Bifidobacterium longum subsp. infantis uses two different β-galactosidases for selectively degrading type-1 and type-2 human milk oligosaccharides

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Received on July 1, 2011; revised on August 11, 2011; accepted on August 13, 2011

The breast-fed infant intestine is often colonized by particular bifidobacteria, and human milk oligosaccharides (HMOs) are considered to be bifidogenic. Recent studies showed that Bifidobacterium longum subsp. infantis can grow on HMOs as the sole carbon source. This ability has been ascribed to the presence of a gene cluster (HMO cluster-1) contained in its genome. However, the metabolism of HMOs by the organism remains unresolved because no enzymatic studies have been completed. In the present study, we characterized β-galactosidases of this subspecies to understand how the organism degrades type-1 (Galβ1-3GlcNAc) and type-2 (Galβ1-4GlcNAc) isomers of HMOs. The results revealed that the locus tag Blon_2016 gene, which is distantly located from the HMO cluster-1, encodes a novel β-galactosidase (Bga42A) with a significantly higher specificity for lacto-N-tetraose (LNT; Galβ1-3GlcNAcβ1-3Galβ1-4Glc) than for lacto-N-biose I (Galβ1-3GlcNAc) and lacto-N-tetraosyl galactosides. The proposed name of Bga42A is LNT β-1,3-galactosidase. The Blon_2334 gene (Bga2A) located within the HMO cluster-1 encodes a β-galactosidase specific for Lac and type-2 HMOs. Real-time quantitative reverse transcription–polymerase chain reaction analysis revealed the physiological significance of Bga42A and Bga2A in HMO metabolism. The organism therefore uses two different β-galactosidases to selectively degrade type-1 and type-2 HMOs. Despite the quite rare occurrence in nature of β-galactosidases acting on type-1 chains, the close homologs of Bga42A were present in the genomes of infant-gut associated bifidobacteria that are known to consume LNT. The predominance of type-1 chains in HMOs and the conservation of Bga42A homologs suggest the coevolution of these bifidobacteria with humans.

Keywords: human milk oligosaccharides / infant-gut associated bifidobacteria / lacto-N-tetraose / β-galactosidase

Introduction

Human milk contains oligosaccharides (the degree of polymerization between 3 and 14) as the third most abundant solid component (~20 g/L) after lactose (Lac) and lipids (Newburg and Neubauer 1995; Bode 2006; Urashima et al. 2011). These oligosaccharides (human milk oligosaccharides, HMOs) have diverse structures and more than 100 molecular species have been identified (Kunz et al. 2000; Kobata 2010; Urashima et al. 2011). HMOs are resistant to host gastrointestinal digestion, and thus the majority of the HMOs reach the colon (Gnoth et al. 2000). The structures of HMOs mimic the sugar chains of the host epithelial cell surfaces, and consequently, HMOs act as decoys to inhibit the binding of pathogens and toxins to the host intestinal cells (Morrow et al. 2004). In addition, HMOs are believed to promote the growth of particular bifidobacteria in the infant gut, and the resulting bifidobacteria-rich gut ecosystem is thought to be important for infant health (Penders et al. 2006).

In the genus Bifidobacteria, Bifidobacterium bifidum, B. breve, B. longum subsp. infantis and B. longum subsp. longum are frequently isolated from infant feces (Benno et al. 1984). Recently, Marcobal et al. (2010) showed that B. longum subsp. infantis is an avid consumer of HMOs and can use HMOs as the sole carbon source, whereas most of the other intestinal bacteria examined were unable to assimilate HMOs. The genome of B. longum subsp. infantis ATCC15697 contains a large gene cluster that comprises several glycosidases and ATP-binding cassette (ABC) transporters predicted to be involved in the metabolism of HMOs (Sela et al. 2008). A comparative genomic survey showed that the occurrence of the cluster is correlated with the survival of this subspecies on HMOs (LoCascio et al. 2010). Consequently, LoCascio et al. (2010) suggested that the HMO-consuming phenotype (HMO+) of the subspecies can...
be attributed to the presence of this cluster (designated as the HMO cluster-1). However, the characteristics of the encoded enzymes have not been determined.

The core structures of HMOs consist of Lac at the reducing ends, that is elongated by β-(1 → 3)-linked lacto-N-biose I (LNBI, type 1; Galβ1-3GlcNAc) and/or β-(1 → 3,6)-linked N-acetyllactosamine (LacNAc, type 2; Galβ1-4GlcNAc) (Kunz et al. 2000; Kobata 2010). A prominent feature of HMOs is the predominance of the type-1 chain over the type-2 chain, as lacto-N-tetraose (LNT; Galβ1-3GlcNAcβ1-3Galβ1-4Glc) represents the most abundant core structure. Such type-1-rich compositions have not been observed in the milk of other mammals (Urashima et al. 2011).

We have recently elucidated that B. bifidum, which is another consumer of HMOs (Turroni et al. 2010), has a dedicated pathway for degrading type-1 HMOs (Kitaoka et al. 2005; Wada et al. 2008). B. bifidum uses a secretory lacto-N-biosidase to hydrolyze LNT into LNB and Lac. The liberated LNB is then incorporated into the cells by an ABC transporter specific for LNB and galacto-N-biose (GNB; Galβ1-3GalNAc) (the GNB/LNB transporter; Suzuki et al. 2008). The disaccharide is subsequently phosphorolyzed by a GNB/LNB phosphorylase to produce galactose-1-phosphate and N-acetylglucosamine (GlcNAc) (Kitaoka et al. 2005) and further metabolized (Nishimoto and Kitaoka 2007a). This pathway (the GNB/LNB pathway), which involves LNB as a key intermediate, occurs in B. bifidum and some strains of B. longum subsp. longum (Wada et al. 2008), but apparently does not work in B. longum subsp. infantis because all of the genomes determined so far do not contain lacto-N-biosidase homologs (LoCascio et al. 2010). B. longum subsp. infantis possesses a GNB/LNB phosphorylase, but this enzyme exclusively acts on the disaccharide and never on LNT (Kitaoka et al. 2005; Hijikawa et al. 2009). Therefore, how the organism degrades the type-1 HMOs and what enzymes are involved remain unresolved.

In this study, we showed that B. longum subsp. infantis directly incorporates LNT and hydrolyzes it inside the cell by a specific β-galactosidase (suggested name: LNT β-1,3-galactosidase) encoded by the locus tag Blon_2334 gene. β-Galactosidase (Blon_2334) encoded in the HMO cluster-1 was specific for Lac and type-2 chains. The results indicate that the organism uses two different β-galactosidases to selectively degrade the type-1 and type-2 HMOs and also indicated that the HMO- phenotype of this subspecies should not be attributed solely to the presence of the HMO cluster-1. Interestingly, LNT β-1,3-galactosidase homologs were also present in other infant-related bifidobacteria.

Results

Difference in the LNT degradation pathway between B. longum subsp. infantis ATCC15697 and B. bifidum JCM1254

Sela et al. (2008) suggested, through genomic analysis, that B. longum subsp. infantis ATCC15697 imports HMOs in their intact forms. Garrido et al. (2011) recently determined the substrate specificities of 20 solute-binding proteins of ABC transporters of the strain and found that the gene product of Blon_2177 binds LNT, the most abundant HMO core structure. When LNT was anaerobically incubated with the cells and the culture supernatant was analyzed by thin-layer chromatography (TLC), the spot corresponding to LNT gradually disappeared (20–90 min, Figure 1A). LNT remained un consumed when verapamil, an inhibitor of ABC transporters (Li et al. 1996), was included in the reaction mixture. In both cases, no sugar spot other than LNT was detected during the cultivation period. The inhibition of the LNT uptake was not observed when the proton uncoupler carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was added to the reaction mixture (data not shown). On the other hand, when B. bifidum JCM1254 were incubated with LNT (Figure 1B), the spots corresponding to Lac and Gal appeared with a concomitant disappearance of LNT. In the presence of verapamil, LNB and Glc were accumulated in the culture supernatant. The results indicated that, while B. bifidum JCM1254 uses the GNB/LNB pathway to degrade LNT, B. longum subsp. infantis ATCC15697 directly incorporates LNT using an ABC transporter.

Degradation of LNT by the cell-free extract of B. longum subsp. infantis

We then incubated LNT with the cell-free extract of B. longum subsp. infantis and analyzed the reaction products (Figure 1C). The spots corresponding to Gal and a trisaccharide appeared immediately (5 min), and the trisaccharide was subsequently hydrolyzed into its constituent monosaccharides galactose (Gal), glucose (Glc) and GlcNAc (30–180 min). A faint spot corresponding to Lac was also observed. No spot corresponding to LNB, which is formed if the organism possesses lacto-N-biosidase, or a spot corresponding to galacto-1-phosphate, which is formed if the GNB/LNB phosphorylase acts on LNT, was detected. No degradation occurred when the heat-inactivated cell-free extract was included in the reaction mixture (data not shown). The results showed that LNT is sequentially hydrolyzed by β-galactosidase and β-N-acetylhexosaminidase inside the cells.

Characterization of β-galactosidase (Blon_2334) encoded in the HMO cluster-1

The HMO- phenotype of B. longum subsp. infantis has been attributed to the HMO cluster-1 (LoCascio et al. 2010). Initially, the gene product of Blon_2334 (located within the cluster) was analyzed as a candidate β-galactosidase acting on HMOs. The gene encodes a protein of 1023 amino acid (aa) residues and does not code for a signal peptide or a cell wall-anchoring motif. A Pfam search (Finn et al. 2010) revealed that the protein belongs to the glycoside hydrolase (GH) family 2 (Cantarel et al. 2009). The purified protein (designated as Bga2A) that was expressed in an Escherichia coli ΔlacZ background migrated as a single band in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; Supplementary data, Figure S1A). The apparent molecular mass (120 kDa) agreed with the calculated mass of the protein (115 kDa). Size-exclusion chromatography indicated that the native molecular mass of the protein is 248 kDa, indicating that Bga2A forms a dimer in solution. The enzyme required 1 mM Mg2+ for maximum activity (Goulas et al. 2011).
2009), and the optimal pH was 6.0 (Supplementary data, Figure S1B). The enzyme was stable in the pH range of 5.5–9.0 and below 45°C for 30 min (Supplementary data, Figure S2). Bga2A hydrolyzed 4-nitrophenyl-β-D-galactopyranoside (pNP-β-Gal) efficiently ($k_{cat}/K_m = 1800 \text{ mM}^{-1} \text{s}^{-1}$) and also hydrolyzed pNP-β-D-fucopyranoside (pNP-β-Fuc) at a lower catalytic rate ($k_{cat}/K_m = 0.4 \text{ mM}^{-1} \text{s}^{-1}$), but did not hydrolyze other pNP-monosaccharides (Table I). Among the HMO-related oligosaccharides examined, Bga2A most efficiently hydrolyzed Lac with $K_m$ and $k_{cat}$ values of 1.4 mM and 600 s$^{-1}$, respectively. LacNAc and lacto-N-neotetraose (LNnT; Galβ1-4GlcNAcβ1-3Galβ1-4Glc) (type-2 chains) were also effectively hydrolyzed ($k_{cat}/K_m = 86$ and 20 mM$^{-1}$ s$^{-1}$, respectively); however, the enzyme did not act on type-1 structures. The catalytic efficiency for LNnT (0.0045 mM$^{-1}$ s$^{-1}$) was 19,000- and 4000-fold lower than those for Lac and LNnT, respectively. Despite the significantly lower $k_{cat}$ values for LN and LNT than those for Lac, LacNAc and LNnT, the $K_m$ values were comparable among these substrates (1.4–8.7 mM). The results revealed that Bga2A is responsible for degrading...
Lac and type-2 HMOs and essentially cannot be involved in the degradation of type-1 HMOs.

Locus tag Blon_2016 gene encodes LNT β-1,3-galactosidase

Due to the above results, we scrutinized the genome of the organism with the aid of the Carbohydrate-Active enZYmes (CAZy) database (Cantarel et al. 2009) and found the gene products of Blon_0268 (606 aa, GH2), Blon_2016 (691 aa, GH42), Blon_2123 (720 aa, GH42), Blon_2411 (326 aa, GH42), and Blon_2416 (706 aa, GH42) as possible enzymes hydrolyzing β-galactosides. All of these genes were predicted to encode intracellular enzymes. These proteins were similarly expressed in an E. coli ΔlacZ background and purified. The products of Blon_0268 and Blon_2411 did not exhibit any detectable activity toward the tested substrates, and these proteins were excluded from further investigation. The products of Blon_2016 (designated as Bga42A), Blon_2123 (Bga42B) and Blon_2416 (Bga42C) gave single bands on SDS-PAGE with apparent molecular masses of 75.5, 79.5 and 73.5 kDa (Supplementary data, Figure S1A), which were consistent with their calculated masses (77.4, 80.2 and 77.4 kDa), respectively. The native molecular masses of Bga42A, Bga42B and Bga42C were estimated to be 229, 233 and 312 kDa, respectively; therefore, Bga42A and Bga42B should form trimers, whereas Bga42C may form a tetramer in solution. The enzymes hydrolyzed pNP-β-Gal with catalytic efficiencies of 1600 (Bga42A), 120 (Bga42B) and 14 mM⁻¹ s⁻¹ (Bga42C). The activities of Bga42A and Bga42B toward pNP-β-Fuc were ~1% of those toward pNP-β-Gal, whereas the activity of Bga42C toward pNP-β-Fuc was similar to that observed for pNP-β-Gal. The other pNP-sugars were not digested by these enzymes.

Optimal pHs of the three enzymes were between 5.5 and 6.0 (Supplementary data, Figure S1B). The enzymes were stable between pH 5.0 and pH 8.0. Bga42A retained 80% activity after 30 min incubation at 55°C, whereas Bga42B and Bga42C lost their activities under the same conditions (Supplementary data, Figure S2). All enzymes were stable in the presence of 0.05% polyoxyethylene sorbitan monolaurate (Tween-20) for at least 3 weeks, but rapidly lost their activities in the absence of the detergent.

Bga42A released Gal from LNT with a high catalytic efficiency (120 mM⁻¹ s⁻¹), but interestingly, the activity drastically decreased when LNB was used as a substrate (3 mM⁻¹ s⁻¹). The enzyme also acted on Lac, LacNAc and LNnT; however, the $k_{cat}/K_m$ values for these substrates were 20–300-fold lower than that for LNT. No saturation curve for LNnT was observed up to 20 mM. Bga42A appears to have a high affinity for LNT compared with the other HMO-related substrates (Table I). Bga42B showed low activity on Lac and was essentially inactive on both type-1 and type-2 HMOs. Bga42C hydrolyzed Galβ1-4Glc/GlcNAc linkages with very low catalytic efficiencies and did not hydrolyze type-1 chains. The results revealed that Bga42A is a β-galactosidase highly specific for LNT and is essentially the sole β-galactosidase acting on type-1 HMOs.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Structure</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$k_{cat}/K_m$ (mM⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bga2A(Blon_2334)</td>
<td>pNP-β-Gal</td>
<td>Galβ1-4Glc</td>
<td>1.7 (±0.1) x 10⁻¹</td>
<td>3.0 (±0.0) x 10⁷</td>
<td>1.8 (±0.1) x 10⁸</td>
</tr>
<tr>
<td></td>
<td>pNP-β-Fuc</td>
<td>Galβ1-4Glc</td>
<td>1.4 (±0.1) x 10⁰</td>
<td>6.0 (±0.2) x 10⁸</td>
<td>4.2 (±0.2) x 10⁹</td>
</tr>
<tr>
<td>Lac</td>
<td>Galβ1-4GlcNac</td>
<td>5.3 (±0.2) x 10⁰</td>
<td>4.6 (±0.1) x 10⁸</td>
<td>8.6 (±0.3) x 10⁹</td>
<td></td>
</tr>
<tr>
<td>LacNAc</td>
<td>Galβ1-4GlcNac</td>
<td>6.0 (±0.7) x 10⁰</td>
<td>1.2 (±0.1) x 10⁹</td>
<td>2.0 (±0.1) x 10¹⁰</td>
<td></td>
</tr>
<tr>
<td>LNnT</td>
<td>Galβ1-4GlcNac</td>
<td>8.7 (±0.9) x 10⁰</td>
<td>3.0 (±0.1) x 10⁸</td>
<td>3.4 (±0.2) x 10⁹</td>
<td></td>
</tr>
<tr>
<td>LNT</td>
<td>Galβ1-3GlcNac</td>
<td>4.3 (±0.1) x 10⁰</td>
<td>1.9 (±0.0) x 10⁸</td>
<td>4.5 (±0.1) x 10⁹</td>
<td></td>
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</tbody>
</table>

Assays were performed in 50 mM sodium phosphate buffer (pH 6.0) containing 0.05% Tween-20. These enzymes did not act on pNP-β-GalNAc, pNP-β-GlcNac, pNP-α-Ara, pNP-β-Glc, pNP-β-GlcA and pNP-β-Xyl. n.d., not detected.
Relative amounts of the transcripts were presented as the percentages of those of the *dnaA* gene, and the values represent the mean ± standard deviations.

**Fig. 2.** Real-time quantitative RT–PCR analysis of the four β-galactosidase genes of *B. longum* subsp. *infantis*. Total RNAs were isolated from the cells grown in the HMOs (white bars) and Glc (gray bars) containing media. Relative amounts of the transcripts are presented as the percentages of those grown in the HMOs (white bars) and Glc (gray bars) containing media.

*Comparison of the expression levels of the four β-galactosidase genes*

To examine the physiological relevance of these β-galactosidases, the transcription levels were quantitated by real-time reverse transcription (RT)–polymerase chain reaction (PCR) using the cells grown in the HMO or Glc medium. The absence of contaminating DNA was confirmed by the RT-omitted reactions (data not shown). The amounts of the transcripts were shown as percentages relative to those of the *dnaA* transcript (Figure 2). The *bga2A* gene was most abundantly expressed, and its mRNA level was 3-fold higher than that of the housekeeping *dnaA* gene. The amounts of *bga42A* transcripts were comparable with those of the *dnaA* gene. On the other hand, the level of the *bga42B* expression was considerably lower, and in the case of *bga42C*, the transcription was negligible. The expression of *bga2A* was marginally increased when the cells were grown in the presence of HMOs, whereas the transcription of *bga42A* was not influenced by the type of carbon source.

**Discussion**

The HMO⁺ phenotype is a unique feature of particular infant-gut associated bifidobacteria with a few exceptions (Marcobal et al. 2010). Nevertheless, two representative HMO⁺ species, *B. bifidum* and *B. longum* subsp. *infantis*, appear to have evolved different pathways to degrade HMOs. *B. bifidum* hydrolyzes LNT, the most abundant core structure, outside the cell by lacto-N-biosidase into LNB and Lac and then imports LNB by a specific ABC transporter (Suzuki et al. 2008). Lac is hydrolyzed by a secretory β-galactosidase (Miwa et al. 2010), and the liberated Glc is preferentially consumed. In contrast, *B. longum* subsp. *infantis* directly incorporated LNT and hydrolyzed it into monosaccharides inside the cells.

GHS acting on β-galactosides occur in GH1, GH2, GH35, GH42 and GH43 (Cantarel et al. 2009). *B. longum* subsp. *infantis* ATCC15697 possesses two GH2 proteins (Blon_0268 and 2334), three GH42 proteins (Blon_2016, 2123 and 2416) and one GH43 protein (Blon_2411). The genes corresponding to GH1 and GH35 enzymes were not found. The Blon_2334 gene is located within the HMO cluster-1, whereas the other genes are positioned at different loci. To elucidate which gene(s) is responsible for the degradation of HMOs, we characterized these gene products (Table 1). The results revealed that the gene product of Blon_2016 (Bga42A) is responsible for the degradation of type-1 HMOs, whereas the gene product of Blon_2334 (Bga2A) is involved in the degradation of Lac and type-2 HMOs. Given that LNT is present at the concentration of 5 mM (Kunz et al. 2000; Urashima et al. 2011), the specific activity (v/[E]₀) of Bgn2A toward LNT is calculated to be 0.005% of that of Bga42A (Table 1 and Supplementary data, Figure S3). The HMO⁺ phenotype of this subspecies therefore should not be ascribed solely to the presence of the HMO cluster-1. The *bga2A* and *bga42A* genes were transcribed constitutively; however, this is not surprising, because such “enzymatic preparedness” was previously demonstrated for a human commensal bacterium (Sonnenburg et al. 2005). Bga42B and Bga42C should act on substrates other than HMOs.

β-Galactosidases acting on type-1 chains are rarely found in nature. To our knowledge, only two enzymes that belong to GH35 have been isolated from *Xanthomonas* species (Taron et al. 1995; Yang et al. 2003). GH42 contains more than 400 sequences, and several members including bifidobacterial enzymes have been characterized (Sheridan and Brenchley 2000; Hung et al. 2001; Moller et al. 2001; Hinz et al. 2004; Hu et al. 2007; Di Lauro et al. 2008; Goulas et al. 2009), and one crystal structure has been determined (Hidaka et al. 2002). However, most studies have focused on the synthesis of galactooligosaccharides (GOSs) using the transglycosylation activity and have not examined the substrate specificities of the enzymes. In a few cases, high activities toward galactans and GOSs with β-(1→4)- or β-(1→6)-linkages were demonstrated (Hinz et al. 2004; Goulas et al. 2009), which led to the assumption that GH42 enzymes may be involved in plant cell wall degradation. The present study on Bga42A is therefore the first that shows the occurrence of a type-1 chain-specific enzyme in GH42, and also the first study to reveal that a GH42 enzyme is physiologically involved in the degradation of the human-related glycan. The high specificity of Bga42A for LNT rather than LNB indicates the presence of a (+) subunit(s) to recognize the reducing end of LNT (Gal and/or Glc residues). The proposed name for this enzyme is LNT β-1,3-galactosidase.

Based on these finding, we performed a phylogenetic analysis using GH42 enzymes from bifidobacteria (Figure 3). The enzymes come from eight different bifidobacterial species/subspecies (http://www.cazy.org/GH42_bacteria.html; Cantarel et al. 2009), and the sequence identities vary from 20 to 100%. Bga42A, Bga42B and Bga42C fall into distant clades that reflect their different substrate specificities. Interestingly, the close homologs of Bga42A (aa identity >95%) that constitute one clade are found to come from infant gut-related species, i.e. *B. breve*, *B. longum* subsp. *infantis* and *B. longum* subsp. *longum*, all of which are known to consume LNT (Ward et al. 2007). The conservation of the type-1-specific β-galactosidase together with the predominance of the type-1 chain in HMOs lead us to postulate the coevolution of these organisms with humans. *B. bifidum* and some strains of *B. longum* subsp. *longum* may have acquired
lacto-N-biosidases alternatively to process type-I HMOs (Wada et al. 2008).

Materials and methods

Chemicals
Gal and Glc were purchased from Nacalai Tesque (Kyoto, Japan). LacNAc, LNnT and LNT were obtained from Dexta Laboratories (Reading, UK). pNP-glycosides, GlcNAc, Lac, verapamil hydrochloride and CCCP were from Wako Pure Chemical Industries (Osaka, Japan). LNB was synthesized as described previously (Nishimoto and Kitaoka 2007b).

Bacterial strains and growth conditions
B. longum subsp. infantis ATCC15697 and B. bifidum JCM1254 were obtained from the American Type Culture Collection and the Japan Collection of Microorganisms, respectively. The strains were anaerobically grown at 37°C in GAM medium (Nissui, Tokyo, Japan) or in basal medium (Wada et al. 2008). The basal medium was supplemented with a 1% carbon source (Glc or HMOs) prior to inoculation. Preparation of HMOs from human milk was performed as described previously (Sumiyoshi et al. 2003) with slight modifications (S Asakuma, T Urashima, M Kitaoka and T Katayama, in preparation).

Transport assay
The bifidobacteria were grown in the GAM media, harvested by centrifugation and suspended in the basal medium containing 1 mM LNT as a carbon source. The suspensions were incubated at 37°C in the presence and the absence of 5 mM verapamil or 50 μM CCCP. Samples were taken at the indicated times and the reaction products were analyzed by TLC using a silica gel 60 aluminum sheet (Merck, Darmstadt, Germany). The plate was developed in a solvent system of 1-buthanol/acetic acid/water (2/1/1). Sugars were visualized as described previously (Anderson et al. 2000).

Degradation of LNT by the cell-free extract of B. longum subsp. infantis
The cells grown in the GAM medium were suspended in 50 mM sodium phosphate buffer (pH 7.0) and disrupted by sonication. The cell-free extract was incubated with 5 mM LNT at 37°C and the reaction products were analyzed by TLC as described in the previous paragraph (Transport assay).

Enzyme preparation
The locus tag Blon_0268, 2016, 2123, 2334, 2411 and 2416 genes were amplified by PCR involving PrimeSTAR Max polymerase (Takara Bio, Shiga, Japan) using the genomic DNA of B. longum subsp. infantis as a template and the primer pairs listed in Supplementary data, Table S1. The amplified fragments were inserted into the pET23b vector to...
generate C-terminally histidine-tagged proteins. After the confirmation of the nucleotide sequences, the plasmids were introduced into a ΔlacZ derivative of E. coli BL21 (DE3) (Miwa et al. 2010). Expression and purification of the proteins using Ni-nitrotriacetic-acid affinity chromatography were performed according to the manufacturer’s instructions (Qiagen, MD). The proteins were further purified using a Superdex 200 10/300 GL column (GE Healthcare Bio-Sciences, Uppsala, Sweden). The elution was performed using 20 mM Tris–HCl buffer (pH 8.0) containing 150 mM NaCl and 0.05% Tween-20. The molecular masses of the proteins were determined using the same column. Thyroglobulin (669 kDa), ferritin (443 kDa), aldolase (158 kDa), conalbumin (75 kDa) and ovalbumin (43 kDa) were used as the standards.

Enzyme assay

Assays were performed in 50 mM sodium phosphate buffer (pH 6.0) containing 0.05% Tween-20 in a total volume of 300 μL. The substrates used were pNP-N-acetyl-β-D-galactosaminide (pNP-β-GalNAc), pNP-N-acetyl-β-D-glucosaminide (pNP-β-GlcNAc), pNP-α-L-arabinofuranoside (pNP-α-Ara), pNP-β-Fuc, pNP-β-Gal, pNP-β-D-glucopyranoside (pNP-β-Glc), pNP-β-D-glucuronide (pNP-β-GlcA) and pNP-β-D-xylopyranoside (pNP-β-Xyl). The reaction was initiated by adding the enzyme, and the mixtures were incubated for 5–120 min, depending on the substrates used. The reaction was stopped by adding 300 μL of 1 M Na₂CO₃. The amounts of released p-nitrophenol were determined by measuring the absorbance at 405 nm. When Lac, LacNAc, LNB, LNT and LNT were used as the substrates, the reactions were carried out in a total volume of 100 μL and terminated by heating (90°C, 5 min), under conditions that no chemical decomposition of LNB was observed (Chiku et al. 2010). The amounts of Gal released were determined using a galactose dehydrogenase-neocuproine method (Cohenford et al. 1989). Initial velocity of hydrolysis was determined in the range where the linearity of the reaction rate was observed. The kinetic parameters were calculated by curve fitting the experimental data with the Michaeli–Menten equation, using KaleidaGraph 4.0 (Synergy Software). In the case that no saturation was observed, only the $k_{cat}/K_m$ value was determined at the low substrate concentration.

The temperature and pH stabilities were determined by measuring the residual pNP-Gal-hydrolyzing activities after incubating the enzymes (1 μM) in 50 mM sodium phosphate buffer (pH 6.0) for 30 min at various temperatures and after dialyzing the enzyme overnight against various 10 mM buffers at 4°C, respectively. The buffers used were citrate phosphate (pH 3.5–6.5), sodium phosphate (pH 5.5–8.0) and Tris–HCl (pH 7.5–9.0). The optimal pHs for hydrolyzing pNP-Gal (0.5 mM) were determined using the same buffers (10 mM). Note that 1 mM MgCl₂ was added to the reaction mixture for assaying the activity of Bga2A throughout the study.

Real-time quantitative RT–PCR

Real-time quantitative RT–PCR was carried out for the four β-galactosidase genes (Blon et al. 2016, 2123, 2334 and 2416). The cells were grown in the basal medium supplemented with 1% Glc or HMOs to the mid-exponential phase and the total RNAs were isolated using a RiboPure-Bacteria kit (Applied Biosystems, CA). Purified RNA (120 ng) was reverse-transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems), and the reaction products were directly used for the TaqMan-based assay. The serial dilutions of the genomic DNA were used to construct the standard curves for the respective genes. Assays were performed in duplicate using RNAs isolated from two separate cultures and the relative amounts of the transcripts were presented as mean ± standard deviations. The dnaA gene was used as the reference gene. The primers and probes were designed using the Primer Express software (Applied Biosystems; Supplementary data, Table S2).

Ethical consideration

This study was approved by the Ethics Committee of the University of Shiga Prefecture and followed the Declaration of Helsinki. The informed consents were obtained from all milk donors.

Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org.

Funding

This work was supported in part by a Grant-in-aid for Scientific Research by Young Scientists (B) 22780072 from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and a Grant-in-aid from the Urakami Foundation.

Conflict of interest

None declared.

Abbreviations

aa, amino acid; ABC, ATP-binding cassette; CAZy, Carbohydrate-Active enZYmes; CCCP, carbonyl cyanide m-chlorophenyl hydrazzone; Gal, galactose; GH, glycoside hydrolase; Glc, glucose; GlcNAc, N-acetylgalcosamine; GNB, galacto-N-biose; GOS, galactooligosaccharide; HMO, human milk oligosaccharide; Lac, lactose; LacNAc, N-acetyllactosamine; LNB, lacto-N-biose I; LNT, lacto-N-neotetraose; LNT, lacto-N-tetraose; PCR, polymerase chain reaction; pNP-α-Ara, pNP-α-L-arabinofuranoside; pNP-β-Fuc, pNP-β-D-fucopyranoside; pNP-β-Gal, 4-nitrophenyl-β-D-galactopyranoside; pNP-β-GalNAc, pNP-N-acetyl-β-D-galactosaminide; pNP-β-Glc, pNP-β-D-glucopyranoside; pNP-β-GlcA, pNP-β-D-glucuronide;
nPN-β-GlcNAc, nPN-β-acetyl-β-d-glucosaminide; nPN-β-Xyl, nPN-β-xylorospanosidase; RT, reverse transcription; SDS-PAGE, sodium dodecyl sulfate-polyacylamide gel electrophoresis; TLC, thin-layer chromatography.

References


