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Bifidobacterial enolase, a cell surface receptor for human plasminogen involved in the interaction with the host

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The interaction with the host plasminogen/plasmin system represents a novel component in the molecular cross-talk between bifidobacteria and human host. Here, we demonstrated that the plasminogen-binding bifidobacterial species *B. longum*, *B. bifidum*, *B. breve* and *B. lactis* share the key glycolytic enzyme enolase as a surface receptor for human plasminogen. Enolase was visualized on the cell surface of the model strain *B. lactis* BI07. The His-tagged recombinant protein showed a high affinity for human plasminogen, with an equilibrium dissociation constant in the nanomolar range. By site-directed mutagenesis we demonstrated that the interaction between the *B. lactis* BI07 enolase and human plasminogen involves an internal plasminogen-binding site homologous to that of pneumococcal enolase. According to our data, the positively charged residues Lys-251 and Lys-255, as well as the negatively charged Glu-252, of the *B. lactis* BI07 enolase are crucial for plasminogen binding. Acting as a human plasminogen receptor, the bifidobacterial surface enolase is suggested to play an important role in the interaction process with the host.

INTRODUCTION

Bifidobacteria represent one of the most important health-promoting groups of human intestinal microbiota (Schell *et al.*, 2002; Ventura *et al.*, 2009). Even though numerous health-promoting activities have been related to the presence of bifidobacteria in the human gastrointestinal tract (Guarner & Malagelada, 2003), knowledge of the mechanisms of interaction with the host is still in its infancy. The understanding of the *Bifidobacterium*–host interaction process, as well as its impact on human health, could be clarified by the identification and characterization of the bacterial proteins involved. In particular, representing the first line of contact with the intestinal epithelium, the proteins of the bacterial cell surface may play a critical role in the early interaction between microbes and the host (Klijn *et al.*, 2005). Recently, a proteomic approach identified five highly conserved cytoplasmic proteins in the cell wall fraction of *Bifidobacterium lactis* BI07 as putative human plasminogen (Plg) receptors: DnaK, glutamine synthetase, enolase, bile salt hydrolase and phosphoglycerate mutase (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 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 of plasmin is involved in fibrinolysis (Collen &

Abbreviations: 2-PGE, 2-phosphoglycerate; EACA, ε-aminocaproic acid; PEP, phosphoenolpyruvate; Plg, plasminogen.

The GenBank/EMBL/DDBJ accession number for the enolase sequence of *B. lactis* BI07 is DQ117970.

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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 gastrointestinal tract (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.*, 2008). With the recruitment of human Plg on the bacterial cell surface, and its subsequent conversion to plasmin, the micro-organism acquires a surface-associated and host-derived proteolytic activity, useful for facilitating the migration across physical and molecular barriers and for responding to the nutritional demands during the colonization process (Lähteenmäki *et al.*, 2005). Several bacterial receptors for human Plg have been characterized (Lähteenmäki *et al.*, 2001). Interestingly, most of them have other important functions besides Plg binding, such as adhesion, movement, enzymic activity or nutrient uptake. In particular, glycolytic enzymes including glyceraldehyde-3-phosphate dehydrogenase and enolase interact with Plg, as shown for different bacterial species (Bergmann & Hammerschmidt, 2007). In spite of the absence of peptides required for secretion and membrane anchorage, these key cytoplasmic enzymes are displayed on the bacterial cell surface, where they acquire a secondary 'moonlighting' function (Jeffery, 1999) that has been shown to be important in the bacteria–host interaction process.

A dose-dependent human Plg-binding activity was recently demonstrated in four bifidobacterial strains belonging to the species *B. lactis*, *B. bifidum* and *B. longum* (Candela *et al.*, 2007). The complete inhibition of Plg recruitment on the bifidobacterial cell surface in the presence of the lysine analogue ε-aminocaproic acid (EACA) suggested that the binding of human Plg to these bifidobacteria was strongly dependent on lysine residues of surface-exposed Plg receptors (Candela *et al.*, 2007). However, bifidobacterial receptors for human Plg have never been characterized. In an attempt to provide insights into the interaction between bifidobacteria and human-derived Plg, we assessed here the role of the bifidobacterial enolase as a surface-displayed Plg receptor in four bifidobacterial species with Plg-binding activity. The localization of enolase on the bacterial cell surface was demonstrated by immunoelectron microscopy in the model strain *B. lactis* BI07. The recombinant His-tagged enolase protein was purified and its Plg-binding activity was characterized with respect to the dissociation constant and the mechanism of Plg binding. By functioning as a surface receptor for human Plg, enolase may play an important role in the bifidobacterial–host interaction process.

METHODS

Bacterial strains, media, and growth conditions. Four bifidobacterial strains were studied. *B. bifidum* S16 and *B. longum* S123 were isolated from human faeces, whereas *B. breve* BBSF and *B. lactis* BI07 were isolated from dairy products. Bifidobacteria were 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 cultures were grown for 18 h until they reached the stationary phase. *Escherichia coli* strains OneShot TOP10, BL21Star(DE3) and XL10-Gold 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 a selective agent when appropriate.

Extraction of bifidobacterial cytoplasmic proteins. Cytoplasmic proteins were extracted from 50 ml bacterial culture in the stationary growth phase. Cells were collected by centrifugation for 10 min at 3800 ***g***, 4 °C and then 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 Inhibitor Solution (Roche) were added. The suspension was sonicated for 8 min, power 30 W, pulse frequency 20 % in a Branson Sonifier 250 and centrifuged for 10 min at 22 000 ***g***, 4 °C. The supernatant was collected and centrifuged for 2 h at 176 000 ***g***, 4 °C by using a Beckman Ultracentrifuge L7-55. The supernatant, containing cytoplasmic proteins, was stored at −20 °C.

Resolution of *Bifidobacterium* cell wall proteins by 2D-electrophoresis and Plg overlay assay. Experiments were carried out as reported by Candela *et al.* (2007). Briefly, 40 μg bifidobacterial cell wall proteins was resolved by 2D-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. For the Plg overlay assay, the resolved proteins were blotted onto a nitrocellulose membrane (Pure nitrocellulose membrane, Bio-Rad) by using a Trans-Blot Electrophoretic Cell (Bio-Rad). After blocking, the membrane was incubated with 4 μg human Plg ml^{-1} (Sigma-Aldrich) in PBS for 1 h at 25 °C. Captured Plg was detected by incubating the membrane with goat anti-Plg IgG antibody (Kordia) and a peroxidase-conjugated anti-goat IgG (Sigma-Aldrich) as secondary antibody. To determine the role of the lysine-binding site(s) in Plg binding, the experiment was repeated in the presence of EACA. Primary and secondary antibody alone did not result in non-specific background binding (data not shown).

Protein identification using MALDI-TOF MS. The selected protein spots were excised from the acrylamide gel and subjected to in-gel tryptic digestion and extraction of peptides (Shevchenko *et al.*, 1996). The extracted peptides were purified with ZipTip (Millipore). Peptide mass fingerprinting maps of tryptic peptides were generated by MALDI-TOF MS with a Voyager-DE Pro Biospectrometry work station (Applied Biosystems), as reported by Candela *et al.* (2007). Aldente (http://www.expasy.org/tools/aldente) and ProFound (http://prowl.rockefeller.edu/prowl-cgi/profound.exe) database search algorithms were used for the identification of the proteins.

Electron microscopy and immunoblot analysis. In order to visualize bifidobacterial enolase on the *B. lactis* BI07 cell surface, we performed pre-embedding immunogold experiments using intact bacterial cells. Bifidobacterial cells recovered from a stationary-phase culture were washed in PBS and adjusted to a concentration of 1×10^9 c.f.u. ml^{-1}. Cells were resuspended in 100 μl rabbit polyclonal anti-pneumococcal enolase antiserum (Bergmann *et al.*, 2003) diluted 1 : 250 in PBS/1 % BSA, and incubated for 1 h at 25 °C under constant agitation. After two washes 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.

Processing for transmission electron microscopy was carried out as reported by Candela *et al.* (2007). Electron microscopic experiments were repeated four times. For immunoblot analysis, *B. lactis* BI07 proteins (10 μg) were subjected to SDS-PAGE with 12 % polyacrylamide and blotted onto a nitrocellulose membrane by using a 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 rabbit polyclonal anti-streptococcal enolase antiserum (Bergmann *et al.*, 2003). 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 a PhosphorImager Storm system (GE Healthcare).

Cloning, expression and purification of recombinant *B. lactis* BI07 enolase. In order to analyse the nucleotide sequence of the *B. lactis* BI07 enolase gene, chromosomal DNA was used as a template for PCR and the enolase gene was amplified with the primer pair L-FEno (5′-GAAACTCACGCCTTTACGGGCGTT-3′) and R-FEno (5′-TCAAGATACACAACCGTTTTAAGGAGT-3′), designed against the nucleotide regions downstream and upstream of the *B. longum* NCC2705 enolase gene (1299 bp, GeneID:1022550), respectively. The PCR product obtained was cloned into the pCRII-TOPO cloning vector (Invitrogen), following the protocol supplied by the manufacturer, and the DNA insert was sequenced using the primer set T7 and T7-reverse. For cloning and expression, the *B. lactis* BI07 enolase gene was amplified by PCR using the primer set EnoTOPO-L (5′-CACCATGGCAGTAATTGAAAGCGTGT-3′) and EnoTOPO-R (5′-TCACTTGGCCAGGTACTTCT-3′), and the PCR product was cloned into the expression vector pET200/D-TOPO (Invitrogen) to obtain the construct pENOwt. Cloning and expression of the *B. lactis* BI07 enolase gene were carried out in *E. coli* TOP-10 and *E. coli* BL21Star(DE3) (Invitrogen), respectively. The His-tagged fusion *B. lactis* BI07 enolase was purified by affinity chromatography under native conditions on Ni-NTA resin, according to the manufacturer's instructions (Invitrogen). The purified proteins were dialysed at 4 °C using Spectra/Por membranes 6000–8000 kDa (Spectrum Laboratories) and 20 mM Tris, 120 mM NaCl as dialysis buffer. The expression of the His-tagged recombinant protein His_6-enolase was verified by Western blot analysis with polyclonal anti-pneumococcal enolase antiserum (Bergmann *et al.*, 2001) and anti-HisTag (Sigma) antibody.

Site-directed mutagenesis. The QuikChange Multi Site-Directed Mutagenesis kit (Stratagene) was used to obtain a mutant plasmid library from pENOwt. Plasmids harbouring mutants of the *B. lactis* BI07 *enolase* gene, deleted of nucleotides encoding the C-terminal lysine (pENObs1), mutated in the internal Plg-binding site (pENObs2), or with both the deletion and the internal mutation (pENOdouble), were created. Primer ENOmutBS1 (5′-AAGAAGTACCTGGCCTGATGAAAGGGCGAGCTCAACG-3′) was designed to replace the C-terminal lysine with a stop codon. Primer ENOmutBS2 (5′-GAGTTCTACAACTTGGGGACCGGCTTGTACC-GCTTCGACGG-3′) was designed to replace Lys-251 and Lys-255 with leucine, and Glu-252 with glycine, as performed by Bergmann *et al.* (2003) on *Streptococcus pneumoniae* enolase. Equal amounts of each primer were added to a 50 μl PCR, together with 200 ng pENOwt as DNA template. The mutagenesis reaction was carried out in a thermocycler with a first denaturation step at 95 °C for 1 min, followed by 30 cycles at 95 °C for 1 min, 55 °C for 1 min and 65 °C for 14 min. The DNA template was removed by *Dpn*I treatment at 37 °C for 2 h. The mutant plasmid library was transformed into the *E. coli* XL10-Gold strain and a number of clones were sequenced to isolate the three mutants. Mutant proteins carrying the desired amino acid substitutions or deletion were expressed and purified as described above.

Enolase activity of bifidobacterial whole cells and purified *B. lactis* BI07 enolase protein. In order to determine the enolase activity of intact bifidobacterial cells, a direct enzyme assay was performed (Pancholi & Fischetti, 1998). Briefly, 1×10^9 bifidobacterial cells were washed three times in 100 mM HEPES buffer pH 7 and equal amounts were resuspended in reaction buffer [100 mM HEPES (pH 7), 10 mM $MgCl_2$, 7.7 mM KCl, 3 mM 2-phosphoglycerate (2-PGE)] or in control buffer [100 mM HEPES (pH 7), 10 mM $MgCl_2$, 7.7 mM KCl]. The bacterial suspensions were incubated for 3 min at 37 °C and subsequently centrifuged for 10 min at 19 000 ***g***, 4 °C. The supernatants were recovered and centrifuged for a further 10 min at 19 000 ***g***, 4 °C. After the second centrifugation step, the supernatants were recovered and phosphoenolpyruvate (PEP) concentration was determined by measuring the absorbance at 240 nm in a Jasco spectrophotometer (model 7800/V-520). The enzyme activity of the purified His-tagged enolase protein was determined in a single enzyme assay. Briefly, different amounts of purified recombinant His-tagged enolase protein were incubated for 3 min at 37 °C in reaction buffer containing 15 mM 2-PGE. The release of PEP was measured spectrophotometrically as described above. For kinetics studies, different amounts of 2-PGE (0.5–15 mM) were used in a single enzyme assay carried out with 5 μg purified His-tagged enolase protein. The K_m value was calculated from the Lineweaver–Burk plot.

Structural model of *B. lactis* BI07 enolase. The homology model of *B. lactis* BI07 enolase was calculated as reported by Zambelli *et al.* (2009). Using the tool BLAST (Altschul *et al.*, 1990, 1997), the amino acid sequence of *B. lactis* BI07 enolase (Swiss-Prot accession no. Q45RT9) was used to search for bacterial enolase structures with a high identity score. Four enolases with known protein structures were selected: *Strep. pneumoniae* enolase (PDB code 1W6T), *Enterococcus hirae* enolase (PDB code 1IYX), *Methanococcus jannaschii* enolase (PDB code 2PA6), and *Escherichia coli* enolase (PDB code 1E91). Multiple sequence alignment was carried out using CLUSTAL W (Thompson *et al.*, 1994). The prediction of the *B. lactis* BI07 enolase secondary structure was carried out using the JPRED tool (Cuff *et al.*, 1998) and the alignment was manually optimized based on the secondary structure information. Model structure was calculated with the program MODELLER 9v5 (Marti-Renom *et al.*, 2000), using the above indicated enolases as templates. The best model was selected on the basis of the lowest value of the MODELLER objective function. Structure validation was performed using PROCHECK (Laskowski *et al.*, 1993). The program UCSF Chimera (Pettersen *et al.*, 2004) was used for protein visualization.

Plg-binding analysis. Human Plg (Sigma-Aldrich) was biotinylated using the EZ-Link Micro Sulfo-NHS-SS Biotinylation kit (Pierce), following the manufacturer's instructions. 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 enolase (50 μl in 0.1 M $NaHCO_3$) and incubated at 4 °C overnight. After washing with PBS, the plates were blocked with 200 μl BSA 2 % in PBS for 1 h at 37 °C. After three PBS washes, increasing concentrations of biotinylated Plg in PBS (2, 6, 18, 36, 55, 110, 166 and 250 nM) were added to the plates, in the presence or absence of a 50-fold molar excess of unlabelled Plg. Plg was allowed to bind to immobilized proteins for 2 h at room temperature, then the plates were washed three times with PBS, and 50 μl ExtrAvidin horseradish-peroxidase-conjugated antibodies (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 using a Victor^{3}V 1420 Multilabel Counter scanner (Perkin Elmer) and the software Wallac 1420 WorkStation. As a negative control for Plg binding, BSA-coated plates were utilized. For each of the recombinant

proteins, Plg-binding experiments were repeated four times, whereas for a given Plg concentration, each measurement was repeated in triplicate. To assess the involvement of lysine residues in the enolase–Plg interaction, experiments were repeated in the presence of 0.5 M EACA. For analysis, data were normalized against the highest and lowest luminescence values, and nonlinear regression analysis was carried out using Graph Pad Prism (version 5.0, Graph Pad Software). For calculation of the equilibrium dissociation constant (K_D) one- and two-site binding analysis was performed and the best-fit curve was chosen.

RESULTS

Enolase is a conserved putative Plg receptor in *Bifidobacterium*

In order to screen the putative surface Plg receptors in *Bifidobacterium*, the cell wall fractions from the Plg-binding strains *B. longum* S123, *B. bifidum* S16 (Candela *et al.*, 2007) and *B. breve* BBSF (data not shown) were purified and a Plg overlay assay was carried out. To this end, cell wall proteins were resolved in a two-dimensional gel, immobilized on a nitrocellulose membrane, incubated with human Plg, and probed with anti-Plg antibody to identify bound Plg (Fig. 1). For each strain, the coordinates of the major putative Plg-binding proteins detected could be matched to a protein spot on the replica gel stained for proteins. Plg binding was completely inhibited in the presence of EACA (data not shown). Among the putative Plg-binding proteins, two proteins with an apparent molecular mass of 70 and 50 kDa and a pI of 4.5 and 4.7, respectively, were found to be conserved in the different bifidobacterial strains analysed. For each strain, the corresponding spots were excised from the gel and subjected to trypsin digestion and MALDI-TOF MS analysis for protein identification. The peptide fingerprints obtained were scanned with the searching tools Aldente and ProFound, and an unambiguous identification was obtained: the protein of 70 kDa and pI 4.5 was identified as DnaK and the protein of 50 kDa and pI 4.7 as enolase. DnaK and enolase had been previously identified as putative Plg receptors in a cell wall fraction of the Plg-binding strain *B. lactis* BI07 (Candela *et al.*, 2007). To investigate the role of bifidobacterial enolase as a receptor for human Plg, *B. lactis* BI07 was selected as model strain.

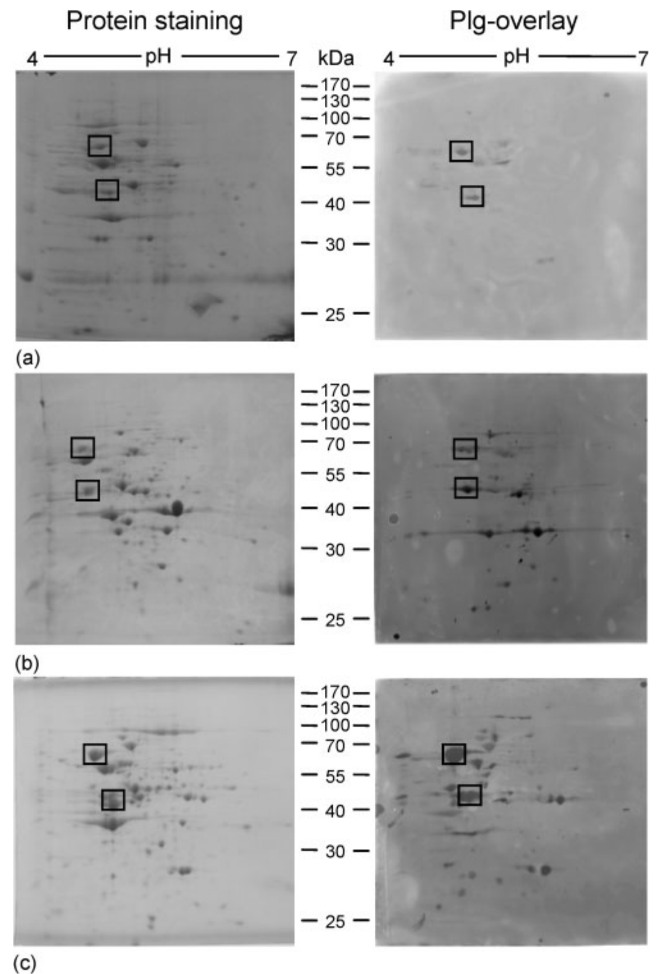

Fig. 1. Plg overlay assay carried out with the cell wall fraction of *B. longum* S123 (a), *B. bifidum* S16 (b) and *B. breve* BBSF (c). For each strain, the 2D-gel stained for proteins and the replicate 2D-gel used for Plg overlay are shown. Four micrograms Plg per ml was applied as the Plg overlay. Plg-binding proteins were detected with anti-Plg antibody and peroxidase-conjugated secondary antibody. The black squares indicate two proteins with an apparent molecular mass of 70 and 50 kDa and a pI of 4.5 and 4.7, which are conserved among the different bifidobacterial strains.

Localization of enolase protein on the *B. lactis* BI07 cell surface

At first, the distribution of enolase in cytoplasmic and cell wall fractions of *B. lactis* BI07 was evaluated by a Western blot experiment carried out with a cross-reactive polyclonal anti-pneumococcal enolase antiserum (anti-Eno) (Bergmann *et al.*, 2003). Enolase was detected in both the cytoplasmic and cell wall fractions of *B. lactis* BI07 (Fig. 2). In order to visualize the enolase protein on the *B. lactis* BI07 cell surface, an immunoelectron microscopy experiment was carried out. Intact *B. lactis* BI07 cells were incubated under pre-embedding labelling conditions with the anti-Eno antiserum followed by the secondary antibody labelled with 10 nm gold particles. Analysis of ultrathin sections at a magnification of $\times 22\,000$ (Fig. 3) detected the enolase (black dots) directly in the bacterial cell wall region. Non-specific binding of the secondary antibody was not detected (data not shown). The binding pattern of the anti-Eno antiserum suggests either that the distribution of enolase is in the form of a cluster, or that its epitopes are not uniformly exposed on the cell surface. The enzyme functionality of the surface enolase was evaluated by monitoring the conversion of 2-PGE to PEP, when supplied to viable bifidobacterial cells. Intact cells of *B. lactis* BI07 showed a dose-dependent enolase activity (data not shown).

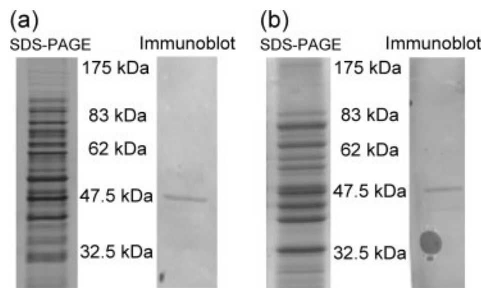

Fig. 2. Immunoblot analysis using anti-enolase antiserum of cytoplasmic (a) and cell wall (b) fractions of *B. lactis* BI07 resolved by SDS-PAGE.

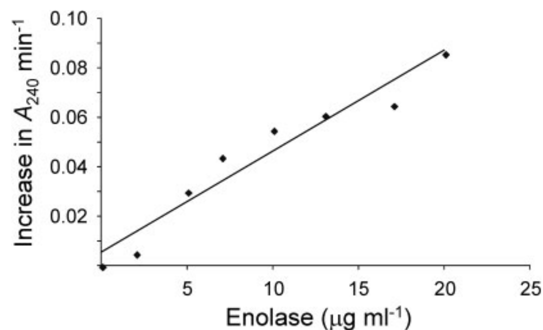

Fig. 4. Enzymic activity of the recombinant His$_6$-enolase of *B. lactis* BI07. The activity was determined using a direct enzyme assay: different amounts of the purified recombinant His$_6$-enolase were incubated with 15 mM 2-PGE. The release of PEP was evaluated by measuring the increase in absorbance at 240 nm.

Analysis of Plg binding to *B. lactis* BI07 enolase

In order to characterize the Plg-binding activity of *B. lactis* BI07 enolase, the gene was sequenced, cloned, and the recombinant His$_6$-tagged enolase protein was purified by affinity chromatography. The nucleotide sequence of *B. lactis* BI07 enolase (GenBank accession no. DQ117970) revealed 98 % identity to the enolase gene of *B. longum* NCC2705 (GenBank accession no. AE014295). In Fig. 4 we show the dose-dependent enzymic activity for the recombinant His$_6$-tagged *B. lactis* BI07 enolase. 2-PGE was converted to PEP with a specific activity of 1.35 μmol PEP synthesized per min per mg protein. These data indicate that *B. lactis* BI07 enolase was purified in the native 3D structure. To evaluate the specific Plg-binding activity of *B. lactis* BI07 enolase, a solid-phase Plg-binding assay (Sanderson-Smith *et al.*, 2007) was carried out. The saturation binding analysis of biotinylated Plg to immobilized recombinant *B. lactis* BI07 enolase is reported in Fig. 5. Non-specific binding was determined in the presence of a 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 a negative control, BSA-coated wells were utilized. According to our data, Plg binding to immobilized enolase was dose-dependent and saturable binding was achieved with 250 nM Plg. The best-fit nonlinear regression analysis allowed calculation of an equilibrium dissociation constant (K_D) for the interaction of enolase with Plg of 42 nM. The binding was completely inhibited in the presence of 0.5 M EACA, proving the crucial role of enolase lysine(s) in Plg binding.

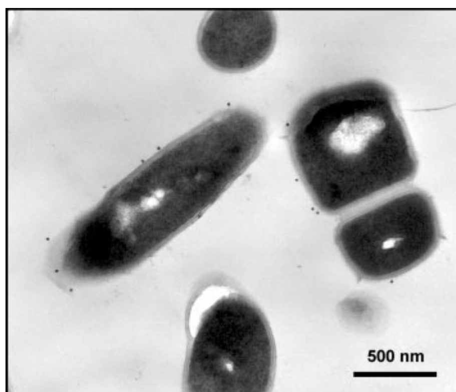

Fig. 3. Immunoelectron microscopic localization of enolase on the cell wall of *B. lactis* BI07. Enolase was detected on the bacterial surface by anti-enolase antiserum and a secondary antibody coupled to 10 nm gold particles in pre-embedding experiments. An ultrathin section of *B. lactis* BI07 cells at a magnification of ×22 000 shows enolase (black dots) localized directly in the cell wall region.

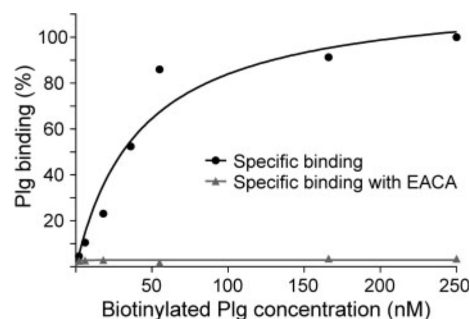

Fig. 5. Saturation binding analysis of biotinylated Plg to immobilized recombinant His-tagged *B. lactis* BI07 enolase. Specific Plg binding (•) was calculated at each concentration of biotinylated Plg by subtracting non-specific binding obtained in the presence of a 50-fold molar excess of unlabelled 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).

Homology model of *B. lactis* BI07 enolase and identification of the putative Plg-binding sites

In an attempt to identify the putative Plg-binding site(s) of *B. lactis* BI07 enolase, a homology model of the protein was generated using the coordinates of four enolases of known structure: the enolases of *Strep. pneumoniae* (PDB code 1W6T) and *Enterococcus hirae* (PDB code 1IYX), which share 55 % identity with *B. lactis* BI07 enolase, and the enolases of *Escherichia coli* (PDB code 1E9I) and *M. jannaschii* (PDB code 2PA6), which show 52 % identity with *B. lactis* BI07 enolase (Fig. 6a). In Fig. 6(b), a model of the *B. lactis* BI07 enolase dimer is represented. The Ramachandran Plot analysis (Willard *et al.*, 2003) of the model for *B. lactis* BI07 enolase indicated a good stereochemical quality (data not shown). The monomer consists of two domains: the N-terminal domain contains a three-stranded antiparallel β-sheet followed by six α-helices, and the C-terminal domain is composed of an α/β-barrel structure. *B. lactis* BI07 enolase possesses a C-terminal lysine at amino acid position 432, which constitutes a homologue of the pneumococcal Plg-binding site BS1 (Bergmann *et al.*, 2003). In order to evaluate whether the surface loop L3 of *B. lactis* BI07 enolase contained a peptide region homologous to the internal Plg-binding site BS2, located on the surface-exposed loop L3 of *Strep. pneumoniae* enolase (residues 248–256; Ehinger *et al.*, 2004), the protein structures were superimposed. A 0.4 Å (0.04 nm) root-mean-square distance (r.m.s.d.) between the Cα atom positions of the *B. lactis* BI07 and pneumococcal enolases demonstrated an overall structural similarity between these two proteins. In particular, the enolases of *B. lactis* BI07 and *Strep. pneumoniae* shared a significant structural similarity in the loop L3 (r.m.s.d. of 0.64 Å, 0.064 nm) (Fig. 6c). Residues 248–256 within the L3 region of *B. lactis* BI07 enolase and the BS2 region of pneumococcal enolase displayed high structural homology.

Impact of BS1 and BS2 homologues on Plg binding to *B. lactis* BI07 enolase

In order to determine the role of the putative Plg-binding sites BS1 and BS2 of *B. lactis* BI07 enolase in Plg binding, three genetic mutants of the corresponding enolase (*eno*) gene were constructed: (i) the eno^{BS1} mutant gene, deleted of the BS1 pneumococcal homologue, obtained by the deletion of the codon encoding the C-terminal lysyl residue at position 432; (ii) the eno^{BS2} mutant gene, obtained by site-directed mutagenesis of the amino acids homologous to those that were previously demonstrated to be essential in Plg-binding to the BS2 site of the pneumococcal enolase (Bergmann *et al.*, 2003; Ehinger *et al.*, 2004), 251 (Lys→Leu), 252 (Glu→Gly) and 255 (Lys→Leu); and (iii) the eno^{double} mutant gene constructed by deleting the C-terminal lysine from the eno^{BS2} mutant. The recombinant His-tagged $enolase^{BS1}$, $enolase^{BS2}$ and $enolase^{double}$ proteins were purified and their specific Plg-binding activity was evaluated in a saturation binding analysis of biotinylated Plg to the immobilized recombinant proteins (Fig. 7). The interaction of $enolase^{BS1}$ with Plg was dose-dependent and saturable, with a K_D of about 46.2 nM, a value comparable to that shown by the wild-type *B. lactis* BI07 enolase protein. In contrast, neither $enolase^{BS2}$ nor $enolase^{double}$ showed specific and saturable interaction with human Plg, proving the crucial role of the BS2 homologue in Plg binding to *B. lactis* BI07 enolase.

DISCUSSION

Plg-binding bifidobacterial strains belonging to the human-associated species *B. longum*, *B. bifidum* and *B. breve* (Ventura *et al.*, 2007), as well as the Plg-binding probiotic strain *B. lactis* BI07 (Candela *et al.*, 2007), share the enolase enzyme as one of their putative surface Plg receptors. Enolase is an essential glycolytic enzyme catalysing the formation of PEP from 2-PGE. Localized on the cell surface, it is one of the best-characterized human Plg receptors in prokaryotes and eukaryotes (Pancholi & Fischetti, 1998; Bergmann *et al.*, 2001; Crowe *et al.*, 2003; Schaumburg *et al.*, 2004; Lähteenmäki *et al.*, 2005; Antikainen *et al.*, 2007b; Knaust *et al.*, 2007). A single enolase gene is present in the published genomes of the bifidobacterial species *B. animalis* subsp. *lactis* (accession no. NC_011835), *B. longum* (accession no. NC_004307), *B. longum* subsp. *infantis* (accession no. NC_011593), *B. adolescentis* (accession no. NC_008618) and *B. bifidum* (accession no. NZ_ABQP00000000). Thus, analogously to *Streptococcus pyogenes*, *Strep. pneumoniae* and *Staphylococcus aureus* (Antikainen *et al.*, 2007a), the same enolase gene product is both an essential glycolytic enzyme in the cytoplasm and, localized on the cell surface, a receptor for human Plg. As reported for several prokaryotes and eukaryotes that display enolase on the cell surface (Pancholi, 2001), the bifidobacterial enolase lacks either predicted or detectable protein-sorting elements for secretion and anchorage onto the bacterial cell wall. The mechanisms of secretion and surface localization of this essential glycolytic enzyme are still under debate. Recently for *Strep. pneumoniae*, scavenging of cytoplasmic proteins released through allolysis was proposed to account for the presence of enolase on the cell surface (Claverys & Havarstein, 2007). On the other hand, according to Boël *et al.* (2004), the export of enolase may depend on the modification of the enzyme by covalent binding of 2-PGE to Lys-341. The role of enolase as a surface Plg receptor was further studied using *B. lactis* BI07 as a model strain. In a Western blot analysis we confirmed that *B. lactis* BI07 enolase is present in the cytoplasm and in the cell wall fractions. Moreover, providing direct evidence for the presence of enolase on the bifidobacterial cell surface, enolase was visualized on the cell surface of *B. lactis* BI07 by immunoelectron microscopy. Similarly to *Strep. pyogenes* (Pancholi & Fischetti, 1998), *Strep. pneumoniae* (Kolberg *et al.*, 2006) and *Strep. suis* (Esgleas *et al.*, 2008), the *B. lactis* BI07 enolase is displayed on the bacterial cell surface

(a)

```
              1        10        20        30        40        50        60        70        80        90       100
              |         |         |         |         |         |         |         |         |         |         |
BI07 ----------MAVIESVYARQILDSRGNPTVQVVLDTEDGAQGLGLVPSGASTGEAEAWERRDGDKSVYGGKGVLNAVKAVNEVIAPKVIGMDAADQRALDDLMIELDGTPNK
1IYX ----------MSIITDVYAREILDSRGNPTIEVEVYTESGAFGRGMVPSGASTGEYEAVELRDGDKARYGGKGVTKAVDNVNNIIAEAIIGYDVRDQMAIDKAMIALDGTPNK
1W6T MRGSHHHHHHMSIITDVYAREVLDSRGNPTLEVEVYTESGAFGRGMVPSGASTGEHEAVELRDGDKSRYGGLGTQKAVDNVNNIIAEAIIGYDVRDQQAIDRAMIALDGTPNK
2PA6 ---MLYNMDERFEIKDIVAREVIDSRGNPTVEVEVITKGNGYGSAIVPSGASTGTHEALELRDKEK-RFGGKGVLMAVENVNSIIRPEILGYDARMQREIDTIMIELDGTPNK
1E9I -----------SKIVKIIGREIIDSRGNPTVEAEVHLEGGFVGMAAAPSGASTGSREALELRDGDKSRFLGKGVTKAVAAVNGPIAQALIGKDAKDQAGIDKIMIDLDGTENK
                * .: .*:::*******::. :  :..  * . .*******  ** * ** :*  : * *.  **  **  *   ::* *.  *  :*  ** **** **

       110       120       130           140       150       160       170       180       190        200       210
         |         |         |             |         |         |         |         |         |          |         |
BI07 GKLGANAILGVSLAALYASAESAGLPLYRYIGGTN----GHILPVPNMNIMNGGAHADFATDIQEYMISPYGFDTYSEALRAGVEVYHTLKNVLKKE-GLNTG-LGDEGGFAP
1IYX GKLGANAILGVSIAVARAAADYLEVPLYHYLGGFN----TKVLPTPMMNIINGGSHADNSIDFQEFMIMPVGAPTFKEALRMGAEVFHALAAILKSR-GLATS-VGDEGGFAP
1W6T GKLGANAILGVSIAVARAAADYLEIPLYSYLGGFN----TKVLPTPMMNIINGGSHSDAPIAFQEFMILPVGAPTFKEALRYGAEIFHALKKILKSR-GLETA-VGDEGGFAP
2PA6 SRLGANAILAVSLAVAKAAAATAKIPLYKYLGGFN----SYVMPVPMMNVINGGKHAGNDLDLQEFMIMPVGATSISEAVRMGSEVYHVLKNVILEKYGKNAVNVGDEGGFAP
1E9I SKFGANAILAVSLANAKAAAAAKGMPLYEHIAELNGTPGKYSMPVPMMNIINGGEHADNNVDIQEFMIQPVGAKTVKEAIRMGSEVFHHLAKVLKAK-GMNTA-VGDEGGYAP
     .::******.**:*    *:*    :*** ::.  *        :*.* **::*** *:.      :**:** * *   : .**:* * *::* *   ::   . *  :  :*****:**

          220       230        240       250          260       270       280       290       300       310
            |         |          |         |            |         |         |         |         |         |
BI07 KMKSNEDSLKYIMDAISAAGYEPGKQIGICLDVASSEFYNKETGKYR---FDGE---ERDSAYMLDYYENLINEYPIVSIEDPFNEEGWEDWAAITARLGDRLQFVGDDLLVT
1IYX NLGSNEEGFEVIIEAIEKAGYVPGKDVVLAMDAASSEFYDKEKGVYVL-ADSGE--GEKTTDEMIKFYEELVSKYPIISIEDGLDENDWDGFKKLTDVLGDKVQLVGDDLFVT
1W6T RFEGTEDGVETILAAIEAAGYVPGKDVFLGFDCASSEFYDKERKVYDYTKFEGEGAAVRTSAEQIDYLEELVNKYPIITIEDGMDENDWDGWKALTERLGKKVQLVGDDFFVT
2PA6 PLKTSREALDLLTESVKKAGYED--EVVFALDAAASEFY-KD-GYYYV-----EG-KKLTREELLDYYKALVDEYPIVSIEDPFHEEDFEGFAMITKEL--DIQIVGDDLFVT
1E9I NLGSNAEALAVIAEAVKAAGYELGKDITLAMDCAASEFY-KD-GKYVL---AGEGNKAFTSEEFTHFLEELTKQYPIVSIEDGLDESDWDGFAYQTKVLGDKIQLVGDDLFVT
      :  . :..  :  ::. ***    :: : :* *:**** *: * *     *           .: : * .:***::*** :.*..::.:      * *   :*:****::**

      320       330       340       350       360       370       380       390       400       410       420       430
        |         |         |         |         |         |         |         |         |         |         |         |
BI07 NPARLQKAIDLGAANSLLVKLNQIGSVTETLDAIELATANGYTSMVSHRSGETPDTTISDLAVAKNTRQIKTGAPARGERVAKYNRLLEIEEELGSTAQYAGYSAFKACKKYLAK
1IYX NTQKLSEGIEKGIANSILIKVNQIGTLTETFEAIEMAKEAGYTAVVSHRSGETEDSTISDIAVATNAGQIKTGSLSRTDRIAKYNQLLRIEDQLGEVAEYKGLKSFYNLKAA---
1W6T NTDYLARGIQEGAANSILIKVNQIGTLTETFEAIEMAKEAGYTAVVSHRSGETEDSTIADIAVATNAGQIKTGSLSRTDRIAKYNQLLRIEDQLGEVAEYRGLKSFYNLKK----
2PA6 NVERLRKGIEMKAANALLLKVNQIGTLSEAVDAAQLAFRNGYGVVVSHRSGETEDTTIADLSVALNSGQIKTGAPARGERTAKYNQLIRIEQELG-LSKYAGRN-FRCPF-----
1E9I NTKILKEGIEKGIANSILIKFNQIGSLTETLAAIKMAKDAGYTAVISHRSGETEDATIADLAVGTAAGQIKTGSMSRSDRVAKYNQLIRIEEALGEKAPYNGRKEIKGQA-----
     *   * ..*:     **::*:*.****:::*:. * ::*     **     ::******* *:**:*::*.  : *****: :* :* ****:*:.**: **  : * *   :
```

(b)

N-term (A)

N-term (B)

C-term (B)

C-term (A)

Lys255A

Lys251A

Lys251B

Lys255B

Lys255A

Lys251A

Plg binding site BS1:

S. pneumoniae BS1: -KK (C-term)

B. lactis BS1: -AK (C-term)

Structural homologue of Plg binding site BS2:

S. pneumoniae BS2: 248-FYDKERKVY-256

B. lactis BS2 homologue: 248-FYNKETGKY-256

(c)

1W6T

BI07

Lys255

Lys251

Fig. 6. (a) Multiple sequence alignment of the sequences of *Bifidobacterium lactis* BI07 (BI07), *Enterococcus hirae* (1IYX), *Streptococcus pneumoniae* (1W6T), *Methanococcus jannaschii* (2PA6) and *Escherichia coli* (1E91) enolases, with the secondary structure indication (α-helix, yellow; β-strand, cyan) derived from the JPRED prediction for *B. lactis* BI07 enolase and from the PDB structure for all the remaining sequences. (b) Ribbon scheme of dimeric *B. lactis* BI07 enolase model structure, derived using the sequence alignment reported in (a). In the bottom image the ribbons are rotated 90 ° around the long horizontal axis. Ribbons are coloured from blue, in the proximity of the N-terminus, to red at the C-terminus. (c) Detailed view of the superimposed L3 regions of *B. lactis* BI07 enolase (blue) and pneumococcal enolase (yellow). Lysines 251 and 255 and the pneumococcal homologue lysines 251 and 254, crucial for Plg binding, are represented in ball and stick notation. The molecular graphic images were produced using the UCSF Chimera package.

with relatively low abundance. The ability of intact *B. lactis* BI07 cells to convert 2-PGE to PEP in a direct enzyme assay demonstrated that the surface enolase still retains its functional enzyme activity. The His-tagged *B. lactis* BI07 enolase was purified and characterized with respect to its enzyme activity and, most importantly, its secondary 'moonlighting' function as a Plg receptor. In a direct enzyme assay, the K_m for 2-PGE was calculated to be 0.73 mM. This value is in the range reported for other bacterial enolases, including the *Strep. pyogenes* (1.492 mM) (Pancholi & Fischetti, 1998), *Strep. pneumoniae* (4.5 mM) (Bergmann *et al.*, 2001) and *Bacteroides fragilis* (0.210 mM) (Sijbrandi *et al.*, 2005) enolases. The affinity of *B. lactis* BI07 enolase for human Plg was calculated in a saturation binding analysis. The purified His-tagged *B. lactis* BI07 enolase showed a high affinity for human Plg, with a K_D value in the nanomolar range (42.8 nM). Compared to the eukaryotic enolase, which possesses a K_D value of about 0.1–2 μM (Redlitz *et al.*, 1995), the *B. lactis* BI07 enolase shows a significantly higher affinity for human Plg. On the other hand, in comparison with bacterial enolases from pathogenic micro-organisms, *B. lactis* BI07 enolase shows a slightly lower affinity to human Plg. *Strep. pyogenes*, *Strep. pneumonia*e and *Strep. suis* enolases demonstrated K_D values for human Plg of 1–4, 0.55 and 14 nM, respectively (Pancholi & Fischetti, 1998; Bergmann *et al.*, 2003; Esgleas *et al.*, 2008). The best-fit nonlinear regression of data obtained in the saturation binding analysis to Plg indicated that the *B. lactis* BI07 enolase possesses only one site of binding to human Plg. Our data are in general agreement with those reported for *Strep. pyogenes* (Derbise *et al.*, 2004) and *Strep. pneumoniae* enolases (Bergmann *et al.*, 2005). However, while in the case of *Strep. pyogenes* the C-terminal lysines have been indicated as the Plg binding site, for pneumococcal enolase Plg-binding mainly depends on the internal Plg-binding site BS2 (residues 248–256) located on the surface-exposed loop L3 (Bergmann *et al.*, 2003, 2005; Ehinger *et al.*, 2004), while the C-terminal lysine residue(s) (BS1) is either not or only marginally involved. In particular, within the Plg-binding site BS2 of pneumococcal enolase Lys-251, Lys-254 and Glu-252 are critical for Plg binding (Ehinger *et al.*, 2004). The homology model of the *B. lactis* BI07 enolase indicated that the protein possesses a structural homologue of both the internal Plg-binding site BS2 of pneumococcal enolase and the C-terminal lysine that constitutes the Plg-binding site BS1. Site-direct mutagenesis of Lys-251, Glu-252 and Lys-255 within the BS2 homologue of *B. lactis* BI07 enolase impaired its Plg-binding activity. In contrast, the deletion of the *B. lactis* BI07 enolase C-terminal lysine

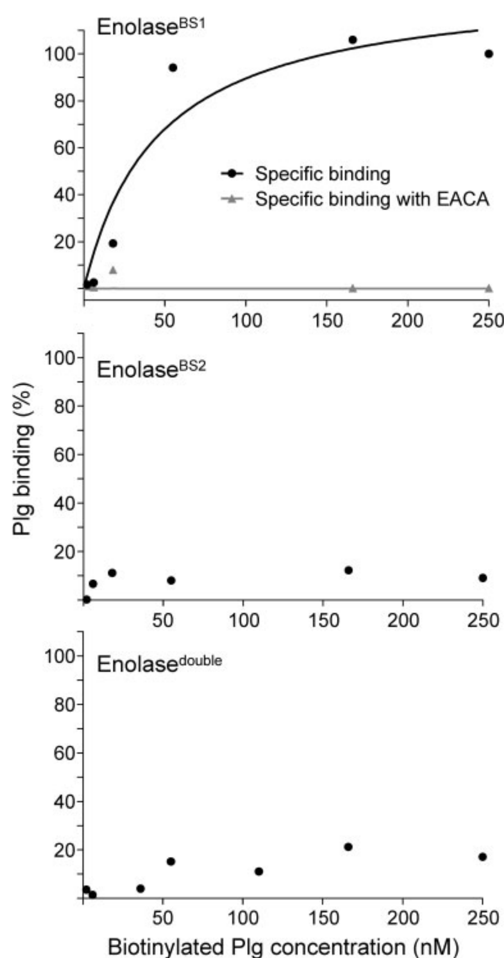

Fig. 7. Saturation binding analysis of biotinylated Plg to immobilized recombinant His-tagged *B. lactis* BI07 Enolase^{BS1}, Enolase^{BS2} and Enolase^{double} proteins. At each concentration of biotinylated Plg, specific binding (●) was calculated by subtracting the non-specific binding obtained in the presence of a 50-fold molar excess of unlabelled Plg from the total binding. For Enolase^{BS1}, 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).

did not have any impact on Plg interaction. Taken together, our data demonstrate that the interaction between the *B. lactis* BI07 enolase and human Plg involves the internal Plg-binding site homologous to the BS2 site of pneumococcal enolase. In particular, as in the case of pneumococcal enolase (Ehinger *et al.*, 2004), the positively charged residues Lys-251 and Lys-255, and the negatively charged Glu-252, are vital for Plg interaction. Disruption of the enolase gene would provide *in vivo* proof of its role as a Plg receptor; however, the presence of only one bifidobacterial enolase gene, which is essential for bacterial survival, renders the construction of a viable enolase-defective *B. lactis* BI07 mutant impossible.

There is a growing number of reports concerning the expression of cytoplasmic housekeeping proteins on the bacterial cell surface of Gram-positive micro-organisms (Pancholi, 2001; Pancholi & Chhatwal, 2003; Schaumburg *et al.*, 2004; Sijbrandi *et al.*, 2005; Lee *et al.*, 2006; Antikainen *et al.*, 2007a; Knaust *et al.*, 2007). Although their mechanism of secretion still remains unclear, localized on the bacterial cell surface these multifunctional proteins acquire a 'moonlighting' function (Jeffery, 1999), with a role in the interaction process with the host. We believe that this is the first report of the localization of enolase on the cell surface of four species of *Bifidobacterium*, a health-promoting member of the human intestinal microbiota. Interestingly, the presence of surface enolase has been recently reported for two species belonging to the gut commensal genus *Lactobacillus* (Antikainen *et al.*, 2007a; Castaldo *et al.*, 2009). However, while the *Lactobacillus crispatus* surface enolase is a receptor for human Plg, the *Lactobacillus plantarum* surface enolase is a fibronectin-binding protein. Acting as a human Plg receptor, the bifidobacterial surface enolase may play a role in the interaction with the host. Although it will be necessary to screen for the presence of surface enolases in other bifidobacterial species, our findings raise the question of the impact of glycolytic enzymes in the biology of the *Bifidobacterium*–host interaction. In particular, concerning enolase, further studies are justified to investigate its capability to interact with extracellular matrix proteins such as laminin and fibronectin, as already reported for the enolases from *Lactobacillus*, *Streptococcus* and *Staphylococcus* (Antikainen *et al.*, 2007a).

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