhibition of the plasmin formation of the *B. animalis* subsp. *lactis* BI07 cell

surface, proving the essential role of the Plg lysine-binding sites for its recruitment to the bacterial cell surface (Fig. 8).

DISCUSSION

In this work we demonstrate the importance of *B. animalis* subsp. *lactis* BI07 DnaK as a surface receptor for human Plg. DnaK is a highly conserved primary cytoplasmic protein of 67 kDa belonging to the heat-shock protein 70 family (HSP70) (Bukau & Horwich, 1998). HSP70s are chaperones with an important role in protein folding and transport. By immunoelectron microscopy we documented the presence of DnaK on the cell surface of *B. animalis* subsp. *lactis* BI07. Similarly to the surface-located *B. animalis* subsp. *lactis* BI07 enolase (Candela *et al.*, 2009), DnaK lacks predicted protein-sorting elements and anchorage on the bacterial cell wall. Mechanisms of secretion and surface localization of these cytoplasmic enzymes are still under debate. However, for *Streptococcus pneumoniae*, scavenging of cytoplasmic proteins released through allolysis was proposed as a possible mechanism for surface localization (Claverys & Havarstein, 2007). Strengthening our findings, DnaK had been previously shown to be present on the cell surface of several pathogens such as *Neisseria meningitidis*, *Listeria monocytogenes* and *Mycobacterium tuberculosis* (Schaumburg *et al.*, 2004; Knaust *et al.*, 2007; Xolalpa *et al.*, 2007), as well as the probiotic member of the human intestinal microbiota *Lactobacillus salivarius* (Kelly *et al.*, 2005). Although for *Lb. salivarius* no information had been provided concerning the DnaK function on the cell surface, in the case of *N. meningitidis*, *L. monocytogenes* and *M. tuberculosis* its role as receptor for human Plg was proved. The His-tagged *B. animalis* subsp. *lactis* BI07 DnaK protein was purified and its affinity for human Plg was evaluated in a saturation binding analysis. According to our data, the *B. animalis*
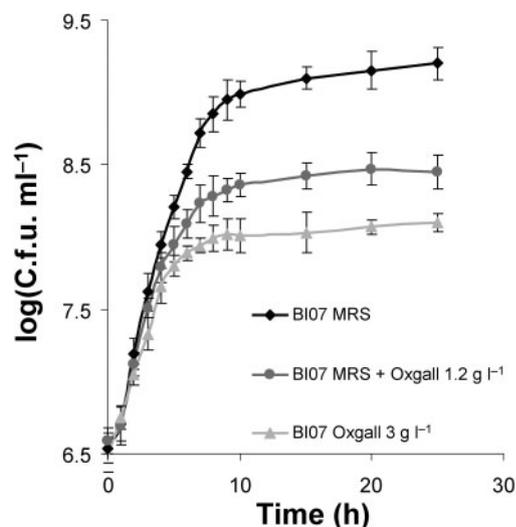

Fig. 5. Growth curves of *B. animalis* subsp. *lactis* BI07 in MRS with 0 (black), 1.2 (dark grey) and 3 g l^{-1} bile salts (light grey). For all the experimental conditions growth curves were performed in three biological replicates and values represent the mean ± SD.
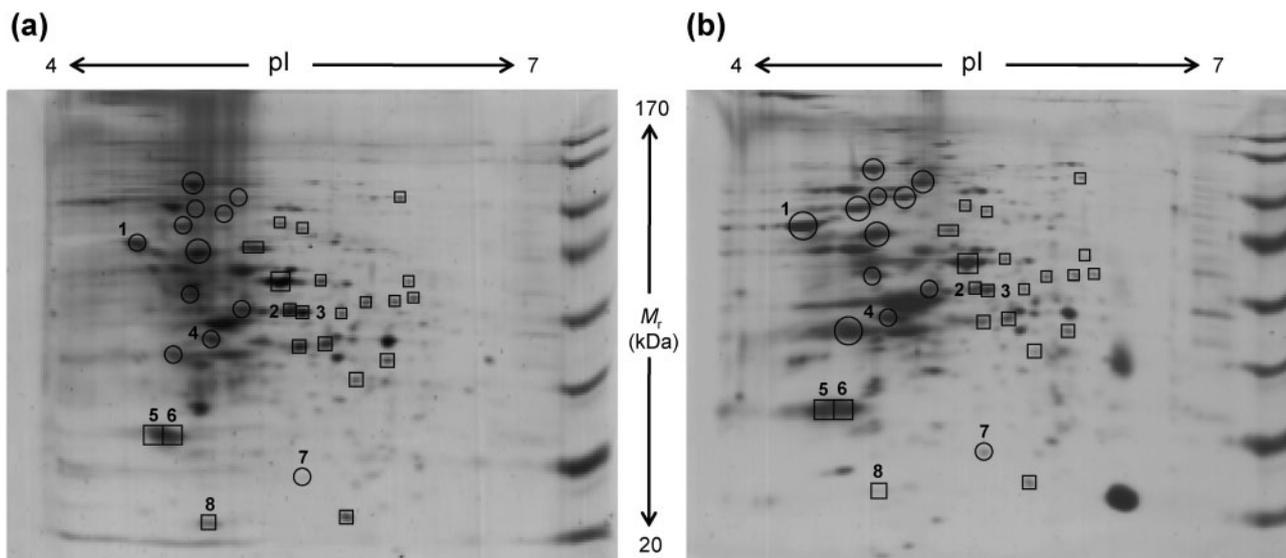
Fig. 6. Cell-wall proteome of stationary-phase *B. animalis* subsp. *lactis* BI07 cells cultured with (b) and without (a) bile salts at 1.2 g l^{-1}. Cell-wall proteins up- and downregulated as a consequence of bile salts adaptation are marked with circles and squares, respectively. Plg-binding proteins are numbered: DnaK (1), unidentified (2), glutamine synthetase (3), enolase (4), BSH (5, 6), phosphoglycerate mutase (7), unidentified (8).

subsp. *lactis* BI07 DnaK shows a high affinity for human Plg with a K_D of about 11 nM, a value significantly higher than that reported for *B. animalis* subsp. *lactis* BI07 enolase ($K_D = 42.8$ nM) (Candela *et al.*, 2009). The complete inhibition of Plg binding in the presence of 0.5 M EACA indicates that DnaK lysine residue(s) are crucial for the Plg interaction. The pretreatment of *B. animalis* subsp. *lactis* BI07 cells with cross-reactive DnaK-specific antiserum had a detrimental impact on the bacterial Plg-binding capacity,

demonstrating the importance of DnaK as a surface Plg receptor. Pre-treatment with anti-Eno also reduced Plg binding to *B. animalis* subsp. *lactis* BI07, but by only 30 %, compared to the 80 % reduction seen with anti-DnaK antiserum. While it would be tempting to speculate that this difference is due to the higher Plg affinity of DnaK compared to enolase, we can not exclude that it is the consequence of a different efficacy in blocking the respective antigens between the two antisera. Moreover, the contribution of a single Plg receptor to Plg recruitment on the bacterial cell surface can be influenced by other variables alongside the mere affinity for Plg, such as its availability on the cell surface and the number of binding epitopes (Knaust *et al.*, 2007).
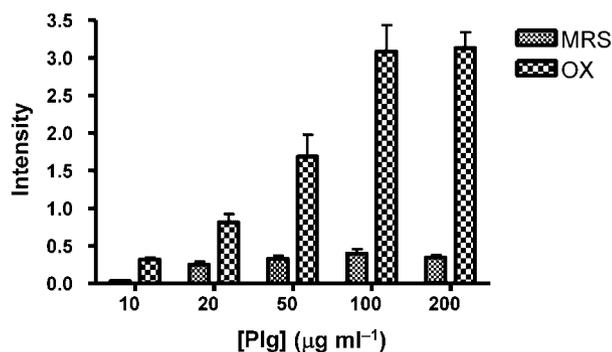
Adaptation of *B. animalis* subsp. *lactis* BI07 to bile salts induces a complex physiological response which influences the cell envelope proteome. Similarly to *B. longum* (Ruiz *et al.*, 2009), the long-term exposure of *B. animalis* subsp. *lactis* BI07 to bile salts results in the upregulation of the important surface Plg receptors DnaK and enolase. Supporting these findings, a recent study of the *B. animalis* subsp. *lactis* protein response to bile salts reported the upregulation of DnaK (Sanchez *et al.*, 2007). In order to investigate if the physiological changes as a consequence of bile salts response could have an impact on the *B. animalis* subsp. *lactis*–Plg interaction, we compared the Plg-binding activity and surface-associated Plg-dependent plasmin activity of *B. animalis* subsp. *lactis* BI07 cells grown with and without 1.2 g bile salts l^{-1}. According to our data, the *B. animalis* subsp. *lactis* BI07 cells grown in the presence of bile salts were about 10 times more efficient in Plg binding
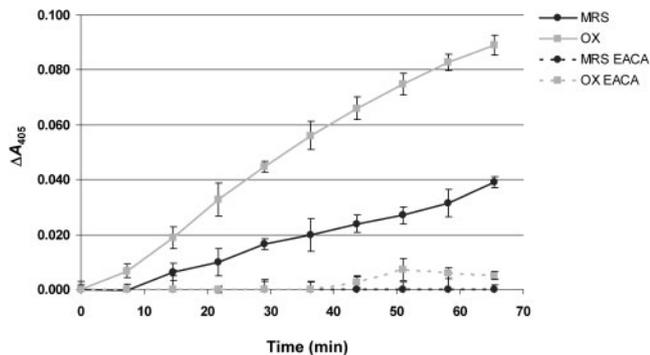

Fig. 7. MALDI-TOF analysis of the Plg-binding activity of stationary-phase cells of *B. animalis* subsp. *lactis* BI07 grown in the presence (OX) or in the absence (MRS) of 1.2 g bile salts l^{-1}. The bars represent the response, expressed as Plg amplitude/ reference signal amplitude (RS amplitude), with the standard deviation, of *B. animalis* subsp. *lactis* BI07 cells incubated with different concentrations of Plg (10, 20, 50, 100, 200 μg ml^{-1}). Reference signal: 23 000 Da.

Fig. 8. Plasmin activity of *B. animalis* subsp. *lactis* BI07 cells grown in the presence (OX) or in the absence (MRS) of 1.2 g bile salts l^{-1}. Plg-pretreated bacterial cells were incubated with uPA and then with the plasmin-specific chromogenic substrate S-2251. The kinetics of plasmin formation was evaluated by measuring the increase in A_{405} at intervals of 7 min during 1 h incubation. Bacterial cells untreated with Plg were used as negative control. Experiments were repeated by incubating the bacteria with Plg in the presence of 0.1 M EACA. Values represent the mean ± SEM of 20 measurements.

compared to cells cultured in standard medium and, after the addition of host uPA, these bacteria exhibited a significantly higher rate of acquired Plg-dependent surface-associated plasmin activity. Taken together, these data prove that bile salts enhance the ability of *B. animalis* subsp. *lactis* BI07 to interact with the host Plg system, and show the potential impact of the GIT bile environment on the *B. animalis* subsp. *lactis*–host interaction process. Even if the upregulation of surface Plg receptors such as enolase and DnaK can partially explain this increase in Plg-binding activity, other mechanisms may be involved. For instance, affecting the accessibility of Plg receptors, changes in the *B. animalis* subsp. *lactis* cell-surface structure may occur in response to bile adaptation (Ruiz *et al.*, 2007) and may influence the bacterial affinity for human Plg (Stie *et al.*, 2009).

Besides the demonstration of the importance of DnaK as a surface Plg receptor, in this work we report for the first time that *B. animalis* subsp. *lactis* significantly increases its affinity for human Plg in response to physiological concentrations of bile salts. Since the ability of *Bifidobacterium* to interact with the plasmin(ogen) system of the human host may have a role in facilitating the colonization of the human GIT (Candela *et al.*, 2008b), our results provide evidence that the adaptation of *B. animalis* subsp. *lactis* to the gut bile environment may favour its capacity to colonize the human GIT. Further studies will be necessary to determine the mechanisms underlying the enhancement of *B. animalis* subsp. *lactis* BI07 Plg affinity in the presence of bile salts, as well as how bile influences the biology of the interaction process between *B. animalis* subsp. *lactis* BI07 and the host enterocytes. However, our findings provide some insights into the adaptation of *B. animalis* subsp. *lactis* to the human gastrointestinal environment. Moreover, the importance of DnaK as a *B. animalis* subsp. *lactis* Plg receptor justifies further studies to attempt to characterize the mechanisms of Plg binding in more detail.

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