RESEARCH LETTER

Evaluation of the ability of *Bifidobacterium longum* to metabolize human intestinal mucus

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Abstract

The ability of *Bifidobacterium longum* to use intestinal mucus as a metabolizable source was characterized. *Bifidobacterium longum* biotype longum NCIMB8809 was grown in a chemically semi-defined medium supplemented with human intestinal mucus, and the cytoplasmic protein profiles and several glycosyl hydrolase activities were analysed and compared with those obtained from the same bacterium grown in the absence of mucus. We were able to identify 22 different proteins in the cytoplasmic fraction, of which nine displayed a different concentration in the presence of mucus. Among the proteins whose concentrations varied, we found specific enzymes that are involved in the response to different environmental conditions, and also proteins that mediate interaction with mucus in bacteria. Significant changes in some glycoside-hydrolysing activities were also detected. In addition, stable isotope labelling of amino acids in cell culture demonstrated that *B. longum* incorporates leucine from the glycoprotein matrix of mucin within its proteins. This study provides the first proteomic data regarding the interaction of *B. longum* with intestinal mucus, and contributes to the understanding of the behaviour of this intestinal species in its natural ecological niche.

Introduction

Microorganisms of the genus *Bifidobacterium* are common inhabitants of the human gastrointestinal tract, constituting one of the predominant microorganisms in the colon during the early stages of life (Harmsen *et al.*, 2000; Lay *et al.*, 2005). Because of the health-promoting effects attributed to some strains of this genus, these bacteria are attractive probiotic candidates and they are routinely included in functional food products, mainly dairy products, in which they are present as adjunct cultures (Masco *et al.*, 2005; Salminen *et al.*, 2005). Furthermore, several studies have demonstrated that apart from being present in the luminal or the faecal community, bifidobacterial populations are also abundant among the mucosa-adherent community (Gueimonde *et al.*, 2007; Leitch *et al.*, 2007; Turroni *et al.*, 2009a, b). Some *Bifidobacterium* strains have been shown to display exocellular glycosidases potentially acting on sugar chains of mucin glycoproteins. In particular, *Bifidobacterium bifidum* possesses an arsenal of enzymatic activities, including endo-α-*N*-acetylgalactosaminidases and α-L-fucosidases, that are likely to be involved in mucus degradation at the intestinal level (Katayama *et al.*, 2004, 2005; Ruas-Madiedo *et al.*, 2008). Some of these enzymes are also present in other *Bifidobacterium* species, such as *Bifidobacterium longum* and *Bifidobacterium breve*, likely contributing to a partial degradation of the glycoprotein matrix of mucus (Ruas-Madiedo *et al.*, 2008).

Bacteria that are able to multiply at the expense of mucus display an adaptative advantage to survive in the colon. In a previous report, we were able to demonstrate that *B. longum* NCIMB8809 was able to partially degrade mucin from porcine stomach (Ruas-Madiedo *et al.*, 2008). In the present study, we aim to analyse the capacity of this strain to use human intestinal mucus as a metabolizable energy source, and to investigate in-depth the proteins and enzymatic activities that could be involved in the interactions between *B. longum* and mucus.

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Materials and methods

Microorganisms and growth conditions

Bifidobacterium longum NCIMB 8809 (National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland, UK), a potential probiotic able to produce antimicrobial substances and originally isolated from nursling stools, was used as a model microorganism for this study (O'Riordan & Fitzgerald, 1998). The preinoculum was obtained by culturing the strain on MRSc agar plates [MRSc: MRS broth (Difco) supplemented with 0.05% (w/v) L-cysteine (Merck)]. Subsequently, an isolated colony was transferred to MRSc broth and grown overnight. The culture was washed three times with a semi-defined medium for *B. longum* (SDMBL) (Couté *et al.*, 2007), and inoculated at 0.05% in the same medium with, or without, human intestinal mucus. For stable isotope labelling of amino acids in cell culture (SILAC) experiments, $^{13}C_6$-leucine was used as the labelled amino acid in the SDMBL medium, and the experiments were carried out exactly as described by Couté *et al.* (2007). The working concentration of mucus in the SDMBL medium was 0.4 mg total protein mL^{-1} (Ouwehand *et al.*, 2002). The human intestinal mucus had been collected from the healthy part of resected colonic tissue as described previously (Ouwehand *et al.*, 2002). The mucus was dissolved in HEPES–Hanks buffer (10 mM HEPES, pH 7.4) and stored at −20 °C until use. Incubations were carried out at 37 °C in an anaerobic chamber (Don Whitley Scientific, West Yorkshire, UK) in an atmosphere of 80% N_2, 10% H_2, 10% CO_2, or, alternatively, in anaerobic jars (Anaerocult A System; Merck). At least three different biological replicates were performed for each condition.

Two-dimensional electrophoresis (2DE) and quantitative proteomic analysis

Variations in the level of cytoplasmic protein concentrations of *B. longum* cells grown in the presence of mucus were analysed by a 2DE study. We used a pI range between 4 and 7, which theoretically allows the separation of about two-thirds of the total proteome of *B. longum* (Sánchez *et al.*, 2008). In all the experiments, cells were collected in the early stationary phase of growth ($OD_{600\,nm}$ 0.5–0.9). Cell-free protein extracts were obtained as described previously (Ruiz *et al.*, 2009a). For 2DE analysis, 500 μg of protein from *B. longum* NCIMB8809 extracts were loaded onto a strip with an immobilized pH range of 4 to 7 (GE Healthcare) and focused at 65 000 V h^{-1}. Separation in the second dimension was carried out in 12.5% polyacrylamide-sodium dodecyl sulphate gels and protein spots were visualized with colloidal Coomassie staining. Proteins were identified by peptide mass fingerprinting and matrix-assisted laser desorption ionization/time of flight/MS at the Proteomics Unit of the

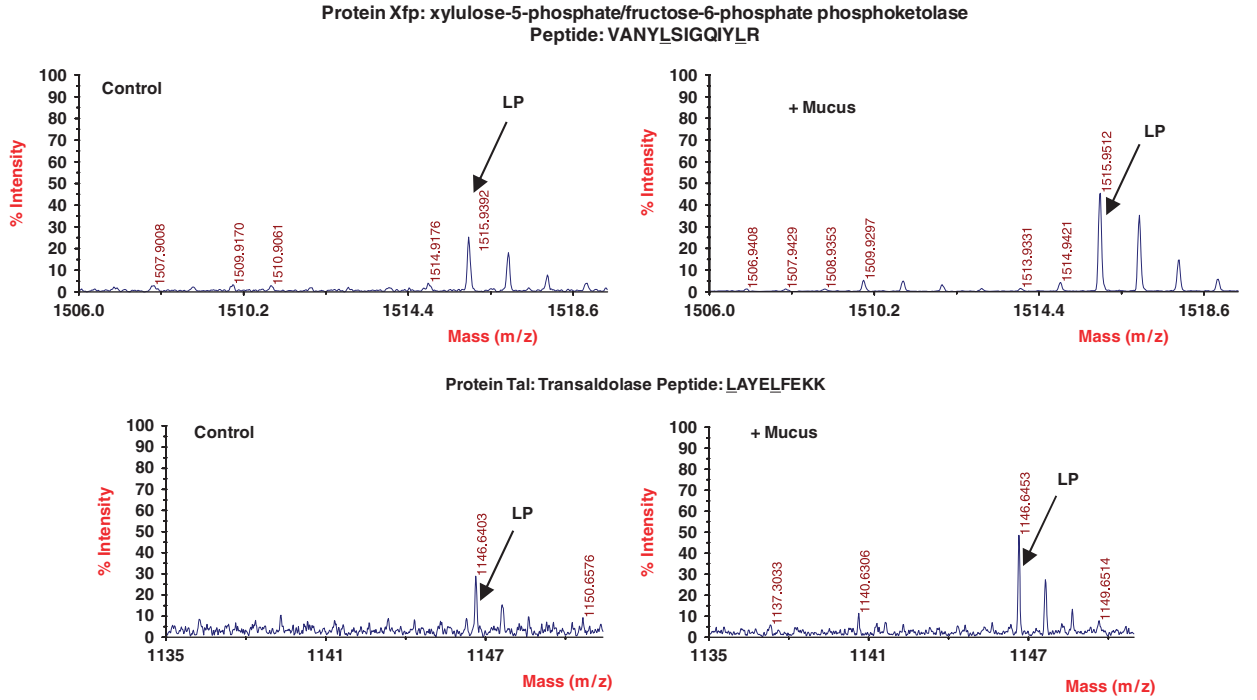

Fig. 1. $^{13}C_6$-leucine incorporation by *Bifidobacterium longum* NCIMB8809 in the presence and absence of mucus. Representative mass spectra from peptides resulting from digestion of the proteins Xfp and Tal are shown. The arrows indicate the presence of light peptide forms (LP) containing one $^{12}C_6$-Leu and one $^{13}C_6$-Leu.

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Parque Científico de Madrid (Cantoblanco, Madrid, Spain). Protein identification was achieved by combining MS data to search a nonredundant protein database (NR; 4.106 entries; National Center for Biotechnology Information) using the MASCOT software (Matrix Science). Spot detection, volume quantification and statistical analysis were performed using IMAGEMASTER PLATINUM software (version 5.00, GE Healthcare). Three protein extracts, coming from three independent cultures, were obtained for each condition (mucus absence or mucus presence), and at least three independent gels (technical replicates) were performed for each extract.

Determination of enzymatic activities and organic acid production

Enzymatic activities were measured for the cytoplasmic fraction and for the secreted fraction. Cell-free protein extracts from the cytoplasmic fraction were obtained as described above. The supernatants were collected by centrifugation from cells grown to the early stationary phase, concentrated 10 times using VivaSpin Columns (cutoff 3000 kDa, Sartorius), and filtered through 0.22-μm sterile filters. Glycosidase activities were measured spectrophotometrically from the release of *p*-nitrophenol (*p*NP) produced by the enzymatic hydrolysis of the corresponding *p*NP-glycoside substrates (Sigma), as described previously (Ruiz *et al.*, 2009b). One unit of enzymatic activity was defined as the amount of protein that releases 1 nmol *p*NP min^{-1}. Specific activities were determined in duplicate for each culture, and expressed as units per milligram protein. For organic acid determination, *Bifidobacterium* cultures, grown in the absence or presence of mucus, were collected by centrifugation. Cells were removed from the suspension, and the supernatant was analysed by HPLC according to Ruas-Madiedo *et al.* (2005), to quantify lactic, acetic and pyruvic acids, as well as glucose and fructose.

Results and discussion

Previous studies demonstrated that *B. longum* NCIMB8809 showed significant differences in growth when cultivated in a chemically defined medium in the presence of porcine mucin, displaying a higher growth after 48 h of incubation when compared with mucin absence conditions (Ruas-Madiedo *et al.*, 2008). This suggested to us that this strain could also display some ability to use human intestinal mucin as a metabolizable source. In fact, when a similar experiment was performed, we showed that, after overnight growth, *B. longum* NCIMB 8809 reached lower ODs at 600 nm in the absence of, rather than in the presence of, mucus (data not shown), suggesting that the presence of mucus in the growth medium provides an extra energy source that allows the bacterium to reach a higher OD.

The human intestinal mucus layer plays an important role in preventing adhesion and binding by enteropathogens and toxins, and it consists mainly of water (*c.* 95%) and glycoproteins (1–10%) (Hamer *et al.*, 2009). The glycoprotein matrix serves as a nutrient for bacterial growth in the intestine, and numerous bacterial species have been shown to display metabolic activities capable of degrading the complex links between carbohydrates and proteins, or within them, including *B. bifidum*, *Bacteroides fragilis* and *Akkermansia muciniphila* (Derrien *et al.*, 2004; Macfarlane *et al.*, 2005; Ruas-Madiedo *et al.*, 2008). In order to determine whether amino acids present in the glycoprotein matrix of mucin can be taken up and incorporated into the proteins synthesized by *B. longum* during growth in SDMBL broth, SILAC experiments were performed as described by Couté *et al.* (2007). *Bifidobacterium longum* NCIMB8809 was grown for 13 generations in SDMBL broth and the presence of heavy and light leucine in *B. longum* proteins was detected by MS. The percentage of light peak height on heavy peak height was 1.30 ± 0.05 times higher with mucus for peptides containing one leucine, and the percentage of

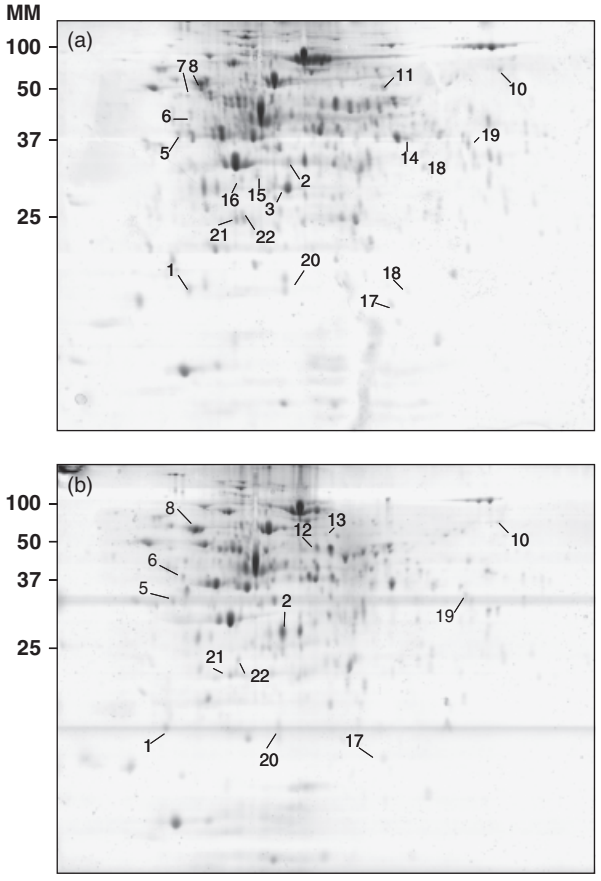

Fig. 2. 2D gels showing the proteins expressed by *Bifidobacterium longum* NCIMB8809 in SDMBL medium without (a) and with (b) human mucus. Spot codes refer to the proteins in Table 1. MM, molecular mass.

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medium peak height on heavy peak height was 1.75 ± 0.09 times higher with mucus for peptides containing two leucines, suggesting that the bacterium is utilizing other leucine sources different from the one provided by the labelled amino acid (Couté *et al.*, 2007). As an example, Fig. 1 shows the spectra of two peptides [from the enzymes xylulose-5-phosphate/fructose-6-phosphate phosphoketolase (Xfp) and transaldolase (Tal)], in which the presence of light peptides (containing one $^{12}C_6$-Leu and one $^{13}C_6$-Leu) is significantly higher when the cells were grown in the presence of human mucus, indicating the incorporation of mucus-derived leucine.

In order to analyse the influence of human intestinal mucus on the cytoplasmic protein profiles of *B. longum* NCIMB8809, a 2DE analysis was carried out. Twenty spots (Fig. 2) were selected on the basis of their apparent variation of cytoplasmic concentrations, and the proteins were identified using MS (Table 1). Among the proteins, whose expression was affected by the presence of mucus, it is worth pointing out the lower concentration of a putative elongation factor Ts. This protein associates with the elongation factor Tu during protein translation in the ribosome, but they can also be displayed on the surface of the bacteria, where they have been reported to act as mediators of adhesion processes to mucins (Granato *et al.*, 2004; Wu *et al.*, 2008). Ketol acid reductoisomerase 1, a protein involved in the synthesis of branched chain amino acids (BCAAs), significantly increased its concentation as a response to the presence of mucus. BCAAs are the most abundant amino acids in membrane proteins, and it is known that many membrane proteins are induced in bacteria as a response to mucus (Ruas-Madiedo *et al.*, 2008; Tu *et al.*, 2008), suggesting an enrichment of BCAA-reach proteins, likely membrane proteins, in the presence of mucus. Furthermore, ribose 5-phosphate isomerase (RpiA) was drastically overproduced in the presence of mucus. RpiA is an enzyme that catalyses the interconversion between ribose-5-phosphate and ribulose-5-phosphate in the pentose phosphate pathway. These data suggest that the carbohydrate preferences of *B. longum* could change when mucus is present in the medium, and could indicate a shift in the carbohydrate catabolism of this bacteria, which prompted us to determine some glycosyl hydrolase activities, the glucose consumption and the abundance of some secondary metabolites.

Table 1. Identification of *Bifidobacterium longum* NCIMB8809 proteins affected by the presence of mucus

Spot no.	Putative function	Gene	Accession no.	Mass	*p*I	No. of peptides matched	Coverage	Normalized volume ratio
BL 1	Hypothetical protein in DPS family		gi|23465202	17765	4.57	10	52%	—
BL 2	Ketol-acid reductoisomerase 1	*ilvC1*	Q8G6V2|ILVC1_BIFLO	38707	5.10	18	54%	↑↑
BL 3	Elongation factor Ts	*tsf*	Q8G485|EFTS_BIFLO	29961	5.07	19	64%	↓↓↓
BL 4 = BL 5	DNA polymerase sliding clamp subunit (PCNA homolog)		gi|23335940	40999	4.54	14	48%	↓↓↓
BL 6	Threonine synthase	*thrC*	gi|23465602	54293	4.63	17	41%	—
BL 7	Hypothetical protein BL0121		gi|23464747	50596	4.66	18	59%	↓↓↓
BL 8	Ribosomal protein S1		gi|23336678	54397	9.19	26	55%	—
BL 10	ABC_NikE_OppD_transporters	*dppD*	gi|23465950	73291	5.91	30	55%	↓
BL 11	CTP synthase	*pyrG*	Q8G5X7|PYRG_BIFLO	61344	5.40	30	62%	↓↓↓
BL 12	Predicted GTPase	*obg*	gi|23336705	61284	5.15	25	52%	—
BL 13	Dihydroxy-acid dehydratase	*ilvD*	Q8G3H2|ILVD_BIFLO	67199	5.28	20	42%	—
BL 14	Acetate kinase	*ackA*	gi|23465540	44287	5.47	22	57%	—
BL 15	Sugar kinases, ribokinase family	*kdgK*	gi|23335430	32547	4.88	25	75%	—
BL 16	Response regulator with putative antiterminator output domain	*amiR*	gi|23336562	29134	4.85	14	49%	—
BL 17	Predicted redox protein, regulator of disulfide bond formation		gi|23336616	15161	5.32		7%	↑
BL 18	Protein-tyrosine-phosphatase	*wzb*	gi|23336613	19944	5.49		8%	↓↓↓
BL 19	Chorismate synthase	*aroC*	gi|23465452	42337	6.06	20	70%	—
BL 20	Elongation factor P	*efp*	gi|23464692	20698	5.05	9	61%	—
BL 21	Ribose 5-phosphate isomerase	*rpiA*	gi|23336290	25281	4.83	13	59%	↑↑↑
BL 22	Methionine aminopeptidase	*map*	gi|23466277	28589	4.91	14	60%	—

Spot numbers refer to the proteins labelled in Fig. 2. Putative functions were assigned from the KEGG pathways for *Bifidobacterium longum* NCC2705. Coverage is given by the number of tryptic peptides observed contributing to the percentage of amino acid coverage. Normalized volume ratios are given for each protein derived from cells cultured in the absence of human mucus, with respect to the protein derived from cells cultured with mucus. One arrow indicates an increase (or a decrease) of 1.7–2.5-fold in spot intensity, two arrows indicate an increase (or decrease) of 2.5–5-fold and three arrows indicate an increase (or a decrease) of more than fivefold or proteins undetected in the presence of mucus. –, not differentially expressed.

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Table 2. Quantification of glycosyl hydrolase activities from cytoplasmic protein extracts and supernatants of *Bifidobacterium longum* NCIMB8809, grown in the presence and absence (control) of mucus

		Specific activity (mean ± SD)	
	Condition	Cytoplasm	Supernatant
α-D-Glucopyranoside	Control	13.13 ± 1.14	0.11 ± 0.02
	Mucus	12.38 ± 1.46	0.12 ± 0.03
β-D-Galactopyranoside	Control	69.94 ± 5.98	0.36 ± 0.10
	Mucus	55.58 ± 6.11**	0.17 ± 0.04*
β-D-Glucopyranoside	Control	0.34 ± 0.20	0.07 ± 0.01
	Mucus	0.47 ± 0.27	0.09 ± 0.02*
α-L-Arabinofuranoside	Control	2.40 ± 0.62	0.28 ± 0.05
	Mucus	3.22 ± 0.68*	0.24 ± 0.04
α-L-Rhamnopyranoside	Control	0.49 ± 0.19	0.18 ± 0.13
	Mucus	0.87 ± 0.48	0.13 ± 0.04
N-α-β-D-glucosamine	Control	0.72 ± 0.16	0.10 ± 0.03
	Mucus	0.32 ± 0.10**	0.19 ± 0.00**
β-D-Fucopyranoside	Control	2.80 ± 0.30	0.18 ± 0.03
	Mucus	2.60 ± 0.23	0.14 ± 0.02
β-D-Glucuronide	Control	0.83 ± 0.25	0.07 ± 0.01
	Mucus	0.62 ± 0.32	0.13 ± 0.05*
α-D-Galactopyranoside	Control	33.28 ± 2.43	0.17 ± 0.02
	Mucus	28.99 ± 2.64*	0.13 ± 0.01**
β-D-Xylopyranoside	Control	0.56 ± 0.01	0.11 ± 0.03
	Mucus	0.43 ± 0.04*	0.12 ± 0.01
α-L-Fucopyranoside	Control	0.40 ± 0.22	0.11 ± 0.02
	Mucus	0.43 ± 0.35	0.11 ± 0.04

*$P < 0.05$,
**$P < 0.01$.

Enzymatic activities were determined for the cytoplasmic fraction and for the secreted fraction (Table 2). We detected some minor changes in β-D-galactopyranosidase, α-L-arabinofuranosidase, *N*-acetyl-β-D-glucosaminidase, α-D-galactopyranosidase and β-D-xylopyranosidase activities. Remarkably, the *N*-acetyl-β-D-glucosaminidase activity showed a reduction in the cytoplasmic protein extracts, and an increase in the extracellular milieu, when the cells were grown in the presence of mucus. Bacterial *N*-acetyl-β-D-glucosaminidases are glycoprotein-degrading enzymes that have been related to the colonization of mucus environments (Homer *et al.*, 1994; Karamanos *et al.*, 1995). The increase of the secreted activity in the presence of mucus could support the possible role of this enzyme in mucus degradation.

Finally, we found a significant increase in the glucose consumption of cells grown in the presence of mucus (295.43 ± 19.38 mg of glucose consumed in 100 mL), in relation to those conditions in which mucus was not present (226.71 ± 23.70 mg of glucose consumed in 100 mL). Consistent with an activation of the glucose catabolism in the presence of mucus, we also detected a higher production of lactic and acetic acids when mucus was present in the growth medium (50.78 ± 5.02 mg 100 mL^{-1} of lactic acid and 85.14 ± 7.96 mg 100 mL^{-1} of acetic acid in the absence of mucus; 78.62 ± 4.95 mg 100 mL^{-1} of lactic acid and 115.13 ± 4.83 mg 100 mL^{-1} of acetic acid in the presence of mucus).

In summary, we found that human mucus influences the metabolism of *B. longum* biotype longum NCIMB8809 in a semi-defined medium. Concomitantly, an increase of glucose consumption was observed, together with a shift of glycosidase activities that could play a role in the degradation of the glycoprotein matrix of mucin. Furthermore, mucin-associated leucine was incorporated into the *B. longum* proteins during growth, and some proteins that are likely to mediate interaction with mucus, as well as some others involved in the response to environmental challenges, were found to be differentially produced. The results shown here will contribute to understanding the interactions between human mucus and intestinal *Bifidobacterium*.

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