Distribution of *In Vitro* Fermentation Ability of Lacto-*N*-Biose I, a Major Building Block of Human Milk Oligosaccharides, in Bifidobacterial Strains^{∇†}

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This study investigated the potential utilization of lacto-*N*-biose I (LNB) by individual strains of bifidobacteria. LNB is a building block for the human milk oligosaccharides, which have been suggested to be a factor for selective growth of bifidobacteria. A total of 208 strains comprising 10 species and 4 subspecies were analyzed for the presence of the galacto-*N*-biose/lacto-*N*-biose I phosphorylase (GLNBP) gene (*lnpA*) and examined for growth when LNB was used as the sole carbohydrate source. While all strains of *Bifidobacterium longum* subsp. *longum*, *B. longum* subsp. *infantis*, *B. breve*, and *B. bifidum* were able to grow on LNB, none of the strains of *B. adolescentis*, *B. catenulatum*, *B. dentium*, *B. angulatum*, *B. animalis* subsp. *lactis*, and *B. thermophilum* showed any growth. In addition, some strains of *B. pseudocatenulatum*, *B. animalis* subsp. *animalis*, and *B. pseudolongum* exhibited the ability to utilize LNB. With the exception for *B. pseudocatenulatum*, the presence of *lnpA* coincided with LNB utilization in almost all strains. These results indicate that bifidobacterial species, which are the predominant species found in infant intestines, are potential utilizers of LNB. These findings support the hypothesis that GLNBP plays a key role in the colonization of bifidobacteria in the infant intestine.

Bifidobacteria are gram-positive anaerobic bacteria that naturally colonize the human intestinal tract and are believed to be beneficial to human health (21, 30). Breastfeeding has been shown to be associated with an infant fecal microbiota dominated by bifidobacteria, whereas the fecal microbiota of infants who are consuming alternative diets has been described as being mixed and adult-like (12, 21). It has been suggested that the selective growth of bifidobacteria observed in breast-fed newborns is related to the oligosaccharides and other factors that are contained in human milk (human milk oligosaccharides [HMOs]) (3, 4, 10, 11, 16, 17, 34). Kitaoka et al. (15) have recently found that bifidobacteria possess a unique metabolic pathway that is specific for lacto-*N*-biose I (LNB; Gal β 1-3GlcNAc) and galacto-*N*-biose (GNB; Gal β 1-3GalNAc). LNB is a building block for the type 1 HMOs [such as lacto-*N*-tetraose (Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc), lacto-*N*-fucopentaose I (Fuc α 1-2Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc), and lacto-*N*-difucohexaose I (Fuc α 1-2Gal β 1-3[Fuc α 1-4]GlcNAc β 1-3Gal β 1-4Glc)], and GNB is a core structure of the mucin sugar that is present in the human intestine and milk (18, 27). The GNB/LNB pathway, as previously illustrated by Wada et al. (33), involves proteins/enzymes that are required for the uptake and degradation of disaccharides such as the GNB/LNB transporter (29, 32), galacto-*N*-biose/lacto-*N*-biose I phosphorylase (GLNBP; *lnpA*)

(15, 24) (renamed from lacto-*N*-biose phosphorylase after the finding of phosphorylases specific to GNB [23] and LNB [22]), *N*-acetylhexosamine 1-kinase (NahK) (25), UDP-glucose-hexose 1-phosphate uridylyltransferase (GalT), and UDP-galactose epimerase (GalE). Some bifidobacteria have been demonstrated to be enzymatically equipped to release LNB from HMOs that have a type 1 structure (lacto-*N* biosidase; *lnbB*) (33) or GNB from the core 1-type O-glycans in mucin glycoproteins (endo- α -*N*-acetylgalactosaminidase) (6, 13, 14). It has been suggested that the presence of the *lnbB* and GNB/LNB pathways in some bifidobacterial strains could provide a nutritional advantage for these organisms, thereby increasing their populations within the ecosystem of these breast-fed newborns (33).

The species that predominantly colonize the infant intestine are the bifidobacterial species *B. breve*, *B. longum* subsp. *infantis*, *B. longum* subsp. *longum*, and *B. bifidum* (21, 28). On the other hand, strains of *B. adolescentis*, *B. catenulatum*, *B. pseudocatenulatum*, and *B. longum* subsp. *longum* are frequently isolated from the adult intestine (19), and strains of *B. animalis* subsp. *animalis*, *B. animalis* subsp. *lactis*, *B. thermophilum* and *B. pseudolongum* have been shown to naturally colonize the guts of animals (1, 2, 7, 8). However, it is unclear whether there is a relationship between the differential colonization of the bifidobacterial species and the presence of the GNB/LNB pathway. In the present study, we investigated the ability of individual bifidobacterial strains in the *in vitro* fermentation of LNB and in addition, we also tried to determine whether or not the GLNBP gene (*lnpA*), which is a key enzyme of the GNB/LNB pathway, was present.

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MATERIALS AND METHODS

Microorganisms. Bifidobacterial strains were obtained from stock cultures maintained in the Morinaga Culture Collection (MCC; Morinaga Milk Industry Co., Ltd., Zama, Japan), the American Type Culture Collection (ATCC; Manassas, VA), Japan Collection of Microorganisms (JCM; Wako, Japan), and the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany). A total of 208 strains comprising 10 species and 4 subspecies originating from human, animals, or commercial products were studied (Table 1). The strains of the series IV (Table 1), which have also been deposited in other public collections, were originally a gift from Tomotari Mitsuoka (University of Tokyo, Tokyo, Japan). These microorganisms were cultured for 16 h at 37°C in ABCM broth (Eiken Chemical Co. Ltd., Tokyo, Japan). Microorganisms were collected by centrifugation, washed once with sterile saline, resuspended in a one-half volume of sterile saline, and used as seed cultures for fermentation studies.

Substrate fermentation. A medium previously developed for carbohydrate substrate fermentation studies (20) was used, with some modifications. The medium contained the following (per liter): Difco Bacto liver (Becton Dickinson [BD], Sparks, MD) extract, 250 ml; Difco proteose peptone no. 3 (BD), 10 g; BBL Trypticase peptone (BD), 5 g; Difco yeast extract (BD), 3 g; Tween 80, 1 g; L-cysteine · HCl · H₂O, 0.2 g; MgSO₄ · 7H₂O, 0.2 g; FeSO₄ · 7H₂O, 0.01 g; NaCl, 0.01 g; MnSO₄ · 4H₂O, 0.0068 g (with the pH adjusted to 7.2 and autoclaved for 20 min at 115°C). For the preparation of Bacto liver extract, 11 g powder was added to 500 ml distilled water and extracted at 50 to 60°C for 1 h with mixing. LNB, which was used as the sole carbohydrate source, was prepared as previously described (26). After being filter sterilized (0.22 µm), it was added to a sterile medium in order to attain a final concentration of 10 g liter⁻¹. The same concentrations (10 g liter⁻¹) of glucose, lactulose, and raffinose were used as the positive controls, while the medium without sugar supplementation was used as the negative control. The media were inoculated with 1.5% of the seed cultures and incubated under anaerobic conditions at 37°C for up to 144 h. Bacterial growth was measured for absorption at 660 nm using a spectrophotometer (Multiskan Spectrum; Thermo LabSystems, Vantaa, Finland) at 24 and 144 h. Growth on each substrate was evaluated on a scale from 0 to 5 based on values of the optical density at 660 nm (OD₆₆₀) measured at 24 h (OD_{24 h}) and 144 h (OD_{144 h}) after subtracting the values for the negative control, as follows: 0, no growth (OD_{24 h} and OD_{144 h}, <0.1); 1, slow and mild growth (OD_{24 h} < 0.1; OD_{144 h} = 0.1 to 0.5); 2, slow but sound growth (OD_{24 h} < 0.1; OD_{144 h} > 0.5); 3, intermediate and mild growth (OD_{24 h} = 0.1 to 0.5; OD_{144 h} = 0.1 to 0.5); 4, intermediate and sound growth (OD_{24 h} = 0.1 to 0.5; OD_{144 h} > 0.5); and 5, fast and sound growth (OD_{24 h} and OD_{144 h} > 0.5). A growth scale of >1 was considered to be positive for utilizing each substrate.

PCR detection of the GLNBP gene (*lnpA*). The presence of *lnpA* was detected by PCR analysis that used the primer pair DPVE-f (5'-GACCGTCGAGATG TACAA-3') and KFPK-r (5'-TCCACGAACCTCGGGAACCTT-3'). The primer, which was designed from sequences previously published or from deduced GLNBP genes of bifidobacteria (15, 24; http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) and from three unpublished bifidobacterial genomic sequences of *B. longum* subsp. *longum*, *B. breve*, and *B. longum* subsp. *infantis* existing in the Food Science and Technology Institute, Morinaga Milk Industry Co., Ltd., Kanagawa, Japan, was expected to result in a product of approximately 760 bp. The temperature program used was as follows: initial denaturation (94°, 5 min); 30 cycles of denaturation (94°C, 20 s), annealing (56°C, 30 s), and extension (72°C, 30 s); and final extension (72°C, 10 min). Electrophoresis on a 1% agarose gel stained with ethidium bromide was used for the visualization of the PCR products.

Detection of enzymatic activity of GLNBP. The enzymatic activity of GLNBP was examined for some strains that with or without GLNBP exhibited LNB utilization. After being cultured for 24 h on glucose or LNB, as described above, cells from 50 ml of each culture broth were collected by centrifugation (8,000 × g, 10 min) and then suspended in 1 ml MOPS (morpholinepropanesulfonic acid) buffer (20 mM, pH 7.5). The suspension was treated with ultrasonic oscillation (output control 4, constant) for 10 min in ice via the use of a sonifier (Branson Sonifier 250; Branson Ultrasonic Corporation, Danbury, CT). After the homogenate was centrifuged at 8,000 × g for 10 min, the supernatant was then collected as a cell extract.

To detect the phosphorolytic activity, 80 µl of a mixture containing 25 mM LNB and 25 mM phosphate in 100 mM MOPS buffer (pH 7.5) was mixed with 20 µl of the cell extract, and the reaction was allowed to proceed at 37°C for up to 18 h. Heat-treated cell extract (boiled for 10 min) served as the negative control. Subsequently, 1 µl of the reaction mixture was spotted on a silica gel thin-layer chromatography (TLC) plate, with the plate being developed using

75% acetonitrile. To visualize the carbohydrates on the plate, each plate was dipped in 5% sulfuric acid in methanol and then heated in a radiation oven.

To detect the synthetic activity of LNB, 80 µl of a mixture containing 25 mM GlcNAc and 25 mM GalIP in 100 mM MOPS buffer (pH 7.5) was mixed with 20 µl of the cell extract. After the reaction mixture was incubated at 37°C for up to 66 h, formation of LNB was detected by TLC, as described above. Some samples were applied to high-performance liquid chromatography with a Corona charged aerosol detector (ESA, Inc., Chelmsford, MA), a Shodex Asahipak NH2P-50 4E column (4.6 mm by 250 mm; Showa Denko K.K., Tokyo, Japan), and a mobile phase of acetonitrile-H₂O (75:25 dilution by volume) at a flow rate of 1 ml/min for the determination of LNB concentration.

RESULTS

Substrate fermentation. Table 1 summarizes the results of fermentation of LNB, lactulose, raffinose, and glucose by the bifidobacterial strains. While all strains of *B. longum* subsp. *longum*, *B. longum* subsp. *infantis*, *B. breve*, and *B. bifidum* were able to grow on LNB, no growth was seen for *B. adolescentis*, *B. catenulatum*, *B. dentium*, *B. angulatum*, *B. animalis* subsp. *lactis*, and *B. thermophilum*. Out of the 61 strains of *B. pseudocatenulatum*, there were 33 that were able to grow on LNB. There also was utilization of LNB by 3 out of the 4 strains of *B. animalis* subsp. *animalis* and by 5 out of the 6 strains of *B. pseudolongum*. Fermentation ability of each carbohydrate source by the 208 individual strains is shown in Table S1 in the supplemental material.

PCR detection of *lnpA*. Like the results for LNB growth, PCR product for *lnpA* was found for all strains of *B. longum* subsp. *longum*, *B. longum* subsp. *infantis*, *B. breve*, and *B. bifidum*, while no product was found for *B. adolescentis*, *B. catenulatum*, *B. dentium*, *B. angulatum*, *B. animalis* subsp. *lactis*, and *B. thermophilum* (Table 1; see also Table S1 in the supplemental material). PCR products were also detected for those strains of *B. animalis* subsp. *animalis* and *B. pseudolongum* that displayed growth on LNB (Table 1). However, no product was found for the strains of *B. pseudocatenulatum*, even though some of the strains had exhibited LNB growth (Table 1).

Detection of enzymatic activity for GLNBP. Strains with or without LNB utilization were examined for the enzymatic activity of GLNBP. Figure 1 shows TLC analysis of LNB-degrading activity for *B. animalis* subsp. *animalis* ATCC 27672 (*lnpA*⁺) and for *B. pseudocatenulatum* ATCC 27677 (lacking *lnpA*). GlcNAc was detected in both lanes, indicating that both strains were able to degrade LNB. GalIP, an indicator of LNB-phosphorolytic activity, was detected only with *B. animalis*. Table 2 summarized the results of GLNBP activity for bifidobacterial strains with or without LNB utilization/PCR detection of *lnpA*. LNB-phosphorolyzing activity of GLNBP, as shown by the detection of GalIP, was observed on extracts from cells cultivated on substrates of LNB for those strains harboring the *lnpA* gene. LNB-phosphorolyzing activity was also observed for strains *B. breve* ATCC 15700^T and *B. bifidum* ATCC 29521^T, with glucose as the carbohydrate substrate. Mild activity was observed for *B. bifidum* ATCC 29521^T, which displayed mild growth on LNB. No enzyme activity of GLNBP was found for those strains of *B. pseudocatenulatum*, regardless of their LNB fermentation ability.

For confirmation, these strains were examined for LNB-forming activity from GalIP and GlcNAc. LNB-forming activity was detected on extracts from those strains exhibiting LNB-phosphorolyzing activity (Table 2), which further supports the notion that *B. pseudocatenulatum* does not have any GLNBP activity.

TABLE 1. Strains of bifidobacteria, fermentation on LNB, and detection of the GLNBP gene *lbpA* by PCR

Species ^a	Strain(s)	Origin	PCR detection of <i>lbpA</i> ^b	Growth on LNB ^b	Growth pattern on ^c :			
					LNB	Lactulose	Raffinose	Glucose
<i>B. longum</i> subsp. <i>longum</i>	IV-51 (same as ATCC 15707 ^T)	Feces of adult	+		5	4	5	5
	ATCC 15708	Feces of infant	+		2	2	4	4
	ATCC BAA-999 (BB536)	Feces of infant	+		5	5	0	5
	MCC strains	Feces of adult or infant	3/3	3/3	2–5	5	4–5	5
<i>B. longum</i> subsp. <i>infantis</i>	IV-8 (same as ATCC 15697 ^T)	Feces of infant	+		5	5	5	5
	MCC strains	Feces of adult, infant, or from commercial products	13/13	13/13	2–5	1–5	0–5	2–5
<i>B. longum</i> subsp. <i>longum/infantis</i>	MCC strains	Feces of adult, infant, or from commercial products	7/7	7/7	2–5	5	5	2–5
<i>B. breve</i>	IV-14 (same as ATCC 15700 ^T)	Feces of infant	+		5	5	5	5
	MCC strains	Feces of infant or from yogurt	8/8	8/8	2–5	3–5	2–5	1–5
<i>B. bifidum</i>	IV-127 (same as ATCC 29521 ^T)	Feces of infant	+		1	3	0	3
	ATCC 15696	Intestine of infant	+		5	5	0	4
	MCC strains	Feces of infant	4/4	4/4	5	5	0	3–5
<i>B. adolescentis</i>	ATCC 15706	Feces of adult	–		0	1	2	2
	ATCC 15705	Feces of adult	–		0	2	0	2
	ATCC 15704	Feces of adult	–		0	1	0	1
	MCC strains	Feces of adult or infant	0/48	0/48	0	0–3	0–4	0–4
<i>B. catenulatum</i>	ATCC 27675	Feces of human	–		0	2	1	2
	JCM7130	Sewage	–		0	4	4	4
	MCC strains	Feces of adult	0/11	0/11	0	1–5	1–5	2–5
<i>B. pseudocatenulatum</i>	IV-152 (same as ATCC 27919 ^T)	Feces of infant	–		4	5	5	5
	ATCC27676	Sewage	–		4	5	5	5
	ATCC27677	Sewage	–		4	4	5	4
	IV-40 (same as JCM7040)	Feces of adult	–		5	5	5	5
	MCC strains	Feces of adult or infant	0/57	29/57	0–4	1–5	3–5	2–5
<i>B. dentium</i>	ATCC 15424	Pleural fluid from adult	–		0	2	2	2
	ATCC 27678	Feces of human	–		0	2	2	4
	ATCC 27679	Human vagina	–		0	1	2	2
	ATCC 27680	Human dental caries	–		0	4	4	5
	ATCC 15423	Lung abscess in adult	–		0	1	4	4
	JCM 7135	Human dental caries	–		0	4	4	4
	MCC strains	Feces of infant or adult	0/10	0/10	0	1–4	2–5	2–5
<i>B. angulatum</i>	ATCC 27535 ^T	Feces of human	–		0–1	4	4	2
	ATCC 27669	Sewage	–		0–1	1	2	1
	ATCC 27671	Sewage	–		0–1	2	4	2
<i>B. animalis</i> subsp. <i>animalis</i>	IV-58 (same as ATCC 25527 ^T)	Feces of rat	–		0	4	5	4
	IV-124 (same as ATCC 27672)	Feces of rat	+		5	5	5	4
	MCC strains	Feces of rat	2/2	2/2	4–5	3	4–5	3–4
<i>B. animalis</i> subsp. <i>lactis</i>	IV-113 (same as ATCC 27673)	Sewage	–		0	5	5	3
	IV-117 (same as ATCC 27674)	Feces of rabbit	–		0	5	5	5
	IV-129 (same as JCM 1253)	Feces of chicken	–		0	5	5	5
	DSM 10140	Yogurt	–		0	4	5	5
	MCC strains	Yogurt	0/3	0/3	0	4–5	4–5	5
<i>B. pseudolongum</i>	IV-70 (same as ATCC 25526 ^T)	Feces of swine	+		5	4	4	4
	MCC strains	Feces of animals	4/5	4/5	0–5	3–4	3–4	3–4
<i>B. thermophilum</i>	IV-29 (same as ATCC 25525 ^T)	Feces of swine	–		0	4	5	3
	MCC strains	Feces of animals	0/4	0/4	0	0–5	5	4–5

^a These microorganisms were identified by cellular morphology, patterns of sugar fermentation, DNA homology, and species- or subspecies-specific PCR.

^b Number of positive strains (growth pattern category ≥ 1)/number of total strains tested.

^c The growth pattern was categorized from 0 to 5 based on OD₆₆₀ measured at 24 h (OD_{24 h}) and 144 h (OD_{144 h}). 0, no growth (OD_{144 h} < 0.1); 1, slow and mild growth (OD_{24 h} < 0.1; OD_{144 h} = 0.1 to 0.5); 2, slow but strong growth (OD_{24 h} < 0.1; OD_{144 h} > 0.5); 3, intermediate and mild growth (OD_{24 h} = 0.1 to 0.5; OD_{144 h} = 0.1 to 0.5); 4, intermediate and strong growth (OD_{24 h} = 0.1 to 0.5; OD_{144 h} > 0.5); 5, quick and strong growth (OD_{24 h} > 0.5).

DISCUSSION

LNB is a building block of the type 1 HMOs that are known to be rich in human milk. In addition, it has also been suggested that the HMOs might be a factor for selective growth of

bifidobacteria (15). Interestingly, the present results indicated that all strains of the species that naturally colonize an infant's intestine, such as *B. longum* subsp. *longum*, *B. longum* subsp. *infantis*, *B. breve*, and *B. bifidum* (21, 28), were able to grow on

FIG. 1. TLC analysis of products from a LNB-phosphorylizing reaction by cell extracts of bifidobacteria in the presence of phosphate. Lane 1, *B. animalis* subsp. *animalis* ATCC 27672; lane 2, *B. pseudocatenulatum* ATCC 27677.

LNB and that they all possessed the GLNBP gene. In contrast, strains of species that are frequently found in the adult intestine or in human dental caries, such as *B. adolescentis*, *B. catenulatum*, and *B. dentium*, or strains of species that are found in the guts of domestic animals, such as *B. animalis* subsp. *lactis* and *B. thermophilum*, are not able to utilize LNB and appear to have no GLNBP gene. In support of these findings is a survey that examined 18 bifidobacterial strains in the Genomic BLAST (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi), which confirmed the presence of highly homologous sequences that have been previously described for GLNBP (15, 24) in the strains of *B. longum* subsp. *longum* (2 strains), *B. longum* subsp. *infantis* (3 strains), *B. bifidum* (1 strain), and *B. breve* (1 strain) but not in those of *B.*

adolescentis (2 strains), *B. angulatum* (1 strain), *B. animalis* subsp. *lactis* (4 strains), *B. catenulatum* (1 strain), *B. dentium* (1 strain), *B. gallicum* (1 strain), and *B. pseudocatenulatum* (1 strain).

Furthermore, among the strains to be tested, enzymatic activity of GLNBP, as indicated by LNB-degrading (release of Gal1P) and -forming activities, was recognized among strains of bifidobacterial species harboring the GLNBP genes when LNB was used as the substrate. Mild activity was observed for *B. bifidum* ATCC 29521^T that displayed mild growth on LNB. GLNBP activity was also observed for strains *B. breve* ATCC 15700^T and *B. bifidum* ATCC 29521^T, with glucose as the carbohydrate substrate. These results suggested that GLNBP is constitutive for the type strains of *B. breve* and *B. bifidum*. Kitaoka et al. previously reported that GLNBP activity was detected for *B. bifidum* JCM1254 but not for *B. longum* JCM1217 (which is ATCC 15707^T) when cultivated in medium containing glucose as the sole carbohydrate source (15). Two genes (*lnpA1* and *lnpA2*) existed in the genome of *B. bifidum* JCM1254, but only *lnpA2* was expressed constitutively (24). The *lnpA* gene of *B. longum* JCM1217 was found to be located at loci similar to those of the *lnpA1* gene of *B. bifidum* JCM1254, which was suggested to be LNB inducible (24).

Some strains of *B. animalis* subsp. *animalis* and *B. pseudolongum*, which are known to be able to colonize the guts of domestic animals, were also found to be able to utilize LNB. Both the *lnpA* gene and GLNBP activity were detected in some strains of *B. animalis* subsp. *animalis* and *B. pseudolongum* (Table 2). Since LNB is supposed to be a building unit specific for HMOs (31), the significance of the presence of the GLNBP gene in these strains is unclear at the current time. However, the GNB/LNB pathway is also related to the use of GNB,

TABLE 2. Detection of enzymatic activity of LNB phosphorylase in bifidobacterial strains

Species	Strain(s)	PCR detection of <i>lnpA</i> ^e	Growth on ^d :		Type of activity ^{c,e}			
			Glucose	LNB	LNB phosphorylizing ^a		LNB forming ^b	
					Glucose	LNB	Glucose	LNB
<i>B. pseudocatenulatum</i>	IV-152 (same as ATCC 27919 ^T)	—	5	5	—	—	—	—
	ATCC 27677	—	5	4	—	—	—	—
	ATCC 27676	—	4	4	—	—	—	—
	MCC 0310	—	5	0	—	ND	—	ND
	MCC 0337	—	5	0	—	ND	—	ND
	MCC 0376	—	5	0	—	ND	—	ND
<i>B. animalis</i> subsp. <i>animalis</i>	IV-58 (same as ATCC 25527 ^T)	—	4	0	—	ND	—	ND
	IV-124 (same as ATCC 27672)	+	4	5	—	+	—	+
<i>B. longum</i> subsp. <i>longum</i>	IV-51 (same as ATCC 15707 ^T)	+	5	5	—	+	—	+
<i>B. longum</i> subsp. <i>infantis</i>	IV-8 (same as ATCC 15697 ^T)	+	5	5	—	+	—	+
<i>B. breve</i>	IV-14 (same as ATCC 15700 ^T)	+	5	5	+	+	+	+
<i>B. bifidum</i>	IV-127 (same as ATCC 29521 ^T)	+	3	1	+	±	+	±
<i>B. pseudolongum</i>	IV-70 (same as ATCC 25526 ^T)	+	4	5	—	+	—	+

^a LNB-phosphorylizing activity was indicated by the detection of Gal1P degraded from LNB in the presence of phosphate by TLC after incubation for 5 to 18 h.

^b LNB-forming activity was indicated by the detection of LNB formation from Gal1P and GlcNAc by TLC after incubation for 24 to 66 h.

^c LNB and glucose were the carbohydrate sources used in cultivating each strain for the preparation of crude cell extract.

^d Growth patterns as described in Table 1.

^e +, presence of *lnpA* or enzymatic activity; —, absence of *lnpA* or enzymatic activity; ±, weak activity; ND, not examined. Crude cell extract boiled for 10 min showed no activity (data not shown).

which is the core structure of the sugar of mucin. In addition, it is also been proposed to be associated with intestinal colonization (5). Besides, different substrate affinity has been described for GLNBP in different microorganisms; the enzyme from *Clostridium perfringens* ATCC 13124 showed a strong preference for GNB over LNB, while LnpA from *B. longum* subsp. *longum* JCM1217 showed identical affinity for the two disaccharides (23). Further studies of the structures and substrate affinity of these enzymes may show some insight into the significance of these microorganisms. The *lnpA* gene and GLNBP activity were not found for *B. pseudocatenulatum*, which was the only species that was able to grow on LNB while being negative for GLNBP. These particular strains seem to have the ability to utilize LNB via the use of a hydrolytic enzyme. LNB-hydrolyzing activity is interesting because all four of the β -galactosidases of *B. bifidum* have been reported to be unable to hydrolyze LNB to any degree (9). In order to clarify and completely understand the LNB utilization mechanism for these *B. pseudocatenulatum* strains, further studies will need to be undertaken.

Recently, Wada et al. cloned LnbB, which is a critical enzyme for the degradation of LNB from HMOs with a type 1 structure (33). Although the number of strains tested was limited, LnbB activity was found only in the strains of *B. longum* and *B. bifidum* and not in any of the other bifidobacteria, including *B. breve*, or in any of the strains belonging to other genera, such as *Bacteroides*, *Clostridium*, and *Lactobacillus* (33). Considering the fact that *B. breve* is generally the dominant species found in an infant's microbiota and that it has been determined to be capable of potentially utilizing LNB, but LNB does not exist in free style in the milk, the possibility exists that some of the LnbB-negative candidates might be able to utilize LNB released by the positive candidates in the intestine of humans. Another possibility is that bifidobacterium factors other than LNB in breast milk are involved in the growth of *B. breve* and other bifidobacteria.

Breastfeeding has long been associated with a microbiota in which bifidobacteria are the predominant bacteria. The current results demonstrate that the bifidobacterial species that are the predominant species in infant intestines can potentially utilize LNB. These findings support the hypothesis that by utilizing LNB released from HMOs, GLNBP may play a key role in the rapid and selective colonization of individual bifidobacterial species that is seen in the intestines of breast-fed infants.

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