Induction of α -L-arabinofuranosidase activity by monomeric carbohydrates in *Bifidobacterium longum* and ubiquity of encoding genes

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Abstract *Bifidobacterium longum* can be isolated from human faeces, some strains being considered probiotics. *B. longum* NIZO B667 produces an exo-acting α -L-arabinofuranosidase, AbfB, previously purified by us, that releases L-arabinose from arabinan and arabinoxylan. This activity was subjected to two–seven-fold induction by L-arabinose, D-xylose, L-arabitol and xylitol and to repression by glucose. Maximum activity was obtained at 48 h incubation except for D-xylose that was at 24 h. High concentrations (200 mM) of L-arabitol also caused repression of the arabinofuranosidase. A unique band of activity showing the same migration pattern as the purified AbfB was found in zymograms of cell free extracts, indicating that the activity was likely due to this sole enzyme. The assessment of the influence of inducers and repressors on the activity of AbfB and on the expression of the *abfB* gene by real time PCR indicated that regulation was transcriptional. DNA amplifications using a pair of degenerated primers flanking an internal fragment within α -L-arabinofuranosidase genes of the family 51 of glycoside hydrolases evidenced that these enzymes are widespread in *Bifidobacterium*. The aminoacidic sequences of bifidobacteria included a fragment of four to six residues in the position 136–141 that was absent in other microorganisms

Keywords *Bifidobacterium* · Arabinofuranosidase · Induction · Repression

Introduction

Arabinofuranosidases are hemicellulose-degrading enzymes that hydrolyze terminal α -L-arabinofuranosyl groups from arabinose-containing oligo and polysaccharides such as arabinans, arabinoxylans, and xylans, which are major components of plant cell walls (Saha 2000). The α -L-arabinofuranosidases are needed together with other hydrolases with endo- and exo-activities for the complete degradation of polymeric carbohydrates (Pitson et al. 1996; Saha 2000). Arabinofuranosidases have potential applications in industrial food processing of fruits, cereals and vegetables (Van Laere et al. 2000).

Arabinofuranosidases are particularly important in the genus *Bifidobacterium*. These microorganisms are natural members of the human intestinal microbiota, where they may be present at concentrations of 10^9 – 10^{10} cells per gram of faeces (Tannock 1995). Some strains of the genus *Bifidobacterium* are considered as probiotics and are being used as active ingredients in functional food products. *Bifidobacterium longum* is one of the bacterial species commonly found in human faeces (Gueimonde et al. in press). Several carbohydrates not digested in the upper gastrointestinal tract (GIT) can act as substrates for bacterial fermentation in the colon (Cumings and Englyst 1995). Among these carbohydrates, those that are selectively fermented by beneficial microorganisms are known as prebiotics (Gibson and Roberfroid 1995). The description of the genome sequence of *Bifidobacterium longum* (Schell et al. 2002) revealed a large number of predicted proteins (more than 8%) that seem to be related to the catabolism of oligo and polysaccharides released from non-digestible plant polymers. Among these many different hypothetical proteins displayed high

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levels of sequence homology with proteins involved in the metabolism of arabinose-containing sugars such as intra- and extracellular α -L-arabinofuranosidases, endo- α -arabinanases, oligosaccharide transporters and transcriptional regulators. In spite of that, to date, only four arabinosidases have been purified and characterized in the genus *Bifidobacterium* (Margolles and de los Reyes-Gavilán 2003; Shin et al. 2003; Van den Broek et al. 2005; Van Laere et al. 1997, 1999) and our current knowledge about the regulation of their activity is limited. Particularly, induction by monosaccharides awaits investigation.

Regulation mechanisms of the biosynthesis of enzymes responsible for the degradation of arabinose-containing polymers are well studied in *Aspergillus*. The arabinase enzymatic set releases monomeric L-arabinose from polymer substrates containing this monosaccharide (Rombouts et al. 1988). L-arabinose is taken up by cells and converted in several steps to D-xylulose-5-phosphate, which enters the pentose phosphate shunt (L-arabinose $\rightarrow$ L-arabitol $\rightarrow$ L-xylulose $\rightarrow$ xylitol $\rightarrow$ D-xylulose $\rightarrow$ D-xylulose-5-phosphate $\rightarrow$ pentose phosphate pathway) (Witteveen et al. 1989). Induction of α -L-arabinofuranosidases and endo-arabinanases is under the control of at least two regulatory systems: pathway-specific induction by pentose sugars and polyols generated through the L-arabinose and D-xylose catabolism and carbon catabolite repression by glucose (Van der Veen et al. 1993, 1994).

The enzyme AbfB from *B. longum* NIZO B667 is an α -L-arabinofuranosidase belonging to the family 51 (CAZy classification by sequence homology) of the glycoside hydrolases and it has been previously purified and characterised by us (Margolles and de los Reyes-Gavilán 2003). In the present work we analysed the induction patterns of the α -L-arabinofuranosidase activity in *B. longum* NIZO B667. We also looked for the presence of genes encoding arabinofuranosidases in some *Bifidobacterium* strains.

Materials and methods

Strains and growth conditions

The strain *Bifidobacterium longum* NIZO B667 (NIZO Food Research, Ede, The Netherlands) was used throughout this study. In addition, twenty-eight *Bifidobacterium* strains, including *B. longum* NIZO B667, were used for the amplification by PCR of arabinofuranosidase genes (Table 1). *Bifidobacteria* were cultured in MRS broth (Merck, Darmstadt, Germany) supplemented with 0.05 % L-cysteine (wt/vol) (Merck) (MRSC), and incubated at 37°C in an anaerobic chamber (Mac500, Down Whitley Scientific, West York-

Table 1 *Bifidobacterium* strains used in this study

Strain	Origin
<i>B. longum</i> CECT 4503	Child intestine ^a
<i>B. bifidum</i> CECT 4549, and CECT4550	Child faeces ^a
<i>B. infantis</i> CECT 4551, CECT4552, and CECT 4553	Child intestine ^a
<i>B. animalis</i> IPLA 4549, and IPLA 658	IPLA collection ^b
<i>B. breve</i> NIZO B658	Child faeces ^c
<i>B. adolescentis</i> NIZO B659	Child faeces ^c
<i>B. longum</i> NIZO B667	Unknown ^c
<i>B. bifidum</i> A1, A8, and A12	Commercial dairy culture ^d
<i>B. bifidum</i> PBT	Probiotic supplement ^d
<i>B. bifidum</i> BBI	Commercial dairy culture ^d
<i>B. animalis</i> BB12	Commercial dairy culture ^d
<i>B. longum</i> BL	Fermented milk ^d
<i>B. longum</i> A72, C55, and C64	Adult faeces ^e
<i>B. bifidum</i> N1, M6, B6, E1, and Y5	Fermented milk ^f
<i>B. animalis</i> H8, and C10	Fermented milk ^f

Superscripts indicate the source of microorganisms

^a CECT: Colección Española de cultivos tipo (Spanish type culture collection), Valencia, Spain

^b IPLA: Instituto de Productos Lácteos de Asturias, Villaviciosa, Spain

^c NIZO: NIZO Food Research Culture Collection, Ede, The Netherlands

^d INLAIN: Instituto de Lactología Industrial, Universidad Nacional del Litoral, Santa Fe, Argentina

^e Human isolates

^f Commercial product isolates

shire, UK) under a 10% (vol/vol) CO₂, 10% (vol/vol) H₂, 80% (vol/vol) N₂ atmosphere.

Induction of the cellular α -L-arabinofuranosidase activity

The α -L-arabinofuranosidase activity levels of *B. longum* NIZO B667 in the presence of arabinose-containing polymers were determined. For this purpose, the microorganism was inoculated (1%, vol/vol) in MRSC and in basal medium according to the formulation of the MRS without glucose (BM) (peptone 10 g l⁻¹, yeast extract 4 g l⁻¹, sodium acetate 5 g l⁻¹, diammonium citrate 2 g l⁻¹, L-cysteine 0.5 g l⁻¹, magnesium sulphate 0.2 g l⁻¹, manganese sulphate 0.05 g l⁻¹, pH 6.4) supplemented with 1% (wt/vol) concentrated solutions of arabinan, arabinoxylan or xylan (Megazyme International Ireland Ltd, Wicklow, Ireland) prepared as indicated by the manufacturer's instructions.

Cultures of *B. longum* NIZO B667 were grown for 48 h in MRSC, washed twice with a saline solution (NaCl 0.05%, wt/vol) and then resuspended in a half volume of BM supplemented with different monomeric

carbon sources. In induction experiments 10 g l^{-1} of the carbon source were added to BM and cultures were incubated for 72 h. In repression experiments, after 48 h of incubation of the strain in BM supplemented with 10 g l^{-1} of the inducer, a second incubation was achieved for a further 8 h following the addition of 10 g l^{-1} glucose to the same culture.

We determined the concentration range over which the carbon sources were effective as inducers of the α -L-arabinofuranosidase activity or whether any of the inducers could also repress the activity depending on the concentration used. For this purpose pentoses (L-arabinose and D-xylose) and polyols (L-arabitol and xylitol) were separately added to the cultures at different concentrations (2 mM, 20 mM, 100 mM and 200 mM) and incubated for 48 h.

The α -L-arabinofuranosidase specific activity of cultures was determined in cell-free extracts as indicated below. Experiments were carried out in duplicate.

Determination of the α -L-arabinofuranosidase activity

The α -L-arabinofuranosidase activity was assayed on cell-free extracts using *p*-NP- α -L-arabinofuranoside as a substrate (Sigma, St. Louis, MO, USA). Cells from 5 ml of cultures were harvested by centrifugation, washed with 100 mM potassium phosphate buffer pH 6.8, and pellets were resuspended in 1 ml of the same buffer. Cells were sonicated for 2 min while cooling on ice, using a CV17 sonicator (VibraCell, Sonics and Materials, Connecticut, USA) and centrifuged to remove cell debris. Extracts obtained were then assayed for α -L-arabinofuranosidase activity. Amounts of 40 μl of cell-free extracts were mixed with 880 μl of 40 mM acetate buffer, pH 5.5 and 80 μl of 20 mM of *p*-NP- α -L-arabinofuranoside. The mixtures were incubated in a water-bath at 37°C for 10 min. After incubation, 1 ml of cold 1 M sodium carbonate was added to stop the reaction. The calculation of micromoles of *p*-nitrophenol (*p*-NP) released was based on the relationship of the $A_{420 \text{ nm}}$ to a standard curve. One unit of enzyme activity was defined as the amount of protein that releases 1 μmol of *p*-NP per min. Protein of cell-free extracts was determined using the BCA protein assay kit (Pierce, Rockford, IL, USA) following the manufacturer's instructions. The activities were given as specific activities (unit mg^{-1} protein) in cell-free extracts.

Visualization of proteins with α -L-arabinofuranosidase activity using zymograms

Five millilitres of BM supplemented with each of the different inducers were inoculated (1% vol/vol) with

the microorganism and incubated for 48 h. Extracts were obtained as indicated above from cells suspended in 1 ml of 100 mM phosphate buffer, pH 6.8. Extracts with the same amount of protein (20 μg) were run in native conditions in polyacrylamide gels (6.0% acrylamide, 0.16% bisacrylamide) by using the MiniProtein IITM system (Bio-Rad, Richmond, CA, USA). Electrophoresis was carried out in electrophoresis buffer (25 mM Tris, 192 mM glycine) at 25 mA for 1 h. Gels were washed with 40 mM acetate buffer pH 5.5 and covered with 1% (wt/vol) low-melting agarose (Gibco BRL, Gaithersburg, USA) added with 50 $\mu\text{g ml}^{-1}$ methylumbelliferyl α -L-arabinofuranoside (MU-ara) (Sigma). After 1 h of incubation at 37°C gels were visualized and photographed under UV light in a Gel Doc 2000 system with the Quantity one software (Bio-Rad).

Effect of inducers on the activity of the purified enzyme and on the expression of *abfB*

To evaluate the effect of inducers on the enzyme, the activity levels of the purified α -L-arabinofuranosidase (AbfB) were determined as previously reported (Margolles and de los Reyes-Gavilán 2003) in 50 mM potassium phosphate buffer (pH 6.0) containing 100 mM of the corresponding monomeric sugar (glucose, L-arabitol, xylitol, D-xylose or L-arabinose). Experiments were carried out in triplicate.

The influence of pentoses, polyols and glucose on the expression levels of *abfB* was assessed. Growth conditions of *B. longum* NIZO B667 were essentially as described before for the study of the arabinofuranosidase activity levels. The microorganism was grown for 48 h in MRSC, washed twice with a saline solution (NaCl 0.05%, wt/vol) and incubated for 48 h in a half volume of BM supplemented with 10 g l^{-1} of the different monomeric sugars (L-arabinose, D-xylose, L-arabitol, and xylitol). An additional incubation of 8 h followed the addition of glucose to these cultures (10 g l^{-1}). Around 500 μl of induced and repressed cultures were resuspended in 1 ml of RNeasy protect bacteria reagent (Qiagen, Hilden, Germany) and RNA was extracted by using RNeasy mini kit (Qiagen) following manufacturer's instructions. Contaminant DNA was removed by on-column digestion using the RNase-free Dnase set (Qiagen). Then, 4 μl of RNA (about 3 μg) were reverse transcribed into cDNA using the cDNA archive kit (Applied Biosystems, Foster City, CA, USA) and the specific oligonucleotide primers AbfBF184 (5'-CGTC TG TTCGGCTCATTCGTA-3') and AbfBR308 (5'-A GCTCCTTGACCAGGTCAAGCA-3'), which amplified a fragment of 124 base pairs within the *abfB* gene. For reverse transcription of 16S rRNA, previously

described PCR primers (Gueimonde et al. 2004) were used. The cDNA was stored at -80°C until analysis.

Real-time RT-PCR was performed in an ABI Prism 7500 machine (Applied Biosystems) using the SYBR green PCR master mix (Applied Biosystems). Thermal cycling consisted of an initial cycle of 95°C 10 min followed by 35 cycles of 95°C 15 s and 60°C 1 min. The 16S rRNA gene was used as endogenous control. The expression levels with the different pentoses and polyols were referred to those obtained in the presence of glucose.

Experiments were carried out in duplicate and analyzed in duplicate in two independent PCR runs.

PCR amplification and sequence analysis of genes coding for arabinofuranosidases

Cells grown for 72 h in MRSC agar were washed and suspended in 0.1 ml TE buffer (Sambrook et al. 1989). The cell suspension was heated to 98°C for 10 min, centrifuged at 1,500 g for 10 min and the supernatant was collected.

Two degenerated primers were designed in conserved regions within gene sequences of arabinofuranosidases belonging to the family 51 of glycoside hydrolases from different bacterial species: ABFLF (5'-CGYTAYCCSGGCGGCAAYTTCG-3') and ABFLR (5'-GATSGGSGCGATGACRTTSAC-3'). Homology searches with sequences in the GenBank database were carried out using software available in the Pôle bio-informatique lyonnais server (Lyon University, France) (<http://www.pbil.univ-lyon1.fr>) and in the National Center for Biotechnology Information (Bethesda, USA) (<http://www.ncbi.nlm.nih.gov>). The amplification with synthetic oligonucleotides ABFLF and ABFLR (Invitrogen, Paisley, United Kingdom) was performed with a PCR Sprint thermocycler (Hybaid Ltd, Middlesex, UK) in 50 μl (final volume) mixtures which contained 0.25 μM of each primer, 200 μM of each dNTP (Amersham Pharmacia Biotech, Uppsala, Sweden), 5 μl of cell lysate and 2.5 units of *Taq* DNA polymerase (Amersham Pharmacia Biotech). The thermal cycle program used consisted of the following time and temperature profile: an initial cycle of 95°C for 5 min, 30 cycles of 94°C 45 s, 56°C 1 min and 72°C 45 s, and a final extension step of 10 min at 72°C . Amplified products were subjected to electrophoresis in 1% (wt/vol) agarose gels and visualized by ethidium bromide staining as indicated above.

PCR products were purified using the GenElute PCR clean up kit (Sigma-Aldrich Química, Madrid, Spain) following the manufacturer's instructions. Automated sequencing of both strands of the PCR

products was done with primers ABFLF and ABFLR at the sequencing unit of the University of Oviedo (Spain) using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA). Amino acidic sequences of an internal fragment predicted from PCR products, as well as the corresponding sequence held in genbank for some other bacteria, were aligned and boxed using the AlignX tool of the Vector NTI Suite 7.1 program (InforMax, Bethesda, USA). A phylogenetic tree was constructed with similarities found in this internal fragment with the Vector NTI Suite 7.1 program using the algorithm neighbour joining of Saitou and Nei (1987).

Nucleotide sequence accession numbers

The partial nucleotide sequences of α -L-arabinofuranosidase genes obtained in this study are available in the genbank database under accession numbers DQ273668 to DQ273677.

Results and discussion

Induction of the α -L-arabinofuranosidase activity

After incubation of *B. longum* NIZO B667 in MRSC and in BM containing arabinan (arabinose 88%, galactose 3%, rhamnose 2%, galacturonic acid 7%), arabinoxylan (arabinose 37%, xylose 61%, other sugars 2%), and xylan (xylose 82%, arabinose 9%, glucose 8%), higher arabinofuranosidase activity levels were obtained in the presence of arabinose and/or xylose-containing polymers (7.36 ± 0.69 , 21.70 ± 1.23 , and 23.22 ± 0.75 units mg^{-1} protein for arabinan, arabinoxylan and xylan, respectively) than in MRSC (2.66 ± 0.51 units mg^{-1} protein). This suggests that the arabinofuranosidase activity could be subjected to induction by arabinose and/or xylose-containing polymers and/or by related oligo and monosaccharides initially present in the polymer samples or released from them during incubation by the set of cellular arabinases (Van der Veen et al. 1991). Particularly, substrates resulting in the highest activity also had the highest xylose and the lowest arabinose content. Subsequently, we focused the work on the study of the capacity to act as inducers of the α -L-arabinofuranosidase activity of D-xylose and L-arabinose and the polyols xylitol and L-arabitol, intermediates together with xylose of the catabolic pathway of L-arabinose in several bacteria and fungi. With this purpose, the microorganism previously grown in MRSC (OD 600 nm about 3–4) was incubated in BM supplemented with glucose and with each of the

four carbon sources indicated above. No changes in the optical densities of cultures were found between 0 and 72 h of incubation with inducers (data not shown). In this situation, the specific α -L-arabinofuranosidase activity clearly rose with D-xylose and polyols whereas the increase was much more moderate in the case of arabinose (Fig. 1), and no increase at all was found with glucose. Our results confirmed the role as inducers of D-xylose, xylitol and L-arabitol, and to a lesser extent of L-arabinose. The α -L-arabinofuranosidase activity peaked at 24 h of incubation in the presence of D-xylose whereas maximum activity was obtained at 48 h of incubation with the other monosaccharides, both times being in the range of those reported in fungi (Ramón et al. 1993; Saha and Bothast 1998). These differences in incubation times for obtaining maximum induction could not be attributed to differences in growth and were more likely related to the different times needed by each inducer to enter the cell and to act on the expression of the gene and/or on the enzymatic activity. Figure 1 also shows a rapid decline in enzyme activity in the later stages of growth, particularly when D-xylose and xylitol were present in the medium. This could be partially explained by a general decline of metabolic activity in old cultures. The sole data available in the genus *Bifidobacterium* on the induction by monosaccharides of enzymes degrading arabinose-containing polymers refers to AXH-d3, an arabinofuranosidase acting on arabinoxylans which was inducible by xylose but not by arabinose (Van Laere et al. 1997). Some enzymes involved in the interconversion of L-arabinose

to D-xylulose-5-phosphate (L-arabinose isomerase and L-ribulose-5-phosphate 4-epimerase, catalyzing the conversion of L-arabinose to L-ribulose and L-ribulose-5-P to D-xylulose-5-P, respectively) have been annotated in the genome of *B. longum* whereas those involved in the arabinose catabolic pathway of fungi seem not to be present in this species (Kyoto encyclopedia of genes and genomes: KEGG, <http://www.genome.jp/kegg/>). In spite of this, the general pattern of regulation by pentoses and polyols of the α -L-arabinofuranosidase activity in *B. longum* NIZO B667 is subjected to an induction system similar to that previously described in other bacteria and fungi (Clinche et al. 1997; Higashi et al. 1983; Ramón et al. 1993; Saha and Bothast 1998; Van der Veen et al. 1993).

When glucose was added to cultures of *B. longum* NIZO B667 previously induced, an abrupt decrease of the α -L-arabinofuranosidase activity was obtained following 8 h of incubation. Thus, the previous activity levels of cultures induced with L-arabinose, D-xylose, L-arabitol, and xylitol were 6.69 ± 1.29 , 8.68 ± 2.12 , 17.81 ± 1.23 , and 16.46 ± 2.03 units mg^{-1} protein, respectively, whereas the activity of the same cultures following the addition of glucose were 0.81 ± 0.08 , 0.90 ± 0.11 , 0.30 ± 0.04 , and 0.11 ± 0.01 units mg^{-1} protein. These results confirmed that the presence of glucose promoted a decrease of the arabinofuranosidase activity as was previously reported for arabinofuranosidases and other arabinases of fungi (Gielkens et al. 1999; Van der Veen et al. 1993, 1994).

We aimed to know the concentration range over which pentoses and polyols were effective as inducers of the α -L-arabinofuranosidase, as well as whether any of the inducing sugars could also repress the activity depending on the concentration. With these purposes the strain previously grown in MRSC was transferred to media containing different concentrations of each compound (from 2.0 to 200 mM). Cultures were incubated for 48 h because this was within the interval of time where the highest activity levels were obtained for most of the inducers in the conditions tested. The maximum activity was obtained at concentrations of 100 mM for L-arabitol and xylitol and from 20 mM for D-xylose (Fig. 2). Only a slight increase of activity was found for L-arabinose. In the concentrations range used, no repression was obtained with D-xylose and xylitol, although the activity lowered slightly at 200 mM. However, L-arabitol caused a clear decrease of the activity at concentrations of 200 mM. Repression of glycosidic activities by high concentrations of the same mono and disaccharides acting as inducers at lower concentrations was previously reported in fungi (Ali and Sayed 1992; De Vries et al. 1999).

Fig. 1 α -L-arabinofuranosidase activity of *B. longum* NIZO B667 during incubation in the presence of 10 g l^{-1} L-arabinose (filled circle), D-xylose (open circle), L-arabitol (filled triangle), xylitol (open triangle), and glucose (+) after the previous growth of the microorganism in MRSC. Results are the mean of two independent experiments, activity measurements were also made in duplicate. Vertical lines indicate standard deviation

Fig. 2 Efficiency of inducers: α -L-arabinofuranosidase activity levels in *B. longum* NIZO B667 after 48 h of incubation in medium with increasing concentrations (2 mM to 200 mM) of L-arabinose (grey bars), D-xylose (sloped line bars), L-arabitol (white bars), and xylitol (black bars). Results are the mean of two independent experiments, activity measurements were also made in duplicate. Vertical lines on the bars represent standard deviation

Detection of proteins with arabinofuranosidase activity in cell-free extracts

Zymograms were carried out with cell free extracts of *B. longum* NIZO B667 in order to detect proteins with α -L-arabinofuranosidase activity against MU-ara after electrophoresis in native conditions in polyacrylamide gels. A unique band of activity showing the same migration pattern in gels as the enzyme AbfB previously purified from *B. longum* NIZO B667 (Margolles and de los Reyes-Gavilán 2003) was detected after incubation in MRSC as well as in the presence of L-arabinose, D-xylose, L-arabitol and xylitol (data not shown). This suggested that the protein displaying α -L-arabinofuranosidase activity in zymograms and AbfB is the same enzyme, and therefore variations on the activity levels likely corresponded to changes in the activity of this sole enzyme. In contrast, various proteins with arabinofuranosidase activity having different isoelectric points have been previously detected in fungi by means of zymograms (De Ioannes et al. 2000; Filho et al. 1996; Luonteri et al. 1995; Van der Veen et al. 1993).

Effect of inducers on the activity of the purified enzyme and on the expression of *abfB* gene

In order to determine whether the induction took place at transcriptional and/or posttranslational level the influence of inducers and glucose on the activity of AbfB, as well as on the expression level of the *abfB* gene, were assessed.

The activity of the purified AbfB was determined in the presence of monosaccharides (glucose, D-xylose

and L-arabinose) and polyols (L-arabitol and xylitol). The specific activity ranged between 5.18 and 6.13 units per μ g of enzyme and did not differ between the control (no sugar added to the buffer) and the different compounds, thus indicating that the presence of monosaccharides and polyols did not modify the activity of the enzyme in these conditions. Interestingly, in Real time PCR experiments the inducers tested caused an increase in the expression of *abfB* when compared to glucose. Thus, the expression levels obtained with L-arabinose, D-xylose, L-arabitol and xylitol were 8.26 ± 2.74 , 4.92 ± 0.72 , 2.55 ± 0.76 and 1.78 ± 0.33 , respectively. These data apparently do not correlate with those of enzymatic activities measured on cells grown in media with the different inducers. In fact, whereas the highest enzymatic activities were reached with L-arabitol and xylitol in the growth medium, the Real time PCR showed the lowest expression levels with polyols and the highest with L-arabinose and D-xylose. This discrepancy could be explained by taking into account that the optimum incubation time of cultures for obtaining the highest enzymatic activity may not be the same as the one used to assess the gene expression. In this way, the physiological state of cells could have been different in both experiments and the expression levels found with Real time PCR did not necessarily correlate with enzymatic activity levels. In addition, following 8 h incubation after the addition of glucose to the same cultures grown with L-arabinose, D-xylose, L-arabitol and xylitol, the expression levels dropped to 1.64 ± 0.26 , 1.14 ± 0.26 , 0.45 ± 0.07 , and 0.63 ± 0.02 , respectively. Our results indicated that induction and repression of the α -L-arabinofuranosidase by pentoses and polyols occurred at transcriptional level. Transcriptional regulation of the expression of α -L-arabinofuranosidases has been previously indicated in other bacteria (Raposo et al. 2004; SaNogueira et al. 1997) and fungi (Carvalho et al. 2003; Mota et al. 1999; Orejas et al. 1999) but not in bifidobacteria, this probably being a common way of regulation for this type of enzymes in many microorganisms.

Ubiquity of α -L-arabinofuranosidases in bifidobacteria

The degenerated oligonucleotides ABFLF and ABFLR were used for the amplification by PCR of internal DNA fragments within arabinofuranosidase genes in 28 *Bifidobacterium* strains (Table 1). The PCR products were sequenced and compared with each other as well as with other known sequences of different microorganisms. In all the strains, excluding *Bifidobacterium adolescentis* NIZO B659, *Bifidobacterium breve* B658 and *Bifidobacterium infantis* CECT 4551,

an amplification fragment with the expected length (1,000 bp) was obtained, the sequence of which showed homology to internal sequences of family 51 arabinofuranosidase genes held in databases (data not shown). Therefore, a phylogenetic tree was constructed based on a central fragment within the amino acid sequences (180–186 amino acids in length) deduced from the PCR products of 11 selected strains belonging to different species (Fig. 3). Some other sequences of arabinofuranosidases from bifidobacteria, as well as from other bacterial genera held in databases, were also included in the comparison (Fig. 4). With respect to the genus *Bifidobacterium*, seven sequences, all of them belonging to strains identified as *Bifidobacterium animalis* and *Bifidobacterium bifidum*, were identical whereas sequences from *B. longum* CECT 4503 and *B. infantis* CECT 4552 differed from each other, and from the former microorganisms, in one amino acid only. This first group shared an 80% similarity with sequences of proteins AbfA1 and AbfA3 from *B. longum* NCC2705 in the second group. Arabinofuranosidases from other bacterial groups appeared clearly differentiated from these two first groups of bifidobacteria and were clustered with the arabinofuranosidase sequence of *B. longum* NIZO B667 and with sequences of other arabinofuranosidases from strains belonging to *B. longum* species. In that way, the arabinofuranosidase AbfA2 from *B. longum* NCC2705 was closer to enzymes from other genera than to the other two arabinofuranosidases, AbfA1 and AbfA3, from the same

strain. The most noticeable difference between sequences of bifidobacteria and other bacteria was a fragment of 4–6 amino acids in the position 136–141 that was present in bifidobacteria but absent in the remaining microorganisms analysed (Fig. 4).

The sequencing of the *B. longum* genome (Schell et al. 2002) allowed the annotation of at least 14 different enzymes with a hypothetical role in the catabolism of arabinose-containing polymers, which corroborated the importance of this type of enzymes and polysaccharides in the metabolism of bifidobacteria. Arabinose-containing polymers are abundant in the diet and reach the colon undegraded. The regulation system of the arabinofuranosidase activity in *B. longum* could allow this bacterium to modulate the activity levels depending on the fluctuation and availability of these substrates in the GIT. Although bifidobacteria do not preferentially degrade long-chain polymers in vitro (Hopkins et al. 1998; Van der Meulen et al. 2004), they could use oligosaccharides released by the activity of other intestinal organisms. In their turn, metabolic products produced by bifidobacteria from these dietary carbohydrates could support the growth of other beneficial microbial population in the colon by a cross-feeding mechanism (Belenguer et al. 2006). Results from the present study represent a contribution to the understanding of metabolic complex systems that allow bifidobacteria to metabolize prebiotic carbohydrates and provide these microorganisms with a selective advantage to survive and colonize the human GIT.

Fig. 3 Phylogenetic tree of the amino acid sequence of a central fragment of 180–186 residues obtained by partial sequencing of α -L-arabinofuranosidase genes from several *Bifidobacterium* strains and other bacteria, generated by the Vector NTI suite 7.1 program (InforMax, Bethesda, USA), using the algorithm neighbour joining. The scale represents the homology distance among sequences. Sequences with an asterisk are from this study, and the remainder are from genbank database (accession numbers within parentheses)

Fig. 4 Multiple alignment of the amino acid sequence of a central fragment (180–186 residues) obtained from the partial sequencing of α -L-arabinofuranosidase genes of several *Bifidobacterium* strains and other bacteria. The boxed sequence identifies amino acids that are identical (black shading) or functionally similar

(grey shading). Sequences with asterisks are from this study, and the remainder are from the genbank database (Fig. 3 for genbank accession numbers). The square includes the zone of four to six aminoacids present in bifidobacteria but absent in the other microorganisms

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References

- Ali S, Sayed A (1992) Regulation of cellulase biosynthesis in *Aspergillus terreus*. *World J Microbiol Biotechnol* 8:73–75
- Belenguer A, Duncan SH, Graham Calder A, Holtrop G, Louis P, Lobley GE, Flint HJ (2006) Two routes of metabolic cross-feeding between *Bifidobacterium adolescentis* and butyrate-producing anaerobes from the human gut. *Appl Environ Microbiol* 72: 3593–3599
- Carvalho M, de Ioannes P, Navarro C, Chavez R, Peirano A, Bull P, Eyzaguirre J (2003) Characterization of an α -L-arabinofuranosidase gene (*abf1*) from *Penicillium purpurogenum* and its expression. *Mycol Res* 107:388–394
- Clinche FL, Piñaga F, Ramón D, Vallés S (1997) α -L-arabinofuranosidases from *Aspergillus terreus* with potential application in enology: induction, purification, and characterization. *J Agric Food Chem* 45:2379–2383
- Cummings JH, Englyst H (1995) Gastrointestinal effects of food carbohydrates. *Am J Clin Nutr* 61:938S–945S
- De Ioannes P, Peirano A, Steiner J, Eyzaguirre J (2000) An α -L-arabinofuranosidase from *Penicillium purpurogenum*: production, purification and properties. *J Biotechnol* 76:253–258
- De Vries RP, van den Broeck HC, Dekkers E, Manzanera P, de Graff LH, Visser J. (1999) Differential expression of three al-

- pha-galactosidase genes and a single beta-galactosidase gene from *Aspergillus niger*. Appl Environ Microbiol 65:2453–2460
- FilhoEX, Puls J, Coughlan MP (1996) Purification and characterization of two arabinofuranosidases from solid-state cultures of the fungus *Penicillium capsulatum*. Appl Environ Microbiol 62:168–173
- Gibson GR, Roberfroid MB (1995) Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J Nutr 125:1401–1412
- Gielkens M, González-Candelas L, Sánchez-Torres P, van de Vondervoort P, de Graaff L, Visser J, Ramón D (1999) The *abfB* gene encoding the major α -L-arabinofuranosidase of *Aspergillus nidulans*: nucleotide sequence, regulation and construction of a disrupted strain. Microbiology 145:735–741
- Gueimonde M, Tölkö S, Korpimäki T, Salminen S (2004) New real-time quantitative PCR procedure for quantification of bifidobacteria in human fecal samples. Appl Environ Microbiol 70:4165–4169
- Gueimonde M, Debor L, Tölkö S, Jokisalo E, Salminen S (2006) Quantitative assessment of faecal bifidobacterial populations by real-time PCR using lanthanide probes. J Appl Microbiol (In press)
- Higashi K, Kusakabe I, Yasui T, Ishiyama T, Okimoto Y (1983) Arabinan-degrading enzymes from *Streptomyces diastatochromogenes* 065. Agric Biol Chem 47:2903–2905
- Hopkins MJ, Cummings JH, Macfarlane GT (1998) Inter-species differences in maximum specific growth rates and cell yields of bifidobacteria culture on oligosaccharides and other simple carbohydrate sources. J Appl Microbiol 85: 381–386
- Luonteri E, Siika-aho M, Tenkanen M, Viikari L (1995) Purification and characterization of three α -L-arabinofuranosidases from *Aspergillus terreus*. J Biotechnol 38:279–291
- Margolles A, de los Reyes-Gavilán CG (2003) Purification and functional characterization of a novel α -L-arabinofuranosidase from *Bifidobacterium longum* B667. Appl Environ Microbiol 69:5096–5103
- Mota LJ, Tavares P, Sá-Nogueira I (1999) Mode of action of AraR, the key regulator of L-arabinose metabolism in *Bacillus subtilis*. Mol Microbiol 33:476–489
- Orejas M, MacCabe AP, Pérez González JA, Kumar S, Ramón D (1999) Carbon catabolite repression of the *Aspergillus nidulans xlnA* gene. Mol Microbiol 31:177–184
- Pitson SM, Voragen AG, Beldman G (1996) Stereochemical course of hydrolysis catalyzed by arabinofuranosyl hydrolases. FEBS Lett 398:7–11
- Ramón D, van der Veen P, Visser J (1993) Arabinan degrading enzymes from *Aspergillus nidulans*: induction and purification. FEMS Microbiol Lett 113:15–22
- Raposo MP, Inacio JM, Mota LJ, Sá-Nogueira I (2004) Transcriptional regulation of genes encoding arabinan-degrading enzymes in *Bacillus subtilis*. J Bacteriol 186:1287–1296
- Rombouts FM, Voragen AGJ, Searle-van Leeuwen MJF, Geraeds CCJM, Schols HA, Pilnik W (1988) The arabinases of *Aspergillus niger*: purification and characterization of two α -L-arabinofuranosidases and an endo-1, 5- α -L-arabinase. Carbohydr Polym 9:25–47
- Saha BC, Bothast RJ (1998) Purification and characterization of a novel thermostable α -L-arabinofuranosidase from a color-variant strain of *Aureobasidium pullulans*. Appl Environ Microbiol 64:216–220
- Saha BC (2000) α -L-Arabinofuranosidases: biochemistry, molecular biology and application in biotechnology. Biotechnol Adv 18:403–423
- Saitou N, Nei M (1987) The neighbor-joining method a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425
- Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. 2nd edn. Cold Spring Harbor Laboratory Press, New York
- Sa-Nogueira I, Nogueira TV, Soares S, deLencastre H (1997) The *Bacillus subtilis* L-arabinose (ara) operon: nucleotide sequence, genetic organization and expression. Microbiology 143:957–969
- Schell MA, Karmirantzou M, Snel B, Vilanova D, Berger B, Pessi G, Zwahlen MC, Desiere F, Bork P, Delley M, Pridmore RD, Arigoni F (2002) The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. Proc Natl Acad Sci USA. 99:14422–14427
- Shin HY, Park SY, Sung JH, Kim DH (2003) Purification and characterization of α -L-arabinopyranosidase and α -L-arabinofuranosidase from *Bifidobacterium breve* K-110, a human intestinal anaerobic bacterium metabolizing ginsenoside Rb2 and Rc. Appl Environ Microbiol 69:7116–7123
- Tannock GW (1995) Microecology of the gastrointestinal tract in relation to lactic acid bacteria. Int Dairy J 5:1059–1070
- Van den Broek LAM, Lloyd RM, Geldman G, Verdoes JC, McCleary BV, Voragen AGJ (2005) Cloning and characterization of arabinoxylan arabinofuranohydrolase-D3 (AXHd3) from *Bifidobacterium adolescentis* DSM20083. Appl Microbiol Biotechnol 67:641–647
- Van der Meulen R, Avonts L, De Vuyst L (2004) Short fractions of oligofructose are preferentially metabolized by *Bifidobacterium animalis* DN-173 010. Appl Environ Microbiol 70: 1923–1930
- Van der Veen P, Flipphi MJA, Voragen AGJ, Visser J (1991) Induction, purification and characterization of arabinases produced by *Aspergillus niger*. Arch Microbiol 157:23–28
- Van der Veen P, Flipphi MJ, Voragen AG, Visser J (1993) Induction of extracellular arabinases on monomeric substrates in *Aspergillus niger*. Arch Microbiol 159:66–71
- Van der Veen P, Arst Jr HN, Flipphi MJA, Visser J (1994) Extracellular arabinases in *Aspergillus nidulans*: the effect of different *cre* mutations on enzyme levels. Arch Microbiol 162:433–440
- Van Laere KM, Beldman G, Voragen AG (1997) A new arabinofuranohydrolase from *Bifidobacterium adolescentis* able to remove arabinosyl residues from double-substituted xylose units in arabinoxylan. Appl Microbiol Biotechnol 47:231–235
- Van Laere KM, Voragen CHL, Kroef T, van den Broek LAM, Beldman G, Voragen AG (1999) Purification and mode of action of two different arabinoxylan arabinofuranohydrolases from *Bifidobacterium adolescentis* DSM 20083. Appl Microbiol Biotechnol 51:606–613
- Van Laere KM, Hartemink R, Bosveld M, Schols HA, Voragen AG (2000) Fermentation of plant cell wall derived polysaccharides and their corresponding oligosaccharides by intestinal bacteria. J Agric Food Chem 48:1644–1652
- Witteveen CFB, Busink R, van de Vondervoort P, Dijkema C, Swart K, Visser J (1989) L-arabinose and D-xylose catabolism in *Aspergillus niger*. J Gen Microbiol 135:2163–2171