Identification of *N*-Acetylhexosamine 1-Kinase in the Complete Lacto-*N*-Biose I/Galacto-*N*-Biose Metabolic Pathway in *Bifidobacterium longum*[▽]

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We have determined the functions of the enzymes encoded by the *lnpB*, *lnpC*, and *lnpD* genes, located downstream of the lacto-*N*-biose phosphorylase gene (*lnpA*), in *Bifidobacterium longum* JCM1217. The *lnpB* gene encodes a novel kinase, *N*-acetylhexosamine 1-kinase, which produces *N*-acetylhexosamine 1-phosphate; the *lnpC* gene encodes UDP-glucose hexose 1-phosphate uridylyltransferase, which is also active on *N*-acetylhexosamine 1-phosphate; and the *lnpD* gene encodes a UDP-glucose 4-epimerase, which is active on both UDP-galactose and UDP-*N*-acetylgalactosamine. These results suggest that the gene operon *lnpABCD* encodes a previously undescribed lacto-*N*-biose I/galacto-*N*-biose metabolic pathway that is involved in the intestinal colonization of bifidobacteria and that utilizes lacto-*N*-biose I from human milk oligosaccharides or galacto-*N*-biose from mucin sugars.

Intestinal colonization by bifidobacteria is considered to be beneficial to human health. Bifidobacteria secrete a variety of hydrolytic enzymes that act on oligosaccharides, enabling them to grow on various prebiotic oligosaccharides that function to increase probiotic bacteria in vivo (6, 7, 22). Lacto-*N*-biose phosphorylase (LNBP) (EC 2.4.1.211) has been found in cell extracts of *Bifidobacterium bifidum* (8). The enzyme specifically phosphorolyzes galacto-*N*-biose (Galβ1→3GalNAc) (GNB) and lacto-*N*-biose I (Galβ1→3GlcNAc) (LNB) to yield α-D-galactose 1-phosphate (Gal1*P*) and the corresponding *N*-acetylhexosamine, suggesting that this enzyme might play a key role in metabolizing mucin sugars (8). After purifying LNBP from *B. bifidum*, we found that its partial amino acid sequences (13) were highly homologous with that of the BL1641 protein of *Bifidobacterium longum* NCC2705, whose entire genomic sequence was available (21). Then, we cloned the LNBP gene, *lnpA*, from the type strain *B. longum* JCM1217, whose sequence was almost identical to that of the BL1641 gene (13). After subsequently cloning and sequencing the LNBP gene from *B. bifidum* (19), we concluded that the deduced amino acid sequences of these LNBPs shared no significant identity with those of any other proteins with known functions.

The gene cluster of *B. longum* NCC2705 consists of the open reading frames BL1638 to BL1644 (Fig. 1). The BL1638 to BL1640 genes were annotated to encode component proteins of an ATP-binding-cassette-type sugar transporter. The BL1641 protein was determined to be LNBP (13), whereas the BL1642, BL1643, and BL1644 proteins were annotated to be mucin desulfatase, galactose-1-phosphate uridylyltransferase (GalT2) (EC 2.7.7.10), and UDP-glucose 4-epimerase (GalE) (EC 5.1.3.2), respectively. The BL1643 and BL1644 proteins

are likely related to the Leloir pathway, which sends galactose to glycolysis (Fig. 2). Promoter-like sequences were observed upstream of the BL1638 gene and between the BL1640 and BL1641 genes, and a palindromic sequence was detected downstream of the BL1644 gene. Since the full set of enzymes involved in the Leloir pathway was not found as a gene cluster in *B. longum* NCC2705, the cluster containing the LNBP gene (*lnpA*) was presumed to code for the major galactose metabolic pathway in this strain (13).

To understand the role of LNBP in the growth of bifidobacteria in the human intestine, it is important to find the possible origins of GNB and LNB in humans. We think that GNB and LNB are abundantly available in mucin and human milk, respectively, as described below.

Fujita et al. found the extracellular endo-α-*N*-acetylgalactosaminidase from *B. longum* that specifically hydrolyzed α-galacto-*N*-bioside, which exists abundantly in mucin as core I (10). The presence of both endo-α-*N*-acetylgalactosaminidase and LNBP in the same organism suggests that the metabolism of GNB by this system is important for the intestinal colonization of bifidobacteria.

The intestinal growth of bifidobacteria is especially important for the health of newborn infants (2). Colonization by bifidobacteria of the intestines of breast-fed infants increases rapidly within 1 week after birth, preventing infection by pathogenic bacteria. This type of rapid growth does not occur in the intestines of bottle-fed infants (3), even when formula is supplemented with prebiotic oligosaccharides, such as lactulose (20). The difference is due to the presence in human milk of oligosaccharides other than lactose (human milk oligosaccharides [HMO]), whereas lactose is the sole saccharide in cow's milk (18). The component of HMO responsible for the growth of bifidobacteria, however, has not yet been identified, since HMO include more than 100 kinds of molecules (14, 18). Lacto-*N*-tetraose (Galβ1→3GlcNAcβ1→3Galβ1→4Glc) and lacto-*N*-fucopentaose I (Fucα1→2Galβ1→3GlcNAcβ1→3Galβ1→4Glc), which contain LNB structure, are predomi-

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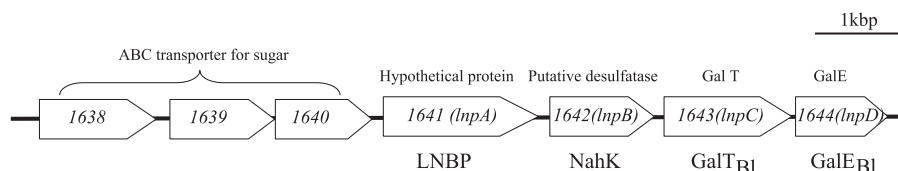
FIG. 1. Schematic structure of the gene cluster between the BL1638 and BL1644 genes.

nant components in HMO, as well as 2′-fucosyllactose (14, 23). Though the presence of lacto-*N*-biosidase has not been proved, α-fucosidase has already been found in *B. bifidum* (12). We therefore suggested that LNB structures present in such oligosaccharides act as the bifidus factor, which enhances the growth of bifidobacteria as a carbon source (13).

In this paper, the enzymes encoded by the BL1642- to BL1644-like genes of *B. longum* JCM1217, the type strain, were overexpressed in *Escherichia coli* and characterized, as was the operon including these genes.

MATERIALS AND METHODS

Materials. The vector pET30 was obtained from Novagen (Madison, WI). *B. longum* JCM1217 was obtained from the Japan Collection of Microorganisms, The Institute of Physical and Chemical Research (Wako, Japan). *E. coli* strains select96 (Promega, Madison, WI) and BL21(DE3) (Novagen) were used as hosts for cloning and expression.

Molecular cloning of the *lnpB*, *lnpC*, and *lnpD* genes. Genomic DNA from *B. longum* JCM1217 was prepared using InstaGene (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. PCR primers were designed from the genomic sequence of *B. longum* NCC2705 (21) and the nucleotide sequence of *lnpA* from *B. longum* JCM1217 (Table 1). PCR was performed with these primers and KOD-plus polymerase (Toyobo, Osaka, Japan), using an amplification program consisting of 25 cycles of denaturation at 95°C for 30 s, annealing at 53°C for 15 s, and extension at 68°C for 2 min. The PCR products were cloned into an appropriate vector using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA), and the nucleotide sequences of the inserts were determined using a BigDye terminator v3.1 cycle-sequencing kit and a 310 Genetic analyzer (Applied Biosystems, Foster, CA). In constructing expression plasmids, PCR was performed using primers to which were added NdeI or XhoI restriction endonuclease sites for ligation to the expression vector (Table 1). After treatment with NdeI and XhoI, the amplified fragments were ligated into the pET30 vector at the NdeI and XhoI sites using a Ligation High DNA ligation kit (Toyobo). All plasmids were designed so that a His$_6$ tag sequence was added to the carboxyl terminus of the protein to facilitate purification. Plasmid DNA was used to transform *E. coli* BL21(DE3) after sequence confirmation.

Purification of recombinant enzymes. Each transformant was cultivated at 30°C with shaking in 100 ml LB medium containing 50 μg/ml kanamycin until the absorbance at 600 nm reached 0.5. Protein expression was induced by the addition of isopropyl-1-thio-β-D-galactoside at a final concentration of 0.5 mM, followed by incubation for an additional 20 h at 30°C with shaking. The cells were

harvested by centrifugation at 15,000 × *g* for 10 min, resuspended in 20 mM MOPS (morpholinepropanesulfonic acid) buffer (pH 7.5), and sonicated using a sonifier (Branson Ultrasonic Corporation, Danbury, CT). The cell debris was removed by centrifugation at 17,000 × *g* for 30 min, and each enzyme was purified on an Ni-nitrilotriacetic acid agarose gel (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The homogeneity of the purified enzymes was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein concentrations were determined using theoretical extinction coefficients calculated from the amino acid sequences of the proteins (11). For the proteins encoded by the *lnpB*, *lnpC*, and *lnpD* genes of *B. longum* JCM1217, these calculated extinction coefficients at 280 nm were 27,390, 77,810, and 48,820 M^{-1} cm^{-1}, respectively.

HPIC. The products of the enzymatic reactions were quantified by high-performance ion chromatography (HPIC) with a pulsed amperometric detector (DX500; Dionex Corporation, Sunnyvale, CA) using a Dionex CarboPac PA1 column (4 mm [inside diameter] by 250 mm) and a linear gradient of 0 to 1 M sodium acetate in 100 mM sodium hydroxide for 20 min at a flow rate of 1 ml/min.

Measurement of enzyme activity. All enzymatic reactions were measured at 30°C. The activity of *N*-acetylhexosamine 1-kinase (NahK) was determined by quantifying the GlcNAc1*P* produced in 30 min in a reaction mixture containing 0.1 M Tris buffer (pH 8.5), 1 mM GlcNAc, 1 mM ATP, 1 mM MgCl$_2$, and various concentrations of enzyme, and the reaction was stopped by boiling the mixture for 5 min. One unit of NahK activity was defined as the amount of enzyme that produced 1 μmol of GlcNAc1*P* per minute under these conditions.

UDP-glucose-hexose 1-phosphate uridylyltransferase (EC 2.7.7.12) (GalT) activity was determined by quantifying the Glc1*P* produced in 30 min in a reaction mixture containing 0.1 M MOPS buffer (pH 7.5), 0.5 mM UDP-Glc, 0.5 mM Gal1*P*, 1 mM MgCl$_2$, and various concentrations of enzyme; the reaction was stopped by boiling the mixture for 1 min. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of Glc1*P* per minute under these conditions.

GalE (EC 5.1.3.2) activity was measured in a reaction mixture containing 0.1 M MOPS buffer (pH 7.5), 1 mM UDP-Gal, and various concentrations of enzyme; the reaction was stopped by boiling the mixture for 1 min. Following the addition of 50 μl of 1 N H$_2$SO$_4$ to hydrolyze UDP sugars, 100 μl of the reaction mixture was boiled for 30 min, and the resultant glucose, the hydrolytic product of UDP-Glc, was quantified by the glucose oxidase method using a glucose CII test (Wako Pure Chemical Industries, Osaka, Japan). One unit of GalE was defined as the amount of enzyme that produced 1 μmol of UDP-Glc per minute under these conditions. When UDP-GalNAc was the substrate, the amount of UDP-GlcNAc produced was quantified by HPIC.

Characterization of NahK. The stability of NahK was measured by determining residual activity after incubation with 1 μM enzyme at various temperatures or in 10 mM of citrate (pH 2.2, 2.7, 3.2, 3.7, and 4.2), acetate (pH 4.3, 4.8, and 5.2), MES (morpholineethanesulfonic acid) (pH 5.7, 6.1, and 6.6), HEPES (pH 7.1, 7.5, and 8.0), Tris (pH 7.8, 8.2, and 8.7), CHES [2-(cyclohexylamino)ethanesulfonic acid] (pH 9.2, 9.6, and 10.1), and glycine (pH 10.0, 10.5, and 11.0) buffers. The optimum temperature and pH were determined by incubating 0.1 μM of enzyme at various temperatures and in 0.1 M acetate (pH 4.3, 4.8, and 5.2), MES (pH 5.7, 6.1, and 6.6), MOPS (pH 6.7, 7.2, and 7.7), HEPES (pH 7.1, 7.5, and 8.0), Tris (pH 7.8, 8.2, and 8.7), CHES (pH 9.2, 9.6, and 10.1), and glycine (pH 10.0, 10.5, and 11.0) buffers. The effects of metal ions were assayed by incubating enzyme in the presence of 1 mM of various metal ions, and substrate specificity was determined by using 1 mM of various substrates. Kinetic parameters were calculated from an *s-v* plot by curve fitting the experimental data to the Michaelis-Menten equation using the GraFit computer program (15).

Structural analysis of NahK products. Following incubation of 20 mM GlcNAc or GalNAc with 22 mM ATP, 10 mM MgCl$_2$, and 70 μg/ml NahK in 0.1 M Tris buffer (pH 8.5) at 37°C for 24 h, the reaction products were purified by Toyopearl HW40F (Tosoh, Tokyo, Japan) gel filtration column chromatography (2.6 cm [inside diameter] by 80 cm) using H$_2$O as the solvent at a flow rate of 1
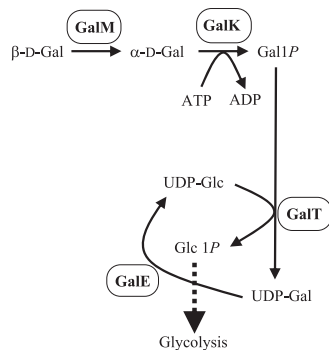

FIG. 2. Scheme of the Leloir pathway.

TABLE 1. Primer sequences for cloning and expression

Gene	Primer	Sequence[a]
lnpB	Cloning forward	GCGACCAACGATTATGGCAAGGGC
	Cloning reverse	GGCGTATACCTCGGTCAGCTGATCGCTCAT
	Expression forward	AACGGACCCC<u>CATATG</u>ACCGAAAGCAATGAAGTTTTATTC
	Expression reverse	GCTGAC<u>CTCGAG</u>CCTGGCAGCCTCCATGATGTCGGCTAC
lnpC	Cloning forward	CATCGTAGCCGACATCATGGAGGCTGCC
	Cloning reverse	CCCGTAACCAGGACAGTGGTCATGTTTCC
	Expression forward	GATATATA<u>CATATG</u>AACGATCAGCTGACCGAGGTA
	Expression reverse	TGACCT<u>CTCGAG</u>CCTAGCGGCAAAACCAAGGCTTTCGA
lnpD	Cloning forward	GGCAACACGGCCGTCTTCAAGCAGAAAGC
	Cloning reverse	GGCAACACGGCCGTCTTCAAGCAGAAAGC
	Expression forward	GATATA<u>CATATG</u>ACTACTGTTCTGGTTACGGGC
	Expression reverse	CTGCTC<u>CTCGAG</u>CTCCGCGTCGCGGAAACCGTTGGGGTTC

[a] Restriction endonuclease sites are underlined.

ml/min. ^{1}H nuclear magnetic resonance spectra were determined in D_2O using an Avance 800 spectrometer (Bruker Biospin, Rheinstetten, Germany) at 298 K and compared with those of authentic α-GlcNAc1*P* and α-GalNAc1*P*.

Nucleotide sequence accession number. The nucleotide and deduced amino acid sequences for *lnpA* to *lnpD* are available in the GenBank sequence database under accession number AB303839.

RESULTS

Molecular cloning of the *lnpB*, *lnpC*, and *lnpD* genes. After amplifying the *lnpB*, *lnpC*, and *lnpD* genes from *B. longum* JCM1217 genomic DNA, as described in Materials and Methods, we sequenced the DNA from the 3′ terminus of the BL1640 gene homolog to the 3′ terminus of the BL1645 gene homolog from *B. longum* JCM1217 containing *lnpA*, which had been cloned previously (13). The sequences were highly homologous, except for the noncoding region between the BL1640 gene homolog and *lnpA*. The deduced amino acid sequences of the *lnpB*, *lnpC*, and *lnpD* genes were 99.2%, 96.7%, and 97.6% identical, respectively, to the deduced amino acid sequences of the BL1642, BL1643, and BL1644 genes, respectively, which had been registered on the database

of Genome Information Broker (http://gib.genes.nig.ac.jp/). The recombinant enzymes encoded by the *lnpB*, *lnpC*, and, *lnpD* genes were purified from *E. coli* transformants as described previously.

Activity of LnpB (the BL1642 homolog). Aside from the activity of BL1642, annotated as a mucin desulfatase, no homology with other desulfatases was observed. BL1642, however, was highly homologous to a hypothetical protein whose gene was adjacent to a mucin desulfatase gene from *Prevotella* sp. strain RS2. Incubation of 4 μM LnpB protein with 10 mM GlcNAc 6-sulfate in 0.1 M MOPS buffer (pH 7.5) at 30°C for 30 min showed that the protein did not have hydrolytic activity, strongly suggesting that LnpB is not a desulfatase. Since a BLAST search showed that BL1642 was moderately similar to some kinases, we assessed the kinase activity of LnpB for GlcNAc in the presence of ATP. Thin-layer chromatography analysis revealed a new spot in the position of a sugar phosphate (data not shown), later confirmed to be α-GlcNAc1*P*, not GlcNAc6*P*, by both HPIC and nuclear magnetic resonance analysis, indicating that the protein encoded by the *lnpB* gene has a novel kinase activity. This protein had similar activity on GalNAc, also yielding α-GalNAc1*P*, and weak activities on several monosaccharides (Table 2). We therefore propose the name NahK for this enzyme. NahK also accepted GTP and ITP as phosphate donors (Table 3). The optimum temperature for this reaction was 40°C (measured for 30 min), and the optimum pH was 8.5. The enzyme was stable at pH 5.0 to 9.5 at 30°C and lost half of its activity during incubation for 30 min at 50°C

TABLE 2. Substrate acceptor specificity of NahK

Acceptor	Sp act ($\mu mol \cdot min^{-1} \cdot mg$ $protein^{-1}$)
N-Acetyl-D-glucosamine	1.49
N-Acetyl-D-galactosamine	0.89
N-Acetyl-D-mannosamine	0.22
D-Talose	0.049
D-Mannose	0.024
2-Deoxyglucose	0.007
D-Glucosamine	0.004
D-Mannosamine	0.002
D-Glucose	0.001
D-Galactosamine	0.0003
D-Galactose	0.0002
D-Allose	ND[a]
D-Lyxose	ND
L-Rhamnose	ND
D-Xylose	ND
D-Ribose	ND
L-Arabinose	ND
L-Fucose	ND

[a] ND, not detected.

TABLE 3. Substrate donor specificity of NahK

Donor	Sp act ($\mu mol \cdot min^{-1} \cdot mg$ $protein^{-1}$)
ATP	1.48
GTP	0.65
ITP	0.48
TTP	0.02
UTP	0.02
CTP	0.005
Polyphosphate	ND[a]

[a] ND, not detected.

TABLE 4. Effects of metal ions on NahK activity

Metal ion (1 mM)	Relative activity (%)
None	0
Mg^{2+}	100
Co^{2+}	21
Zn^{2+}	8
Mn^{2+}	7
Ni^{2+}	4
Ca^{2+}	1

in 0.1 M Tris buffer (pH 8.5). Similar to other kinases, NahK required magnesium ions for activity (Table 4). Its K_m values against GlcNAc and GalNAc were 0.118 and 0.065 mM, respectively, and its k_{cat} values were 1.21 and 0.752 s^{-1}, respectively, making its k_{cat}/K_m values for GlcNAc and GalNAc almost the same (10.3 and 11.6 mM^{-1} s^{-1}). NahK was determined to act by a sequential bi bi (two substrates-two products) mechanism (Fig. 3), with the reaction occurring after the binding of both ATP and *N*-acetylhexosamine. Its K_m value against ATP was 0.172 mM at GlcNAc saturation.

The activities of LnpC and LnpD (BL1643 and BL1644 homologs). BL1643 was annotated to be a GalT2 (EC 2.7.7.10), transferring the UMP unit of UTP to Gal1*P*. Although the protein encoded by the *lnpC* gene did not have this activity, it showed GalT (EC 2.7.7.12) activity, transferring the UMP unit from UDP-Glc to Gal1*P*. Unusually, the enzyme also transferred the UMP unit to GlcNAc1*P* and GalNAc1*P*. The UMP-transferring activities from UDP-Glc to GlcNAc1*P* (2.24 U/mg protein) and GalNAc1*P* (2.30 U/mg protein) were found to be twofold larger (2.24 and 2.30 U/mg protein, respectively) than that to Gal1*P* (1.08 U/mg protein).

LnpD had GalE activity, as well as epimerizing UDP-GalNAc into UDP-GlcNAc, with an activity of 147 U/mg protein, the same level of activity as on UDP-Gal (158 U/mg). We therefore describe the catalytic activities of the LnpC and LnpD proteins as $GalT_{Bl}$ and $GalE_{Bl}$ (GalT and GalE of *B. longum*), respectively.

DISCUSSION

NahK. Our results indicate that the protein encoded by the *lnpB* gene is not a "desulfatase for mucin" but a novel enzyme. Generally, most hexose kinases phosphorylate at the 6 position, with the exception of galactokinase (EC 2.7.1.6) (26) and *N*-acetylgalactosamine kinase (EC 2.7.1.157) (25), both of which recognize only galacto-type substrates. In contrast, NahK phosphorylates both GlcNAc and GalNAc at similar rates and possesses weak activities on various substrates. To our knowledge, NahK is a unique enzyme, as no other kinases at the anomeric position of a gluco-type substrate have yet been described.

$GalT_{Bl}$ and $GalE_{Bl}$. Our results also indicate that LnpC and LnpD are involved not only in general glucose/galactose metabolism, but also in GlcNAc/GalNAc metabolism. $GalT_{Bl}$ is a novel type of GalT, having wide substrate specificity; no other GalT has been found to recognize GlcNAc1*P* or GalNAc1*P*. Based on their amino acid sequences, GalT enzymes have been classified into two groups: class I, which is present in most

species, and class II, which is present only in bacteria (InterPro database; http://www.ebi.ac.uk/interpro/) (17). The amino acid sequence of $GalT_{Bl}$ showed weak homology with class II enzymes. However, it is not clear if the substrate specificity of $GalT_{Bl}$ is the same as those of other class II enzymes, because the difference between classes I and II has not been completely determined.

GalE from *E. coli* catalyzes only the conversion between UDP-Glc and UDP-Gal, whereas GalE from humans converts both UDP-Glc/UDP-Gal and UDP-GlcNAc/UDP-GalNAc. Replacing the Tyr299 residue of *E. coli* GalE with the cysteine residue from human GalE enables the enzyme to convert between UDP-GlcNAc and UDP-GalNAc (24). The tyrosine side chain has been shown to cause steric hindrance for the 2-acetoamide group during the rotation of GlcNAc to GalNAc through the 4-ketose intermediate. The amino acid residue at this position of the $GalE_{Bl}$ protein is cysteine, explaining its ability to epimerize both UDP-Glc and UDP-GlcNAc.

Interpretation of the LNB/GNB pathway. The enzyme activities of LnpA, LnpB, LnpC, and LnpD suggest that its gene cluster functions as an operon to metabolize whole molecules of LNB and GNB. LNB from HMO, liberated by the hydrolysis of lacto-*N*-tetraose by lacto-*N*-biosidase (EC 3.2.1.140), an enzyme that has not been isolated from bifidobacteria, and GNB from mucin, liberated by endo-α-*N*-acetylgalactosaminidase (EC 3.2.1.97) (10), are thought to be transported into the cell by a protein encoded by a putative ABC transporter gene located upstream of this cluster (the BL1638-BL1640 genes). Subsequently, LNB/GNB can be phosphorylated by LNBP to form Gal1*P* and GlcNAc/GalNAc. Gal1*P* is converted to Glc1*P* by the interaction of $GalT_{Bl}$ and $GalE_{Bl}$ and then joins the glycolytic pathway. In contrast, GlcNAc/GalNAc can be phosphorylated by NahK to form GlcNAc1*P*/GalNAc1*P*, which are converted by $GalT_{Bl}$ and $GalE_{Bl}$, with GlcNAc1*P* entering an aminosugar metabolic pathway. Thus, the operon encodes a sufficient number of enzymes to metabolize the entire LNB and GNB molecules to substrates of a subse-
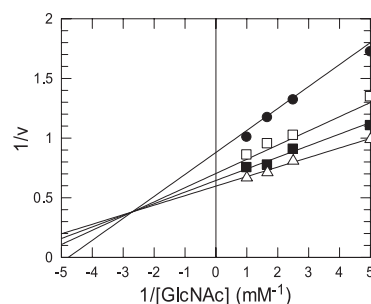

FIG. 3. Lineweaver-Burk plot of the NahK reaction with GlcNAc at ATP concentrations of 0.2 mM (closed circles), 0.4 mM (open squares), 0.6 mM (closed squares), and 1.0 mM (triangles). The kinetic parameters in the equation $v = V[GlcNAc][ATP]/(K_{mATP}K_{iGlcNAc} + K_{mATP}[GlcNAc] + K_{mGlcNAc}[ATP] + [GlcNAc][ATP])$ were calculated to be as follows: $V = 1.90$ μmol/min · mg protein, $K_{mGlcNAc} = 0.10$ mM, $K_{mATP} = 0.13$ mM, and $K_{iGlcNAc} = 0.40$ mM. v, initial reaction rate at the concentrations of the substrates; V, maximum velocity.
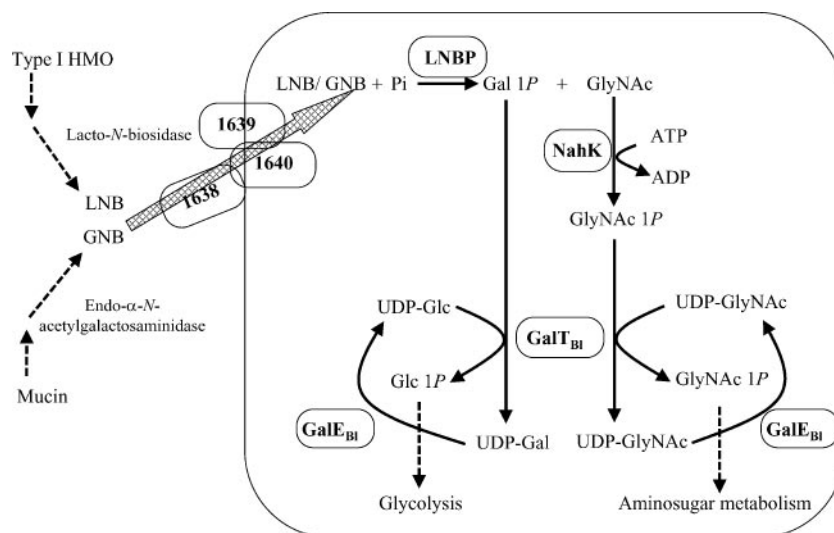
FIG. 4. Scheme of the LNB/GNB pathway in *B. longum*. Solid arrow, reaction catalyzed by an enzyme encoded in the operon; dashed arrow, reaction catalyzed by an enzyme not encoded in the operon.

quent metabolic pathway. We call the metabolic pathway encoded by this operon the "LNB/GNB pathway" (Fig. 4).

Comparison with the Leloir pathway. In most organisms, Gal is metabolized to Glc1*P* by the Leloir pathway (5, 9, 16), which consists of four enzymes (Fig. 2): galactose mutarotase (GalM), which converts β-Gal to α-Gal; galactokinase, which converts α-D-Gal to Gal1*P*; GalT, which converts Gal1*P* to UDP-Gal, coupled with the conversion of UDP-Glc to Glc1*P*; and GalE, which converts UDP-Gal to UDP-Glc, with the resultant UDP-Glc consumed in the reaction of GalT. Although the LNB/GNB pathway resembles the Leloir pathway, the two pathways differ. For example, in the Leloir pathway, α-Gal is phosphorylated by galactokinase, consuming one molecule of ATP, whereas in the LNB/GNB pathway, LNBP produces Gal1*P* without consuming ATP by the phosphorolysis of the galactosides. Utilization of *N*-acetylhexosamine, the other part of LNB/GNB, in the LNB/GNB pathway also differs from that in the Leloir pathway. GalNAc is transformed into GlcNAc1*P* with consumption of ATP, like GalK in the Leloir pathway. No gene cluster encoding the full set of enzymes involved in the Leloir pathway was found in *B. longum* NCC2705. Only a gene cluster containing genes for two of the Leloir enzymes, *galK* (BL1210) and *galT* (BL1211), was found in the genome, except for the *lnpABCD* cluster. In contrast, genes encoding the Leloir enzymes for galactose metabolism, *galM*, *galK*, *galT*, and *galE*, are often found in operons in other species (1, 4, 27). These results suggest that the LNB/GNB pathway is the main metabolic pathway for galactose as an energy source in *B. longum*. Moreover, LNB and GNB may be bifidus factors for intestinal colonization through the metabolism of milk oligosaccharides and mucin carbohydrates. Because colonization of bifidobacteria in the intestine is beneficial for human health, the LNB/GNB pathway is thought to be related to the symbiosis between humans and bifidobacteria.

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