SUPPLEMENT
The Glycobiology of Human Milk Oligosaccharides

Bifidobacterial Enzymes Involved in the Metabolism of Human Milk Oligosaccharides[1–3]

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ABSTRACT

Intestinal colonization of bifidobacteria is important for the health of infants. Human milk oligosaccharides (HMO) have been identified as growth factors for bifidobacteria. Recently, a bifidobacterial enzymatic system to metabolize HMO was identified. 1,3-β-Galactosyl-*N*-acetylhexosamine phosphorylase (GLNBP, EC 2.4.1.211), which catalyzes the reversible phosphorolysis of galacto-*N*-biose (GNB) (Galβ1→3GalNAc)] and lacto-*N*-biose I (LNB) (Galβ1→3GlcNAc), is a key enzyme to explain the metabolism of HMO. Infant-type bifidobacteria possess the intracellular pathway to specifically metabolize GNB and LNB (GNB/LNB pathway). *Bifidobacterium bifidum* possesses extracellular enzymes to liberate LNB from HMO. However, *Bifidobacterium longum* subsp. *infantis* imports intact HMO to be hydrolyzed by intracellular enzymes. Bifidobacterial enzymes related to the metabolism of HMO are useful tools for preparing compounds related to HMO. For instance, LNB and GNB were produced from sucrose and GlcNAc/GalNAc in 1 pot using 4 bifidobacterial enzymes, including GLNBP. LNB is expected to be a selective bifidus factor for infant-type strains. *Adv. Nutr. 3: 422S–429S, 2012.*

Introduction

Bifidobacterium is the genus of gram-positive anaerobic bacteria. Some of its species are typical inhabitants in the human gut. Their intestinal growth is considered to be beneficial for health, and they are often used as probiotics.

Until the early 20th century, formula-fed infants often had pathogenic bacterial infections that caused diarrhea and other infectious diseases (1). Bifidobacteria were first identified in 1899 by Tissier (2) from the feces of a breast-fed infant. Shortly after this discovery, it was found that bifidobacteria were rarely isolated from feces of formula-fed infants; however, they were the predominant intestinal bacteria in breast-fed infants. Intestinal colonization of bifidobacteria is considered to prevent the intestinal growth of pathogenic bacteria and maintain health in breast-fed infants (1).

Investigations have been conducted since then to identify growth-stimulating factors for bifidobacteria (bifidus factor) present in human milk. In 1950s, a GlcNAc-containing sugar was assumed to be the bifidus factor, but subsequent studies revealed that the originators of this idea were misled because of the use of a particular GlcNAc-auxotrophic strain of *Bifidobacterium bifidum* var. *pennsylvanicus* (3,4). Human milk oligosaccharides (HMO)[4] have been considered as the most promising candidates for the real bifidus factor in human milk since then (1).

The predominant carbohydrate in human milk is lactose, which is found at a concentration of 60–70 g/L. Human milk also contains HMO possessing degrees of polymerization

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[4] Abbreviations used: Gal1*P*, α-galactose 1-phosphate; GalE, UDP-glucose 4-epimerase; GalNAc1*P*, α-GalNAc 1-phosphate; GalT, UDP-glucose-hexose 1-phosphate uridylyl transferase; GH, glycoside hydrolase family; GL-BP, galacto-*N*-biose/lacto-*N*-biose binding protein; GlcNAc1*P*, α-GlcNAc 1-phosphate; GLNBP, galacto-*N*-biose/lacto-*N*-biose I phosphorylase; GNB, galacto-*N*-biose; HMO, human milk oligosaccharides; LNB, lacto-*N*-biose I; LNT, lacto-*N*-tetraose.

that are ≥3 at a concentration of 22–24 g/L in colostrum and 12–13 g/L in mature milk (5,6). Lactose is digested by lactase in the small intestine and serves as a nutrient for infants, but HMO are not digestible by intestinal enzymes and reach the large intestine intact, where they are potentially used by bifidobacteria.

Later research revealed that various nondigestible oligosaccharides acted as bifidus factors, and in the early 1980s, prebiotic oligosaccharides were developed in Japan (7). These oligosaccharides reach the large intestine intact and are used there by bifidobacteria to enhance their intestinal growth. Modern milk formulas are supplemented with prebiotic oligosaccharides such as fructooligosaccharides, galactooligosaccharides, and lactulose to allow the growth of intestinal bifidobacteria, and infant health with regard to infectious diseases improves. However, the intestinal microbiota of bottle-fed infants is different from that of breast-fed infants, with a larger population of *Enterobacteriaceae* (8). Thus, an understanding of the bifidus factor in human milk is still necessary.

The complex composition of HMO, which consists of >130 different oligosaccharides (9,10), has made it difficult to understand how HMO acts as a bifidus factor (9,10). Each component of HMO can be assigned 1 of 12 core structures with or without the modifications of fucosylation and/or sialylation (9,10). All the core structures possess a lactose unit at the reducing end. Core structures are classified into types I and II based on the disaccharide unit at their nonreducing ends, i.e., containing lacto-*N*-biose I (LNB) (Galβ1→3GlcNAc) and *N*-acetyllactosamine (Galβ1→4GlcNAc) structures, respectively. Type I oligosaccharides are predominant in HMO, and type II oligosaccharides are minor components. 2′-Fucosyllactose (Fucα1→2Galβ1→4Glc), lacto-*N*-tetraose (LNT) (Galβ1→3GlcNAcβ1→3Galβ1→4Glc), and lacto-*N*-fucopentaose I (Fucα1→2Galβ1→3GlcNAcβ1→3Galβ1→4Glc) are the most abundant components in HMO (11), and the latter 2 are type I oligosaccharides. Such type I predominance in milk oligosaccharides is a specific feature of milk in humans and not in other mammals including anthropoids (12). The Galβ1→3GlcNAc bond in LNB is resistant to most β-galactosidases (13–15). Thus, it is important to discover how bifidobacteria digest this bond to adequately understand the prebiotic effect of HMO.

Two species, *B. bifidum* and *Bifidobacterium longum* subsp. *infantis* can grow in a medium with HMO as the sole carbon source (16–18). In this review, bifidobacterial enzymes related to the cleavage of the core structures of HMO as well as the release of fucosyl and sialyl residues are discussed, especially of the 2 species.

Current status of knowledge

The intracellular galacto-*N*-biose/LNB pathway

Since the discovery of 1,3-β-galactosyl-*N*-acetylhexosamine phosphorylase (19) in the cell-free extract of *B. bifidum*, the intracellular sugar-metabolic pathway specific to galacto-*N*-biose (GNB) (Galβ1→3GalNAc) and LNB, the GNB/LNB pathway (20,21), has been identified from several species of bifidobacteria. The genes encoding the components of the pathway are clustered as shown in **Figure 1**. As explained in the following, the pathway fully describes the metabolism of GNB and LNB.

GNB/LNB-specific transporter. The upstream genes *gltA–C* encode components of an ATP-binding cassette sugar transporter. A prediction was made that *gltA* would encode a solute-binding protein that determines the specificity of the transporter. Research showed that the GltA protein [named GNB/LNB binding protein (GL-BP)] of *B. longum* subsp. *longum* JCM1217 strongly bound GNB (K_d = 10 nM) and LNB (K_d = 87 nM), but did not bind with LacNAc, indicating that *gltA–C* encodes the specific GNB/LNB transporter that allows intake of GNB and LNB generated outside the cell (22,23). GL-BP also showed the capacity to bind to LNT with a higher K_d value (11 μM), suggesting that LNT may also be transported in the absence of GNB and LNB. The residues involved in binding GNB and LNB were identified through a structural analysis of GL-BP (22). Genes encoding for proteins sharing >30% identity with GL-BP have not been found in genomic sequences of intestinal bacteria other than bifidobacteria.

GNB/LNB phosphorylase. 1,3-β-Galactosyl-*N*-acetylhexosamine phosphorylase phosphorolyzes GNB and LNB into α-galactose 1-phosphate (Gal1*P*) and the corresponding HexNAc (GalNAc and GlcNAc, respectively). It is categorized into 3 groups based on the activity on GNB and LNB: GNB phosphorylase (specific to GNB) (24–26), LNB phosphorylase (LNBP, specific to LNB) (27), and GNB/LNB phosphorylase (GLNBP, acting on both GNB and LNB equally) (19,26,28–31). The bifidobacterial enzymes are members of GLNBP, and *lnpA* (Fig. 1) encodes GLNBP.

In the CAZy database (32), GLNBP is classified into the glycoside hydrolase family (GH) 112 (33) as well as GNB phosphorylase, LNBP, and 1,4-β-D-galactosyl-L-rhamnose phosphorylase (29,34), based on structural similarity with β-galactosidase GH42 (35). Mutational analysis of the GLNBP of *B. longum* subsp. *longum* revealed the residues that determined the substrate specificities of GH112 enzymes (36).

The presence of a phosphorylase often suggests that the substrate sugar plays an important role for the energy source especially for anaerobic bacteria (37). Because the phosphorolytic reaction by phosphorylase directly produces a phosphorylated sugar without consuming ATP, the energy obtained from the substrate sugar is considerably higher than that from other sugars, especially under anaerobic conditions in which only 3 molecules of ATP are available via the glycolytic pathway from glucose 6-phosphate.

N-Acetylhexosamine 1-kinase. The LnpB protein was shown to produce α-GlcNAc 1-phosphate (GlcNAc1*P*) from GlcNAc and ATP, indicating that LnpB is a novel anomeric kinase (20). This protein had similar activity on GalNAc,

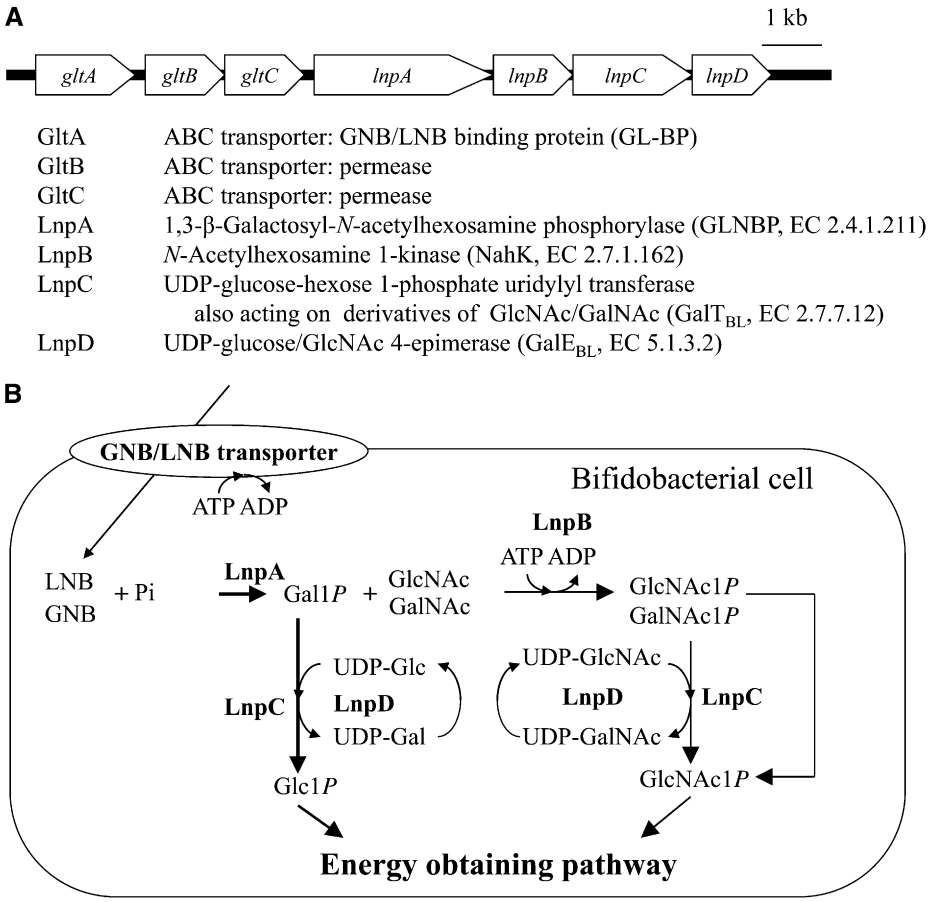

Figure 1 The galacto-*N*-biose (GNB)/lacto-*N*-biose I (LNB) pathway found in bifidobacteria. *A*, The gene cluster encoding the pathway. *B*, Schematic representation of the pathway. GalNAc1*P*, α-GalNAc 1-phosphate; Gal1*P*, α-galactose 1-phosphate; GlcNAc1*P*, α-GlcNAc 1-phosphate; Glc1*P*, α-glucose 1-phosphate.

yielding α-GalNAc 1-phosphate (GalNAc1*P*), and weak activities on several monosaccharides. The enzyme was named *N*-acetylhexosamine 1-kinase, with a new EC number, EC 2.7.1.162. LnpB does not have a signal peptide sequence and is considered to be an intracellular enzyme.

Most kinases acting on hexoses phosphorylate their substrates at the sixth position. Three enzymes—galactokinase (EC 2.7.1.6), *N*-acetylgalactosamine kinase (EC 2.7.1.157), and fucokinase (EC 2.7.1.52)—are known to phosphorylate the anomeric hydroxyl group. An anomeric kinase on a gluco-type sugar had not been reported before *N*-acetylhexosamine 1-kinase, which has been used for preparing various analogs of GlcNAc1*P* and GalNAc1*P* (38,39).

UDP-glucose-hexose 1-phosphate uridylyl transferase (GalT) and UDP-glucose 4-epimerase (GalE). LnpC and LnpD were predicted to be a GalT (EC 2.7.7.12) and GalE (EC 5.1.3.2), respectively, based on their amino acid sequences. GalT and GalE are the enzymes involved in the Leloir pathway for galactose metabolism (40). They act in concert to transfer Gal1*P* to α-glucose 1-phosphate (Glc1*P*), enabling them to enter the glycolytic pathway. Because Gal1*P* is generated from GNB and LNB through phosphorolysis by GLNBP, the presence of GalT and GalE suggested that the galactose part of GNB and LNB is transformed into Glc1*P*, which is then sent to the energy-obtaining pathway within the enzymes encoded in the gene cluster.

GalT and GalE activities were proven experimentally with LnpC and LnpD, and both proteins possessed additional activities (20). LnpC transfers the UMP unit of UDP-Glc to both GlcNAc1*P* and GalNAc1*P*. LnpC is a novel type of GalT, having wide substrate specificity, as no other GalT has been found to recognize GlcNAc1*P* or GalNAc1*P* (20). LnpC was named GalT$_{BL}$. LnpD demonstrated UDP-GlcNAc 4-epimerase activity as well as UDP-glucose 4-epimerase and was named GalE$_{BL}$ (20). These activities explain the transformation of GalNAc1*P* into GlcNAc1*P* by way of the Leloir pathway. Because GalNAc must be transformed to GlcNAc before entering an energy-obtaining pathway, the enzymes encoded by *lnpB-D* explain the metabolism of the HexNAc part of GNB and LNB.

Distribution of the GNB/LNB pathway. The distribution of the GNB/LNB pathway was examined with various species of bifidobacteria by detecting the presence of GLNBP (41). All strains of *B. longum* subsp. *longum*, *B. longum* subsp. *infantis*, *Bifidobacterium. breve*, and *B. bifidum* possess GLNBP. It should be noted that the 4 species are often isolated from infant feces. However, GLNBP has not been found in any strains of *Bifidobacterium adolescentis* and *Bifidobacterium catenulatum*, the major bifidobacterial species in the adult intestine.

It is valuable to find the origins of GNB and LNB in the human intestine to understand the role of the GNB/LNB

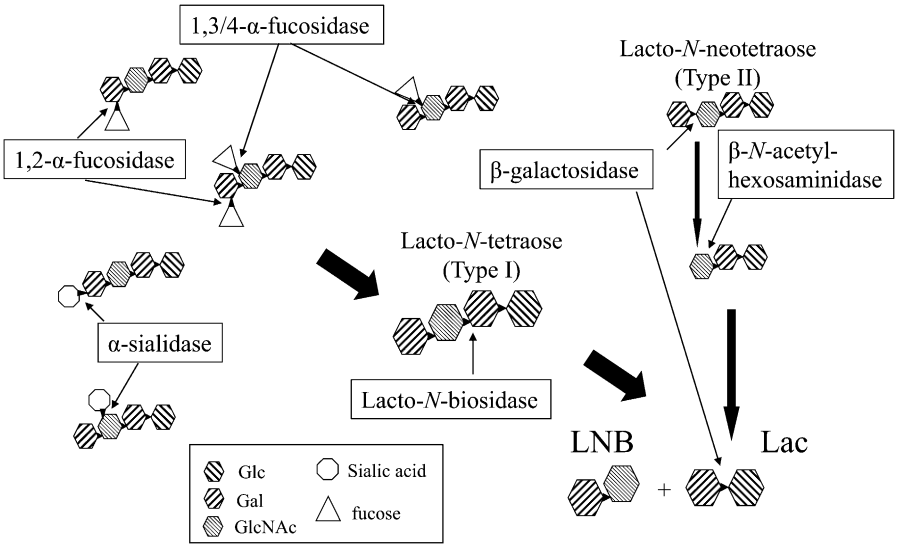

Figure 2 Pathway of the extracellular enzymatic hydrolysis of HMO by *Bifidobacterium bifidum*. Lac, lactose; LNB, lacto-*N*-biose I.

pathway in the intestinal growth of bifidobacteria. GNB exists as a structural component of *O*-linked glycoproteins in mucous membranes. An enzyme that liberates GNB (endo-α-*N*-acetylgalactosaminidase) was found in *B. longum* subsp. *longum* (42,43). In case of LNB, HMO are the serious candidate for the source of LNB because it would be in agreement with the fact that the infant type of bifidobacteria possess the GNB/LNB pathway. The presence of the LNB structure in HMO explains the predominant growth of bifidobacteria if they possess an extracellular enzymatic system to liberate LNB from HMO (LNB hypothesis) (28).

Extracellular enzymes hydrolyzing HMO isolated from *B. bifidum*

An extracellular enzymatic system to liberate LNB from HMO was elucidated from *B. bifidum* JCM1254. Seven enzymes are involved in the extracellular hydrolyses. The possible pathway is illustrated in **Figure 2**. Two α-fucosidases are involved in the cleavage of HMO. One is 1,2-α-fucosidase belonging to GH95 (AfcA) (44,45), which hydrolyzes the fucosyl unit bound to the second position of the galactosyl residue at the nonreducing end of HMO. The specificity was also confirmed with the selective syntheses of 1,2-α-fucoside catalyzed by a glycosynthase mutant of AfcA (46). The other is 1,3/4-α-fucosidase belonging to GH29 (AfcB) (47), which hydrolyzes the fucosyl unit neighboring the β-linked galactose. The enzyme hydrolyzes 1,4-α-fucoside in type I sugar and 1,3-α-fucoside in type II sugar. Two 2,3/6-α-sialidases belonging to GH33 (SiaBB1 and SiaBB2) (48,49) were also isolated. Because major modifications in the core structures of HMO are α-1,2/3/4-fucosylation and α-2,3/6-sialylation, the presence of these enzymes is beneficial for bifidobacteria to remove the branched sugar from the core structure of HMO.

Extracellular lacto-*N*-biosidase belonging to GH20 (LnbB) (50) was also isolated from *B. bifidum*. The LnbB exhibited 38% amino acid identity to the lacto-*N*-biosidase protein from *Streptomyces* sp. (51,52), which was the only lacto-*N*-biosidase isolated before LnbB. The presence of LnbB indicates that *B. bifidum* produces LNB from HMO by the extracellular enzymes. Furthermore, a β-galactosidase belonging to GH2 (Bbg3) and a β-*N*-acetylglucosaminidase (Bbh1) belonging to GH20 were shown to digest type II sugars (53). It should be noted that Bbg3 hydrolyzes neither LNB nor LNT (53).

All the enzymes listed possess a membrane-anchoring motif at the C-terminal, suggesting that they are cell-bound enzymes. Genes encoding these enzymes are also found in the genomic sequences of 2 *B. bifidum* strains (54,55). They may be advantageous for the use of HMO by the *B. bifidum* that hydrolyzes HMO on the surface of the cells.

Metabolism of HMO in *B. longum* subsp. *infantis*

The genomic sequence of *B. longum* subsp. *infantis*, a consumer of HMO, revealed that the strain possesses a 43-kb gene cluster specific for HMO degradation that is not found in other bifidobacterial species (56,57). In the cluster, homologous genes of 1,2-α-fucosidase, 1,3/4-α-fucosidase, and 2,3/6-α-sialidase are present in addition to the genes for β-galactosidase and β-*N*-acetylhexosaminidase, but they are considered to be intracellular proteins because of the lack of signal peptides. The cluster includes genes encoding several sugar transporters that are considered to import HMO intact (58). Although the strain also possesses the GNB/LNB pathway, neither a gene predicted to encode lacto-*N*-biosidase (56) nor lacto-*N*-biosidase activity (50) has been identified. The β-galactosidase in the gene cluster (Bga2A) hydrolyzed type II sugars, but did not hydrolyze type I sugars. Yoshida et al. (59) and Garrido et al. (60) independently identified an intracellular β-galactosidase belonging to GH42 (Bga42A) that hydrolyzed type I sugars. Bga42A hydrolyzes LNT 40 times more effectively than LNB (59). It is concluded that *B. longum* subsp. *infantis* incorporates HMO intact and then degrades them exowisely from the nonreducing ends without using the GNB/LNB pathway.

Practical preparation of LNB using GLNBP

The presence of the GNB/LNB pathway in infant-type bifidobacteria suggests that LNB acts as a promoting factor for the growth of bifidobacteria. A practical preparation method was reported (61) that uses a 1-pot enzymatic reaction to produce LNB and is summarized in **Figure 3**. In this method, sucrose and GlcNAc are converted into LNB and fructose by the concerted reactions of 4 enzymes—sucrose phosphorylase (EC 2.4.1.7), GalT, GalE, and GLNBP—in the presence of catalytic amounts of UDP-glucose and inorganic phosphate. Sucrose is phosphorolyzed to Glc1*P* and fructose by sucrose phosphorylase, and then Glc1*P* is converted to UDP-Glc concomitantly with the conversion of UDP-Gal into Gal1*P* by GalT. Then UDP-Glc is converted to UDP-Gal by GalE, and the resulting UDP-Gal is consumed in the reaction of GalT. Finally, LNB is synthesized from Gal1*P* and GlcNAc by GalHexNAcP.

Starting from a 10-L reaction mixture consisting of 660 mmol/Lsucrose and 600 mmol/L GlcNAc, the concentration of LNB produced reached 500 mmol/L (190 g/L). The yield was 83% based on the amount of GlcNAc used. LNB was isolated by crystallization after fermentation of the reaction mixture by yeast to remove the resultant fructose and unreacted sucrose to obtain 1.8 kg of crystalline LNB (95% purity). Recrystallization of LNB yielded 1.4 kg of crystalline LNB (99.6% purity). This method was carried out with unit processes that were ready for scaling up. The process for the isolation of LNB does not require any chromatography step, which is often difficult to scale up. This method can be easily used in the production of GNB by substituting GlcNAc for GalNAc (62,63).

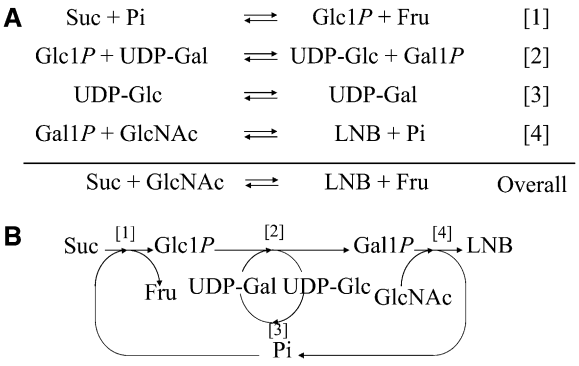

Figure 3 One-pot enzymatic production of lacto-*N*-biose I (LNB). *A*, Principle of the reaction. Four enzymatic reactions are carried out in 1 pot, and the compounds appearing on both sides are recycled in the overall reaction. Thus, the overall reaction is to produce LNB and fructose from sucrose and GlcNAc in the presence of 4 enzymes and catalytic amounts of UDP-Glc and Pi. *B*, Schematic description of the reaction. Catalyzed by sucrose phosphorylase [1], DP-glucose-hexose 1-phosphate uridylyl transferase (GalT) [2], UDP-glucose 4-epimerase (GalE) [3], and Galacto-*N*-biose/LNB phosphorylase (GLNBP) [4]. Gal1*P*, α-galactose 1-phosphate; Glc1*P*, α-glucose 1-phosphate.

LNB as a growth-promoting factor

Because large-scale production of LNB is feasible, experiments that require large amounts of LNB can easily be conducted. Kiyohara et al. (64) examined the in vitro growth-promoting activity of LNB in various intestinal bacteria. LNB showed such activity in several bifidobacteria, including *B. bifidum*, *B. breve*, *B. longum* subsp. *longum*, *B. longum* subsp. *infantis*, and *Bifidobacterium scardovii*, but not in other strains of bifidobacteria or in other intestinal bacteria. Xiao et al. (41) examined the effect of LNB on 203 strains of bifidobacteria and found that LNB promoted the growth of bifidobacterial strains possessing the GNB/LNB pathway except for strains of *Bifidobacterium pseudocatenulatum*. All the infant-type strains of bifidobacteria showed growths on LNB, suggesting that LNB is a possible candidate to be used as a prebiotic for certain strains of bifidobacteria.

Conclusions

Discovery of the GNB/LNB pathway opens up the possibility to systematically investigate HMO metabolism in bifidobacteria. The GNB/LNB pathway was found to be distributed in infant-type bifidobacterial species such as *B. longum* subsp. *longum* (65–68), *B. longum* subsp. *infantis* (56,69), *B. bifidum* (54,55), and *B. breve* (70).

TABLE 1. Location of the enzymes related to the metabolism in HMO by *Bifidobacterium bifidum* JCM1254 and *Bifidobacterium longum* subsp. *infantis* ATCC15697[1]

Enzyme	Family	*B. bifidum*	*B. longum* subsp. *infantis*
1,2-α-Fucosidase	GH95	Cell bound	Cytosol
1,3/4-α-Fucosidase	GH29	Cell bound	Cytosol
Sialidase	GH33	Cell bound	Cytosol
Lacto-*N*-biosidase	GH20	Cell bound	Does not exist
β-Galactosidase	GH2	Cell bound	Cytosol
β-*N*-acetylhexosaminidase	GH20	Cell bound	Cytosol
GNB/LNB transporter		Transmembrane	Transmembrane
GLNBP	GH112	Cytosol	Cytosol
GNB/LNB pathway		Cytosol	Cytosol
Transporters in HMO cluster		Does not exist	Transmembrane
LNT β-galactosidase	GH42	Cytosol	Cytosol

[1] GLNBP, galacto-*N*-biose/lacto-*N*-biose I phosphorylase; GNB/LNB, galacto-*N*-biose/lacto-*N*-biose I; HMO, human milk oligosaccharides; LNT, lacto-*N*-tetraose.

The extracellular enzymatic system of *B. bifidum* that digests HMO to generate LNB has been fully identified. However, the enzymatic system of *B. longum* subsp. *infantis* suggests that the strain uptake of intact HMO followed by intracellular exowise hydrolyses does not use the GNB/LNB pathway in the metabolism of HMO. All the bifidobacterial enzymes related to the metabolism of HMO have finally been identified, as listed in **Table 1**.

It had often been reported that *B. longum* subsp. *infantis* ferments HMO in vitro but *B. bifidum* does not (17,18), even though the species possesses the enzymes to use HMO. A recent study has revealed that the result is probably because of a deficiency of the GNB/LNB transporter in the type strain of *B. bifidum*, and that other strains of *B. bifidum* were able to ferment HMO (16). The consumption pattern of HMO by *B. longum* subsp. *infantis* and *B. bifidum* reflect their pathways. *B. longum* subsp. *infantis* consumed HMO without the accumulation of any oligosaccharides as the intermediate (16). *B. bifidum* temporarily accumulates disaccharides such as lactose and LNB in the medium during the fermentation (16).

It should be noted that *B. breve* is the species most abundantly isolated from infant feces (8,71). However, it has been reported that *B. breve* is incapable of fermenting HMO in vitro, and fucosidase, sialidase, and lacto-*N*-biosidase have not been isolated from *B. breve*. Some mutualism should be assumed to understand the growth of *B. breve* in the gut of breast-fed infants (72). *B. bifidum* may be a candidate for mutualism because it accumulates LNB to be consumed by other bacteria (16).

LNB is expected to function as a specific prebiotic for infant-type bifidobacteria that possess the GNB/LNB pathway. The production of LNB at an industrial scale may allow its use as a functional food ingredient and, perhaps, as a supplement for infant formula milk.

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