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Cooperation of β-galactosidase and β-*N*-acetylhexosaminidase from bifidobacteria in assimilation of human milk oligosaccharides with type 2 structure

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Bifidobacteria are predominant in the intestines of breast-fed infants and offer health benefits to the host. Human milk oligosaccharides (HMOs) are considered to be one of the most important growth factors for intestinal bifidobacteria. HMOs contain two major structures of core tetrasaccharide: lacto-*N*-tetraose (Galβ1-3GlcNAcβ1-3Galβ1-4Glc; type 1 chain) and lacto-*N*-neotetraose (Galβ1-4GlcNAcβ1-3Galβ1-4Glc; type 2 chain). We previously identified the unique metabolic pathway for lacto-*N*-tetraose in *Bifidobacterium bifidum*. Here, we clarified the degradation pathway for lacto-*N*-neotetraose in the same bifidobacteria. We cloned one β-galactosidase (BbgIII) and two β-*N*-acetylhexosaminidases (BbhI and BbhII), all of which are extracellular membrane-bound enzymes. The recombinant BbgIII hydrolyzed lacto-*N*-neotetraose into Gal and lacto-*N*-triose II, and furthermore the recombinant BbhI, but not BbhII, catalyzed the hydrolysis of lacto-*N*-triose II to GlcNAc and lactose. Since BbgIII and BbhI were highly specific for lacto-*N*-neotetraose and lacto-*N*-triose II, respectively, they may play essential roles in degrading the type 2 oligosaccharides in HMOs.

Keywords: Bifidobacterium/GH2/GH20/lacto-*N*-neotetraose/prebiotics

Introduction

Bifidobacteria are Gram-positive anaerobic bacteria that naturally occur in the human intestinal tract. They are considered to be beneficial commensals for human health because they prevent the growth of pathogenic bacteria by lowering the intestinal pH and stimulate the host's immune system to enhance anti-pathogenic and anti-carcinogenic activities. The intestines of breast-fed infants are colonized by bifidobacteria within a week after birth, in comparison with those of bottle-fed infants (Yoshioka et al. 1983; Harmsen et al. 2000). The human milk oligosaccharides (HMOs) are defined as the oligosaccharides in human milk excluding lactose. They seem to be indigestible for the host and most intestinal bacteria but are thought to be assimilated by bifidobacteria and thus to promote the growth of those bacteria (Ward et al. 2006; LoCascio et al. 2007). HMOs are present at a concentration of 10–20 g/L in human milk and are characterized by their highly complex structures: i.e., more than 130 types of HMOs have so far been isolated (Kunz et al. 2000; Asakuma et al. 2008). Most HMOs carry lactose at their reducing end as the core structure. An elongation of the oligosaccharide is achieved by an enzymatic attachment of GlcNAc residue through β1,3-linkage to the Gal residue of lactose, followed by further addition of Gal through either β1,3- or β1,4-linkage to GlcNAc to form two major core tetrasaccharide structures: lacto-*N*-tetraose (LNT; Galβ1-3GlcNAcβ1-3Galβ1-4Glc; type 1 chain) and lacto-*N*-neotetraose (LNnT; Galβ1-4GlcNAcβ1-3Galβ1-4Glc; type 2 chain). These core tetrasaccharides are further elongated or branched by the addition of various sugars such as Gal, GlcNAc, Fuc, and sialic acid.

We have previously identified the unique metabolic pathway for lacto-*N*-tetraose in bifidobacteria. In this pathway, lacto-*N*-tetraose is hydrolyzed into Galβ1-3GlcNAc (lacto-*N*-biose I) and lactose by the extracellular lacto-*N*-biosidase LnbB (Wada et al. 2008). Lacto-*N*-biose I is then incorporated into bifidobacterial cells through a specific ABC-type transporter (Wada et al. 2007; Suzuki et al. 2008) and finally metabolized by intracellular enzymes such as galacto-*N*-biose (Galβ1-3GalNAc)/lacto-*N*-biose I phosphorylase, *N*-acetylhexosamine 1-kinase, UDP-glucose-hexose-1-phosphate uridylyltransferase, and UDP-galactose epimerase (Kitaoka et al. 2005; Nishimoto and Kitaoka 2007; Hidaka et al. 2009). This pathway for assimilation of lacto-*N*-biose I seems to be restricted so far in several bifidobacteria that include the species found in

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the intestinal tract of newborn infants. We have confirmed that lacto-*N*-biose I selectively promoted the growth of several bifidobacteria in vitro (Kiyohara et al. 2009).

In contrast to the metabolic pathway for LNT, that for LNnT in bifidobacteria has not yet been investigated. In this study, we clarified that LNnT was sequentially degraded from its non-reducing terminus by extracellular exo-glycosidases and identified the genes encoding one extracellular β-galactosidase (EC 3.2.1.23) and two extracellular β-*N*-acetylhexosaminidases (EC 3.2.1.52) from *Bifidobacterium bifidum* JCM1254.

Results

Degradation of LNnT by various bifidobacteria

To investigate the degradation of LNnT by bifidobacteria, we incubated LNnT with the cells of various strains grown on GAM medium and analyzed the supernatants by thin-layer chromatography (TLC) (Figure 1). Complete disappearance of LNnT and the remnant monosaccharides such as Gal, Glc, and GlcNAc were observed in the reaction mixtures of various *B. bifidum* strains (lanes 8–10). Weak hydrolysis of LNnT into Gal and a trisaccharide was also found in the mixtures with *Bifidobacterium longum longum* 705, *B. longum longum* JCM7054, and *Bifidobacterium scardovi* JCM12489 (lanes 12, 13, and 18, respectively). However, other subspecies/strains including few *B. longum longum* and *Bifidobacterium longum infantis* as well as the strains of *Bifidobacterium breve*, *Bifidobacterium pseudolongum*, *Bifidobacterium adolescentis*, *Bifidobacterium animalis*, *Bifidobacterium gallicum*, *Bifidobacterium catenulatum*, *Bifidobacterium angulatum*, and *Bifidobacterium pseudocatenulatum* showed no hydrolysis. Then, we chose *B. bifidum* JCM1254 for further analyses and gene cloning because we previously sequenced the genome of this strain.

In order to know the mode of degradation, we analyzed the time course of LNnT degradation by *B. bifidum* JCM1254 (Figure 2). At 10 min incubation, the amount of LNnT was reduced, and a trisaccharide and Gal appeared concomitantly. Then, at 20–30 min, lactose and GlcNAc appeared, which should be produced from the trisaccharide. On the other hand, *N*-acetyllactosamine (LacNAc) was not detected at any time point. Two other strains of *B. bifidum* showed similar results

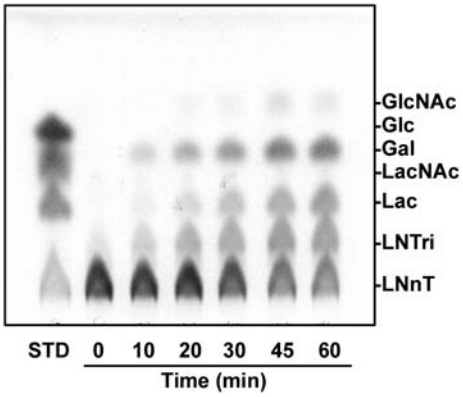

Fig. 2. Time-course analysis of LNnT degradation by the cells of *B. bifidum* JCM1254. LNnT was incubated with the cells for indicated times and analyzed by TLC. The plate was developed twice using two different developing solvents (first, 1-propanol:water = 42.5:7.5, by volume; second, ethyl acetate: acetic acid:methanol:water = 20:7.5:7.5:5, by volume). STD, mixture of standard LNnT, lactose, LacNAc, Gal, and Glc.

in the time course of LNnT degradation (data not shown). These results suggest that LNnT is sequentially degraded outside of the cells of *B. bifidum* by extracellular exo-glycosidases such as β-galactosidase(s) and β-*N*-acetylhexosaminidase(s).

Cloning and expression of β-galactosidase from B. bifidum JCM1254

We searched for the gene of β-galactosidase in the genome of *B. bifidum* JCM1254 and found five candidate open-reading-frames, which encode three glycoside hydrolase (GH) family 2 (GH2) and two GH42 enzymes. Since four genes out of five were nearly identical to the previously identified β-galactosidase genes as *bbgI*, *bbgII*, *bbgIII*, and *bbgIV* from *B. bifidum* NCIMB41171 (Goulas et al. 2007, 2009a), we referred to the genes from *B. bifidum* JCM1254 as the same names (accession numbers: *bbgI*, AB542711; *bbgII*, AB542712; *bbgIII*, AB504520; *bbgIV*, AB542713). We named the fifth one *bbgV* (accession number AB542714) that encodes a putative GH42 enzyme. Among these five genes, only *bbgIII* was predicted to encode an extracellular membrane-bound enzyme possessing

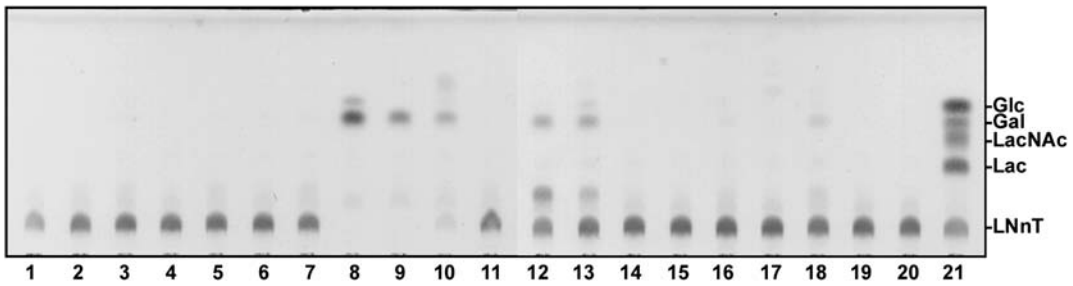

Fig. 1. Hydrolysis of LNnT by various bifidobacterial strains. Bifidobacteria cultured on GAM medium were washed with 50 mM sodium phosphate buffer, pH 7.0. The washed cells (50 μg, wet weight) were incubated with 20 nmol LNnT in 7 μL of the same buffer at 37°C for 2 h. The supernatants of the reaction mixtures were analyzed by TLC. Lane 1, no bifidobacterial cells; lane 2, *Bifidobacterium breve* 203; lane 3, *B. breve* clb; lane 4, *B. breve* JCM1192^T; lane 5, *B. pseudolongum* subsp. *pseudolongum* JCM1205^T; lane 6, *B. longum* subsp. *longum* JCM1217^T; lane 7, *B. longum* subsp. *infantis* JCM1222; lane 8, *B. bifidum* JCM1254; lane 9, *B. bifidum* JCM7004; lane 10, *B. bifidum* JCM1255^T; lane 11, *B. longum* 33R; lane 12, *B. longum* 705; lane 13, *B. longum* subsp. *longum* JCM7054; lane 14, *B. adolescentis* JCM1275^T; lane 15, *B. animalis* subsp. *lactis* JCM10602^T; lane 16, *B. gallicum* JCM8224^T; lane 17, *B. catenulatum* JCM1194^T; lane 18, *B. scardovii* JCM12489^T; lane 19, *B. angulatum* JCM7096^T; lane 20, *B. pseudocatenulatum* JCM1200^T; lane 21, mixture of standard LNnT, lactose, LacNAc, Gal, and Glc.

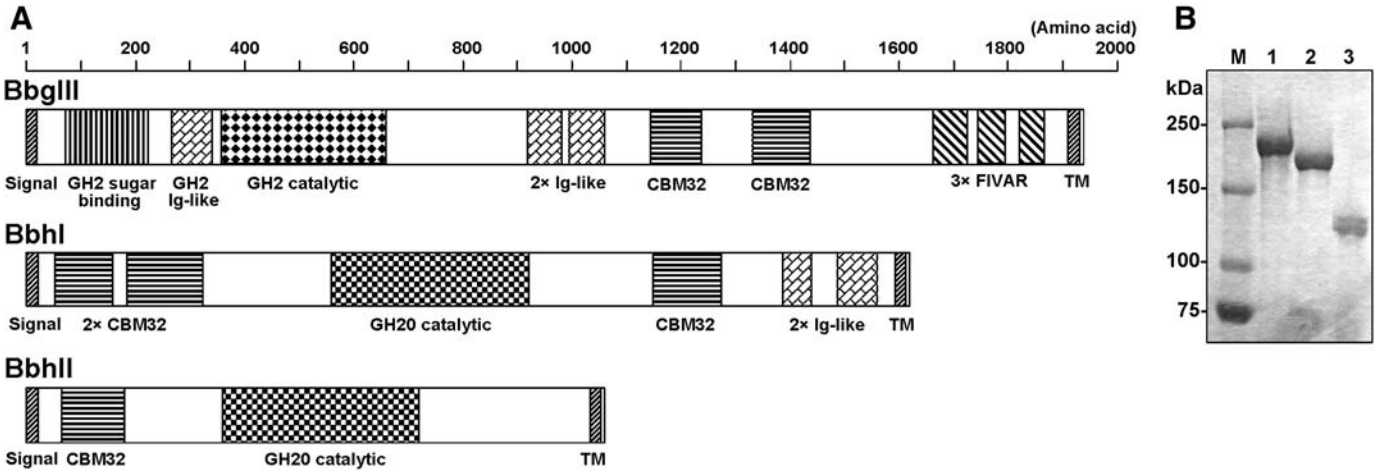

Fig. 3. Molecular cloning and expression of BbgIII, BbhI, and BbhII. (**A**) Domain structures of BbgIII, BbhI, and BbhII from *B. bifidum* JCM1254. (**B**) SDS-PAGE of the purified recombinant proteins expressed in *E. coli*. Lane 1, BbgIII; lane 2, BbhI; lane 3; BbhII. M, marker proteins.

an N-terminal signal sequence and a C-terminal transmembrane region. Two BbgIIIs from *B. bifidum* JCM1254 and *B. bifidum* NCIMB41171 have the same length of 1935 amino acid (aa) polypeptides and share 99% identity in amino acid sequence (1914/1935). Two homologues also share multi-domain structure, namely, they consist of the following putative sequences/domains: an N-terminal signal sequence (aa 1–32), a GH2 sugar binding domain (aa 61–214), a GH2 Ig-like domain (aa 291–334), a GH2 β-galactosidase catalytic domain (aa 340–665), two Ig-like domains (group 4) (aa 924–982, 997–1052), two carbohydrate-binding module (CBM) 32 domains (aa 1134–1237, 1343–1477), three found-in-various-architectures (FIVAR) domains (aa 1666–1719, 1741–1794, 1823–1876), and a C-terminal transmembrane region (aa 1905–1930) (Figure 3A, top panel).

The N-terminally six histidine (His)-tagged BbgIII from *B. bifidum* JCM1254 without the N-terminal signal peptide and the C-terminal transmembrane region was overexpressed in *Escherichia coli* BL21(DE3)Δ*lacZ* using pET vector. The expressed protein was extracted from the cells and purified using a His-tag affinity column chromatography and gel filtration. The purified protein migrated as a single protein band of 200 kDa on reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 3B, lane 1). By gel filtration using Superdex 200 10/300 GE, the molecular weight was estimated to be approximately 400 kDa (data not shown), suggesting that BbgIII is a homodimeric enzyme. The general properties of BbgIII were determined using *p*NP-β-Gal as a substrate as follows: optimum at pH 5.5 and 40°C; stable between pH 3.0–9.0 and less than 50°C at pH 5.5.

Cloning and expression of β-N-acetylhexosaminidases from B. bifidum JCM1254

We found three candidate genes of GH20 β-*N*-acetylhexosaminidases in the genome of *B. bifidum* JCM1254. Two of them encode extracellular membrane-bound enzymes, and the other one encodes an intracellular protein. We cloned and termed the former two genes *bbhI* and *bbhII* (accession numbers AB504521 and AB504522, respectively) and the third one *bbhIII* (accession number AB542715). BbhI is predicted to be a 1627-aa polypeptide and contains the following putative sequences/domains: an N-terminal signal sequence (aa 1–32), two CBM32 domains (aa 42–167, 190–326), a GH20 catalytic domain (aa 566–917), a CBM32 domain (aa 1148–1281), two Ig-like domains (aa 1391–1428, 1487–1567), and a C-terminal transmembrane region (aa 1605–1621) (Figure 3A, middle panel). BbhII has a relatively simple structure: 1060 aa polypeptide containing an N-terminal signal sequence (aa 1–32), a CBM32 domain (aa 52–190), a GH20 catalytic domain (347–714), and a C-terminal transmembrane region (aa 1028–1044) (Figure 3A, bottom panel).

The recombinant BbhI and BbhII were expressed in *E. coli* in the same way as the case of BbgIII. The purified BbhI and BbhII migrated as single protein bands of 170 and 120 kDa, respectively, on reducing SDS-PAGE (Figure 3B, lanes 2 and 3, respectively). By gel filtration using Superdex 200 10/300 GE, the molecular weights of BbhI and BbhII were estimated to be around 350 and 200 kDa (data not shown), respectively, suggesting that both enzymes are homodimeric enzymes as BbgIII. The general properties of BbhI and BbhII were determined using *p*NP-β-GlcNAc as a substrate as follows, respectively: optimum pH, pH 6.0 and pH 5.5; optimum temperature, 50°C and 55°C; stable pH range, pH 4.0–7.5 and pH 5.0–6.0; thermal stability, less than 50°C and 42°C.

Substrate specificities of BbgIII, BbhI, and BbhII

The kinetic parameters of BbgIII toward various β-galactoside substrates were determined (Table I). The affinities toward LacNAc (K_m = 1.11 mM) and LNnT (K_m = 1.39 mM) were much higher than those toward the other disaccharides such as lactose (K_m = 3.49 mM), *allo*-LacNAc (K_m = 5.40 mM), and lactulose (K_m = 6.76 mM). The k_{cat} values for LacNAc (k_{cat} = 281 s^{-1}) and LNnT (k_{cat} = 273 s^{-1}) were also very high. Branched LacNAc structure (lacto-*N*-hexaose; LNH) was a moderately preferable substrate (K_m = 2.26 mM and k_{cat} = 273 s^{-1}). In contrast to β1,4- and β1,6-linked Gal, β1,3-liked Gal in lacto-*N*-biose I and LNT were not hydrolyzed by BbgIII at all. These analytical data suggest that BbgIII is a LacNAcase rather than lactase. Since free LacNAc is not present in human intestines, BbgIII might be responsible for degradation of

Table I. Kinetic parameters of BbgIII β-galactosidase from *B. bifidum* JCM1254

Substrate	Name (abbreviation)	Hydrolytic activity	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
Galβ1-*p*-nitorophenol	*p*NP-β-Gal	+	0.37 ± 0.01	32.8 ± 1.5	89.1
Galβ1-4Glc	Lactose (Lac)	+	3.49 ± 0.45	70.0 ± 4.7	19.8
Galβ1-4GlcNAc	*N*-Acetyllactosamine (LacNAc)	+	1.11 ± 0.32	281.0 ± 27.1	253
Galβ1-4GlcNAcβ1-3Galβ1-4Glc	Lacto-*N*-neotetraose (LNnT)	+	1.39 ± 0.15	273.0 ± 11.0	209
Galβ1-4GlcNAcβ1-3Galβ1-4Glc Galβ1-4GlcNAcβ1 ↘6	Lacto-*N*-hexaose (LNH)	+	2.26 ± 0.03	273.0 ± 14.4	128
Galβ1-6GlcNAc	*allo*-*N*-Acetyllactosamine (*allo*-LacNAc)	+	5.40 ± 0.26	10.1 ± 0.1	1.9
Galβ1-4Frc	Lactulose	+	6.76 ± 0.99	2.3 ± 0.5	0.3
Galβ1-3GlcNAc	Lacto-*N*-biose I (LNB)		nd.	nd.	nd.
Galβ1-3GlcNAcβ1-3Galβ1-4Glc	Lacto-*N*-tetraose (LNT)	−	nd.	nd.	nd.
Galβ1-4Glc Fucα1 ↗3	3-Fucosyllactose	−	nd.	nd.	nd.
Galβ1-4GlcNAc Fucα1 ↗3	Le^x trisaccharide	−	nd.	nd.	nd.
Galβ1-4GlcNAcβ1-3Galβ1-4Glc Fucα1 ↗3	Lacto-*N*-fucopnetaose III	−	nd.	nd.	nd.

The K_m and k_{cat} values represent mean ± SD (n = 3); +, detected; −, not detected; nd, not determined.

LacNAc-containing oligosaccharides such as LNnT and LNH. However, BbgIII could not hydrolyze Fuc-branched oligosaccharides such as Lewis X trisaccharide and 3-fucosyllactose, indicating that the prior removal of α1,3-linked fucose by AfcB α1,3-1,4-L-fucosidase (Ashida et al. 2009) is necessary for degrading these oligosaccharides.

Next, to determine the substrate specificities of BbhI and BbhII, we first tested them using various *p*NP-monosaccharide substrates. Both enzymes specifically hydrolyzed *p*NP-β-GlcNAc and *p*NP-β-GalNAc but not other *p*NP-monosaccharides. Although their specificities for both β-linked GlcNAc and GalNAc are the common characteristics of general β-*N*-acetylhexosaminidases, their hydrolysis rates for *p*NP-β-GlcNAc are extraordinarily higher than that for *p*NP-β-GalNAc: 330-fold and 5-fold in BbhI and BbhII, respectively (Table II). We then tested their activities toward β-GlcNAc-containing oligosaccharides. BbhI hydrolyzed lacto-*N*-triose II (GlcNAcβ1-3Galβ1-4Glc) very rapidly, and the K_m and k_{cat} values for the trisaccharide were estimated to be 0.36 mM and 93 s^{-1}, respectively. BbhI acted, however, very slowly on GlcNAcβ1-2Man, GlcNAcβ1-3Man, and GlcNAcβ1-4GlcNAc (Supplementary Figure 1), and thus the kinetic parameters for these disaccharides could not be estimated. Mucin-type core 2 trisaccharide, GlcNAcβ1-6(Galβ1-3)GalNAc, was also very slowly hydrolyzed, whereas GlcNAcβ1-4MurNAc was completely resistant to BbhI (Table II and Supplementary Figure 1). LNT and LNnT were also resistant (data not shown), indicating BbhI has neither endo-type nor disaccharide-releasing activities like GH20 lacto-*N*-biosidase. In comparison with BbhI, BbhII showed low activity even for *p*NP-β-GlcNAc (its specific activity was 1/70 of that of BbhI) and also a very weak activity for lacto-*N*-triose II and mucin-type core 2 trisaccharide.

Sequential hydrolysis of LNnT and LNH by recombinant BbgIII and BbhI

To demonstrate the hydrolysis of LNnT by bifidobacteria, we incubated LNnT with either or both of the recombinant BbgIII and BbhI and analyzed the reaction mixtures by TLC (Figure 4A). BbgIII hydrolyzed LNnT into Gal and lacto-*N*-triose II (lane 2). However, BbhI did not act on LNnT directly (lane 3), but by co-incubation with BbgIII, LNnT was completely hydrolyzed: BbhI hydrolyzed lacto-*N*-triose II produced from LNnT by BbgIII into GlcNAc and lactose that was immediately hydrolyzed by co-existing BbgIII (lane 4). By contrast, the ad-

Table II. Substrate specificities of BbhI and BbhII β-*N*-acetylhexosaminidases from *B. bifidum* JCM1254

		BbhI				BbhII
Substrate	Name	Specific activity (μmol min^{-1} mg^{-1})	K_m (mM)	k_{cat} (s^{-1})	$k_{cat}{}^1/K_m$ (s^{-1} mM^{-1})	Specific activity (μmol min^{-1} mg^{-1})
GlcNAcβ1-*p*-nitorophenol	*p*NP-β-GlcNAc	22.30 ± 3.08	120 ± 0.18	213.0 ± 12.9	178	0.320 ± 0.005
GlcNAcβ1-*p*-nitorophenol	*p*NP-β-GlcNAc	0.067 ± 0.024	nd.	nd.	nd.	0.062 ± 0.004
GlcNAcβ1-3Galβ1-4Glc	Lacto-*N*-triose II	42.60 ± 1.02	0.36 ± 0.09	93.0 ± 11.9	257	0.007 ± 0.001
GlcNAcβ1 ↘6 Galβ1-3GalNAcα1-*p*-nitorophenol	*p*NP-α-core 2	0.062 ± 0.010	nd.	nd.	nd.	0.34 ± 0.003
GlcNAcβ1-2Man		0.150 ± 0.019	nd.	nd.	nd.	−
GlcNAcβ1-3Man		Trace	nd.	nd.	nd.	−
GlcNAcβ1-4GlcNAc	*N*,*N*′-Diacetylchitobiose	Trace	nd.	nd.	nd.	−
GlcNAcβ1-4MurNAc		−	nd.	nd.	nd.	−

The K_m and k_{cat} values represent mean ± SD (n = 3); −, not detected; nd, not determined.

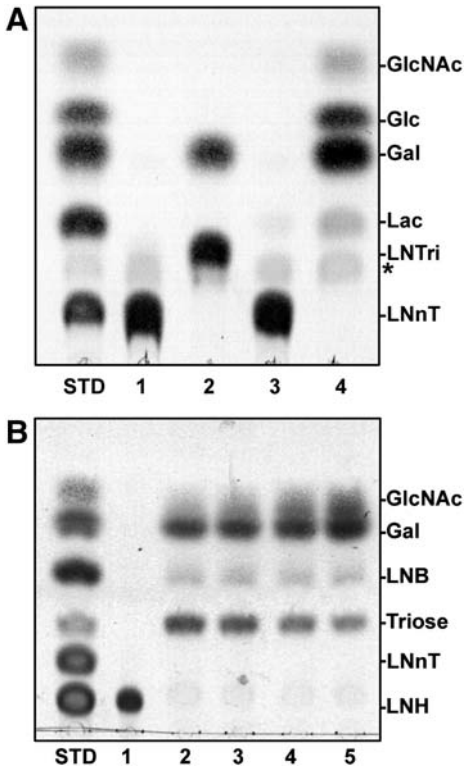

Fig. 4. Hydrolysis of LNnT and LNH by the recombinant BbgIII and BbhI. (**A**) LNnT (40 nmol) was incubated with 1.4 milliunits BbgIII and/or 0.17 milliunits BbhI for 6 h. The reaction mixtures were analyzed by TLC using a developing solvent (1-propanol:water = 85:15, by volume). Lane 1, LNnT only; lane 2, LNnT incubated with by BbgIII; lane 3, LNnT incubated with BbhI; lane 4, LNnT incubated with both BbgIII and BbhI. STD, mixture of standard LNnT, lactose, Gal, Glc, and GlcNAc. Asterisk, an unknown contaminant in commercial LNnT. (**B**) LNH (30 nmol) was incubated with 1.3 milliunits BbgIII, 0.6 milliunits BbhI, and 1.0 milliunits LnbB for indicated periods. The reaction mixtures were analyzed by TLC using a developing solvent (1-butanol:acetic acid:water = 2:1:1, by volume). Lanes 1–5, 0, 0.5, 1, 2, and 4 h. STD, mixture of standard LNH, LNnT, lacto-*N*-triose II, lacto-*N*-biose I, Gal, and GlcNAc.

dition of the same protein amount of BbhII to reaction mixture instead of BbhI caused no hydrolysis of lacto-*N*-triose II (data not shown), suggesting that BbhII may not function in assimilation of LNnT by bifidobacteria.

We further tested the hydrolysis of LNH that is one of the main components of HMOs and contains β1,6-branched LacNAc on Gal of core lactose in LNT. When LNH was incubated with recombinant BbgIII, BbhI, and lacto-*N*-biosidase LnbB, a trisaccharide that was predicted to be GlcNAcβ1-6Galβ1-4Glc was produced, and lacto-*N*-biose I (relatively weak coloration because of the presence of GlcNAc at reducing end) and Gal were concomitantly released from LNH by the action of LnbB and BbgIII, respectively (Figure 4B, lane 2). The β1,6-GlcNAc-linkage of the trisaccharide was then slowly hydrolyzed by BbhI (Figure 4B, lanes 3–5). When BbhI was replaced with BbhII, a similar result was obtained (data not shown), suggesting that BbhII as well as BbhI may function in degradation of LNH. These results suggested that LNH might be completely hydrolyzed by these bifidobacterial enzymes.

Discussion

Cumulative evidences suggest that HMOs have the critical role in the initial colonization of bifidobacteria in the intestines of newborn infants. In HMOs, two types of the tetrasaccharide core structures are present: LNT and LNnT. Previously, we identified the unique pathway for assimilation of LNT involving extracellular lacto-*N*-biosidase (Wada et al. 2008), galacto-*N*-biose/lacto-*N*-biose I transporter (Wada et al. 2007; Suzuki et al. 2008), and intracellular galacto-*N*-biose/lacto-*N*-biose I phosphorylase (Kitaoka et al. 2005; Hidaka et al. 2009). In addition, we characterized two different extracellular α-L-fucosidases acting on fucosylated HMOs (Katayama et al. 2004, 2005; Nagae et al. 2007; Ashida et al. 2009). In this report, we have completed the identification of the degradation pathway of LNnT. We found the candidate genes for five β-galactosidases and three β-*N*-acetylhexosaminidases in the genome of *B. bifidum* JCM 1254 and identified that a β-galactosidase (BbgIII) and a β-*N*-acetylhexosaminidase (BbhI), both of which are predicted to be extracellular membrane-bound enzymes, are responsible for the degradation of LNnT.

Although β-galactosidase is a very common glycosidase present in intestinal bacteria, the presence of membrane-bound β-galactosidases is limited in several bifidobacterial strains. BbgIII of this strain is nearly identical to the previously reported BbgIII from *B. bifidum* NCIMB41171, which was characterized in view of the ability for synthesizing galactooligosaccharides (Goulas et al. 2009b), one of the promising prebiotics. We found that BbgIII hydrolyzed LacNAc and LNnT more rapidly than lactose. Our result strongly suggests that bifidobacterial BbgIII is physiologically involved in the degradation and assimilation of LNnT in HMOs. All the other β-galactosidase genes in the genomes of *B. bifidum* JCM1254 and *B. bifidum* NCIMB41171 encode intracellular enzymes probably acting on lactose. The fact that *bbgIV* is clustered with *lacS* and *lacI* in the genome suggests its function in lactose metabolism (Goulas et al. 2007). Phylogenetic analysis of GH2 enzymes also shows that BbgI/BbgIV and BbgIII are evolutionally far from each other (Figure 5A). Near the clades of BbgI and BbgIV, there are a lot of LacZ homologues that belong to lactase-type β-galactosidases. On the other hand, BbgIII is clustered with BgaA β-galactosidase from *Streptococcus pneumoniae* that acts on the LacNAc moiety of *N*-glycans (King et al. 2006). Thus, the enzymes from *Clostridium* and *Bacteroides* in this cluster may also prefer LacNAc.

In contrast to β-galactosidase, β-*N*-acetylhexosaminidase is a relatively rare glycosidase in intestinal bacteria. So far as we know, this is the first report of bifidobacterial β-*N*-acetylhexosaminidases. BbhI hydrolyzed almost only lacto-*N*-triose II, suggesting that BbhI is evolved for degrading LNnT with cooperation of BbgIII. The recombinant BbgIII and BbhI, both of which were successfully expressed in *E. coli*, degraded LNnT sequentially. On the other hand, BbhII showed only trace activities toward natural substrates tested; however, it practically acted on β1,6-linked GlcNAc that occurs in LNH and mucin core 2-type *O*-glycan. Therefore, BbhII might function in the degradation of these glycans in cooperation with BbhI. The genome of *B. bifidum* JCM1254 includes one more β-*N*-acetylhexosaminidase gene, *bbhIII*, which is predicted to encode an intracellular enzyme. It was successfully expressed in *E. coli* and showed activity toward *p*NP-β-GlcNAc (data not

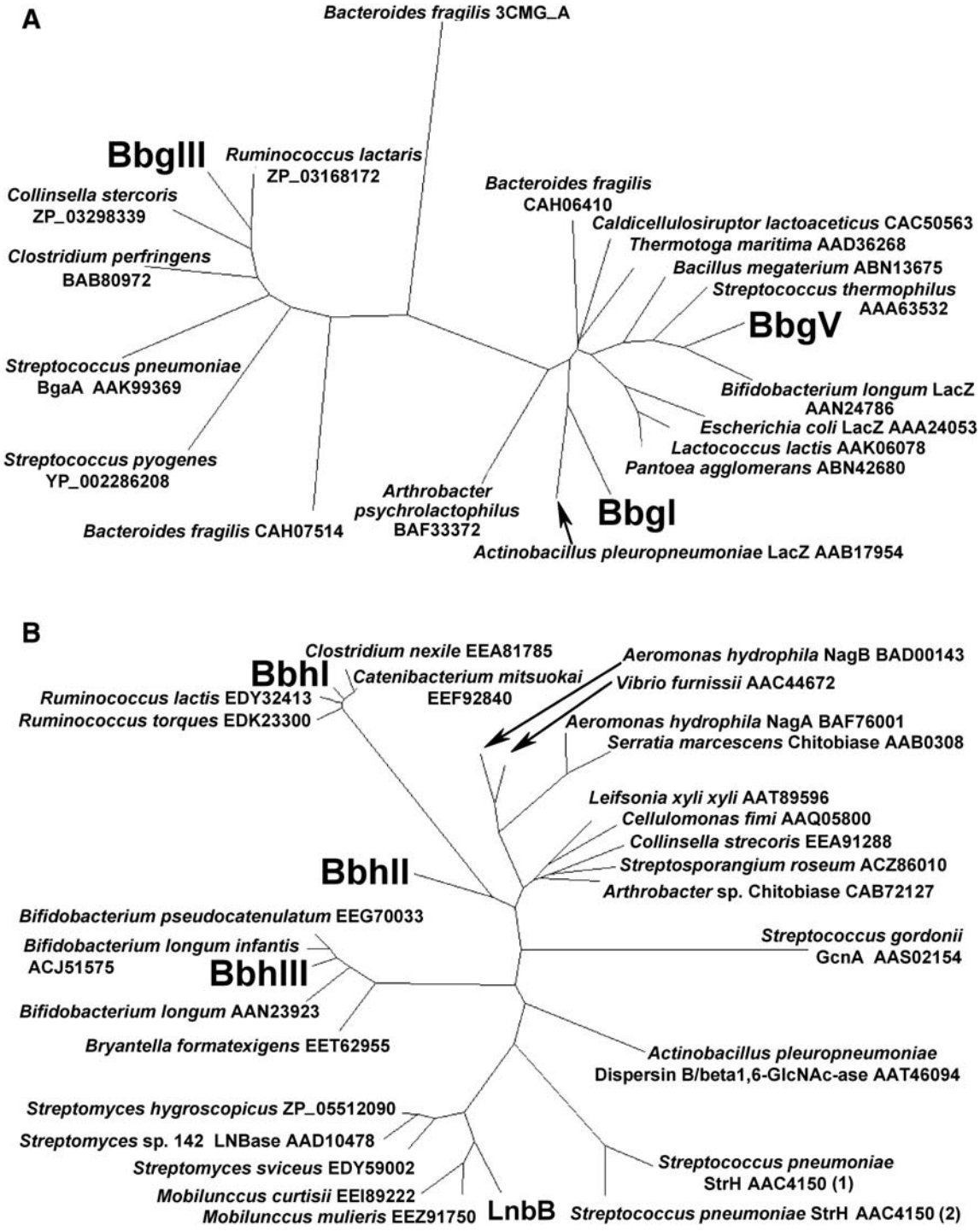

Fig. 5. Phylogenetic trees of GH2 β-galactosidases (**A**) and GH20 β-*N*-acetylhexosaminidases (**B**). Unrooted trees were constructed using the ClustalW program. The amino acid sequences of the conserved GH domains of each protein were used for analyses.

shown), but its biological meaning remains to be elucidated. All these β-*N*-acetylhexosaminidases belong to GH20. We previously identified GH20 lacto-*N*-biosidase (LnbB) from the same bifidobacterial strain, which releases lacto-*N*-biose I from LNT (Wada et al. 2008). Although both BbhI and LnbB recognize the same GlcNAcβ1-3Gal-linkage, the amino acid identity between the GH20 catalytic domains of two enzymes is only 21.6%. The phylogenetic analysis of bacterial GH20 enzymes reveals that BbhI and LnbB locate on the distinct clades (Figure 5B). BbhI is also different from β1,4-*N*-acetylglucosaminidases such as chitobiases and exochitinases (a clade at the upper right of the panel), β1,6-*N*-acetylglucosaminidase/dispersin B from *Actinobacillus pleuropneumoniae* (Kaplan et al. 2004) and StrH β1,2-*N*-acetylglucosaminidase from *S. pneumoniae* (King et al. 2006). BbhI clusters with previously uncharacterized pro-

teins from several intestinal bacteria and may therefore form a novel subfamily. GH20 enzymes as well as GH18 and GH85 enzymes are known to catalyze hydrolysis by a substrate-assisted catalytic mechanism, in which the 2-acetamide group of the substrate GlcNAc acts as a nucleophile, and a single acidic residue in polypeptide functions as an acid/base (Terwisscha van Scheltinga et al. 1995; Williams et al. 2002; Umekawa et al. 2008; Umekawa et al. 2010). In GH20 enzymes, one more acidic residue at one residue upstream of the acid/base is required for orienting and stabilizing oxazolinium ion intermediate. These conserved DE sequences were also found in BbhI, BbhII, and LnbB: DE^{747}, DE^{519}, and DE^{321}, respectively. The D746A and E747A mutants of BbhI and the D518A and E519A mutants of BbhII were generated and confirmed to be their loss of enzyme activity (data not shown).

A LacNAc unit is frequently found not only in HMOs but also various glycoconjugates such as *N*-glycans, *O*-glycans, and glycosphingolipids. These glycoconjugates should be substrates for BbgIII and BbhI. We previously identified endo-α-*N*-acetylgalactosaminidase (EngBF) from bifidobacteria, which releases Galβ1-3GalNAc from the core 1-type *O*-glycan mainly found in gastric mucin (Fujita et al. 2005; Ashida et al. 2008, 2010; Suzuki et al. 2009). The released disaccharide could be specifically assimilated by bifidobacteria through galacto-*N*-biose/lacto-*N*-biose I pathway. However, EngBF hardly acts on *O*-glycans when the core 1-disaccharide is modified by addition of other sugars. BbgIII and BbhI may contribute to remove LacNAc repeats on the core 1-type *O*-glycans, and BbhI and BbhII may function in removal of β1,6-branched GlcNAc in the core 2-type *O*-glycans to expose the core 1-disaccharide.

LNnT was unexpectedly resistant to digestion by many intestinal bacteria we tested, such as *Bacteroides ovatus* JCM5824^T, *Eubacterium limosum* JCM6421^T, *Eubacterium cylindroides* JCM10261^T, *Clostridium hiranonis* JCM10541^T, *Clostridium hylemonae* JCM10539^T, *Clostridium scindens* JCM6567^T, *Enterococcus pseudoavium* JCM8732^T, and *Enterococcus raffinosus* JCM8733^T (data not shown). Thus, LacNAc-containing glycans such as LNnT and mucin *O*-glycans may be potential and specific prebiotics for bifidobacteria. BbgIII and BbhI play an essential role in the assimilation of these prebiotic glycans.

Materials and methods

Bacterial strains and culture

The bifidobacterial strains were obtained from the Japan Collection of Microorganisms (JCM, RIKEN Bioresource Center, Japan). The bacteria were cultured on GAM broth (Nissui Pharmaceutical, Japan) for 16 h at 37°C under anaerobic conditions using Anaeropack (Mitsubishi Chemical, Japan).

Genome sequence of B. bifidum JCM1254

Draft sequencing of the genome of *B. bifidum* JCM1254 was performed using a Genome Sequencer 20 System (Roche Applied Science). The details will be reported elsewhere.

Cloning and expression of bbgIII, bbhI, and bbhII in E. coli

To construct the expression vector for BbgIII, a DNA fragment encoding aa 33–1910 (without N-terminal signal peptide and C-terminal transmembrane region) was amplified by high-fidelity polymerase chain reaction using genomic DNA from *B. bifidum* JCM1254 as a template and the following primers: forward 5′-aaggatccggtcgaggacgccacccga and reverse 5′-ttctcgaggct-cagtgcgctgccatc, digested with *Bam*HI and *Xho*I, and ligated into pET23b(+). Similarly, a DNA fragment encoding aa 33–1604 of BbhI were amplified using the primers, 5′-cgaagcttagc-gatgacaatcttgcactga and 5′-tctcgagggcgacctcgtcaggcg, digested with *Xho*I and *Hin*dIII, and ligated into pET23b(+); a DNA fragment encoding amino acids 33–1027 of BbhII were amplified using the primers, 5′-agctagcgcggcagcggcggaa and 5′-actcga-gaccggtctcggcgacgac, digested with *Xho*I and *Nhe*I, and ligated into the same plasmid. The nucleotide sequences were confirmed by sequencing. The *E. coli* BL21(DE3)Δ*lacZ* strain that lacks β-galactosidase activity was transformed with each of the constructed plasmids and cultured in Luria–Bertani liquid medium containing 100 μg/mL ampicillin at 25°C until the optical density at 600 nm reached 0.5. Then, to induce the expression, isopropyl β-D-1-thiogalactopyranoside was added to the culture at final concentration of 0.5 mM, and the cultivation was carried out for 5 h at 25°C.

Purification of the recombinant enzymes

The transformed *E. coli* cells grown under the induced condition were lysed by BugBuster Protein Extraction Reagent (Novagen). After centrifugation, the supernatant was applied to a HisTrap HP (1 mL, GE Healthcare), and the adsorbed proteins were eluted by a stepwise imidazole concentration gradient in 50 mM sodium phosphate buffer, pH 7.0, containing 250 mM NaCl. The active fraction (1 mL) was applied onto a Superdex 200 10/300 GL (GE healthcare) gel filtration column equipped with ÄKTA explorer (GE Healthcare). Elution was carried out using 50 mM sodium phosphate buffer, pH 7.0, containing 150 mM NaCl. Active fractions were collected, concentrated and desalted using an Amicon Ultra 30K (Millipore).

Enzyme assays

Standard assays for β-galactosidase and β-*N*-acetylhexosaminidase were carried out using the substrates *p*NP-β-Gal and *p*NP-β-GlcNAc, respectively, at 37°C, and quantified the released *p*NP by measuring absorbance at 400 nm. When oligosaccharides were used for substrates, the released Gal and GlcNAc were quantified by the galactose dehydrogenase/neocuproine method (Dygert et al. 1965; Cohenford et al. 1989) and the method of Reissig et al. (1955), respectively. The hydrolyses of oligosaccharide substrates were also analyzed by silica-gel TLC (Merck 5553) with 1-butanol:acetic acid:water (2:1:1, by volume) as a developing solvent, unless otherwise indicated, and visualized using diphenylamine-aniline-phosphoric acid (Anderson et al. 2000). LacNAc, GlcNAcβ1-4GlcNAc, and *p*NP-monosaccharides were purchased from Sigma-Aldrich; GlcNAcβ1-2Man and *p*NP-α-core 2 trisaccharide was from Toronto Research Chemicals (Ontario, Canada); the other oligosaccharides except for lacto-*N*-triose II were from Dextra (Reading, UK). Lacto-*N*-triose II was prepared by BbgIII β-galactosidase digestion of LNnT followed by purification using Sephadex G-15 gel filtration.

Supplementary Data

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

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Conflict of interest statement

None declared.

Abbreviations

CBM, carbohydrate-binding module; FIVAR, found-in-various-architectures; GH, glycoside hydrolase; HMO, human milk oligosaccharide; LacNAc, *N*-acetyllactosamine; LNH, lacto-*N*-hexaose; LNnT, lacto-*N*-neotetraose; LNT, lacto-*N*-tetraose; *p*NP, *para*-nitrophenyl; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

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