Lacto-N-biosidase Encoded by a Novel Gene of Bifidobacterium longum Subspecies longum Shows Unique Substrate Specificity and Requires a Designated Chaperone for Its Active Expression* [5]

Received for publication, May 10, 2013, and in revised form, July 3, 2013. Published, JBC Papers in Press, July 10, 2013, DOI 10.1074/jbc.M113.484733

Haruko Sakurama1, Masashi Kiyohara1, Jun Wada2, Yuji Honda3, Masanori Yamaguchi4, Satoru Fukiya5, Atsushi Yokota6, Hisashi Ashida1, Hidehiko Kumagai1, Motomitsu Kitaoka7, Kenji Yamamoto8, and Takane Katayama4,11

From the 4Research Institute for Bioresources and Biotechnology, Ishikawa Prefectural University, Nozoiichi, Ishikawa 921-8836, the 5Department of Organic Chemistry, Waseda University, Sakaedani, Saitama, 332-8502, the 6Research Faculty of Agriculture, Hokkaido University, Sapporo, Hokkaido 060-8589, the 7Faculty of Biology-Oriented Science and Technology, Kinki University, Kinokawa, Wakayama 649-6493, and the 8**National Food Research Institute, National Agriculture and Food Research Organization, Tsukuba, Ibaraki 305-8642, Japan

Background: Phenotypically lacto-N-biosidase-positive Bifidobacterium longum JCM1217 does not possess a gene homologous to previously identified lacto-N-biosidase.

Results: Hypothetical proteins BLLJ_1505 and BLLJ_1506 encode lacto-N-biosidase and its designated chaperone, respectively.

Conclusion: The enzyme showed unique and unexpected substrate specificity.

Significance: The enzyme is important for understanding how B. longum consumes human milk oligosaccharides and also may serve as a new tool in glycobiology.

Infant gut-associated bifidobacteria possess species-specific enzymatic sets to assimilate human milk oligosaccharides, and lacto-N-biosidase (LNBase) is a key enzyme that degrades lacto-N-tetraose (Galβ1–3GlcNAcβ1–3Galβ1–4Glc), the main component of human milk oligosaccharides, to lacto-N-biose I (Galβ1–3GlcNAc) and lactose. We have previously identified LNBase activity in Bifidobacterium bifidum and some strains of Bifidobacterium longum subsp. longum (B. longum). Subsequently, we isolated a glycoside hydrolase family 20 (GH20) LNBase from B. bifidum; however, the genome of the LNBase+ strain of B. longum contains no GH20 LNBase homolog. Here, we reveal that locus tags BLLJ_1505 and BLLJ_1506 constitute LNBase from B. longum JCM1217. The gene products, designated LnbX and LnbY, respectively, showed no sequence similarity to previously characterized proteins. The purified enzyme, which consisted of LnbX only, hydrolyzed via a retaining mechanism the GlcNAcβ1–3Gal linkage in lacto-N-tetraose, lacto-N-fucopentaose I (Fucα1–2Galβ1–3GlcNAcβ1–3Galβ1–4Glc), and siarylacto-N-tetraose a (Neu5Acα2–3Galβ1–3GlcNAcβ1–3Galβ1–4Gal); the latter two are not hydrolyzed by GH20 LNBase. Among the chromogenic substrates examined, the enzyme acted on p-nitrophenyl (pNP)-β-lacto-N-biose I (Galβ1–3-GlcNAcβ-pNP) and GalNAcβ1–3GlcNAcβ-pNP. GalNAcβ1–3GlcNAcβ linkage has been found in O-mannosyl glycans of α-dystroglycan. Therefore, the enzyme may serve as a new tool for examining glycan structures. In vitro refolding experiments revealed that LnbY and metal ions (Ca2+ and Mg2+) are required for proper folding of LnbX. The LnbX and LnbY homologs have been found only in B. bifidum, B. longum, and a few gut microbes, suggesting that the proteins have evolved in specialized niches.

Infant intestinal microflora develops from a tripartite relationship between mother’s milk, infant, and bacteria, and it is generally accepted that the microbiota in the intestines of breast-fed infants are rich in particular bifidobacterial species, such as Bifidobacterium breve, Bifidobacterium bifidum, and Bifidobacterium longum subsp. longum/infantis (collectively termed infant gut-associated bifidobacteria) (1, 2). These species/subspecies dominate in the gut ecosystem within a week after birth and continue their prevalence until weaning, when the gut microflora become adult-like (1). For this reason, human milk has long been considered to contain bifidogenic compounds (3–6).

Recent research indicates that oligosaccharides (human milk oligosaccharides; HMOs)² are responsible for the bifidogenic effect of human milk. Present at concentrations between 10 and

* This work is supported in part by Grants-in-aid for Scientific Research (C) 24580119 and 24580179 from the Ministry of Education, Culture, Sports, Science, and Technology, Japan; a grant-in-aid from the Asahi Glass Foundation; a grant-in-aid from the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN); and a grant-in-aid from the Institution for Fermentation, Osaka.

† The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) BK008766.

1 This article contains supplemental Table S1 and Figs. S1 and S2.

2 The abbreviations used are: HMO, human milk oligosaccharide; Lac, lactose; GH, glycoside hydrolase; LacNDNAc, N,N’-diacetylatedlactosamine; LNA, lacto-N-biose I; LNBase, lacto-N-biosidase; LNFp, lacto-N-fucopentaose; LNT, lacto-N-tetraose; LST, siarylacto-N-tetraose; LNT, lacto-N-neo-tetraose; LNH, lacto-N-hexaose; LN, lacto-N-hexaose; pNP, p-nitrophenyl; aa, amino acids.
Novel Lacto-N-biosidase from \textit{B. longum}

In contrast to the two species mentioned above, the other infant gut-associated bifidobacteria, \textit{B. longum} subsp. \textit{longum} (referred to as \textit{B. longum}) and \textit{B. breve} have received far less attention, perhaps because they grow poorly on HMO-containing medium (OD$_{600}$ < 0.3) (12, 26). Because more than 70% of HMOs are fucosylated (i.e. derived from secretor and Lewis-positive donors), the poor growth ability of the two species may be attributable to a lack of 1,2-\alpha-1-fucosidase and 1,3-1,4-\alpha-1-fucosidase (5). Accordingly, when \textit{B. breve} JCM1192$^T$ and \textit{B. longum} JCM1217$^T$ are inoculated in HMO-containing media, they consume LNT only (12). In the spent medium of \textit{B. breve} JCM1192 cultures, no degradation products (mono-, di-, and trisaccharides) of LNT have been detected, whereas in the culture supernatant of \textit{B. longum} JCM1217, Lac appears transiently as LNT decreases. Thus, whereas \textit{B. breve} JCM1192 imports intact LNT as does \textit{B. infantis}, LNT consumption by \textit{B. longum} JCM1217 should involve secretory LNBase and the subsequent GNB/LNB pathway (supplemental Fig. S1). Indeed, LNBase activity has been previously detected in \textit{B. longum} strains JCM1217 and JCM7054 (17).

The genomic sequence of \textit{B. longum} JCM1217 was made publicly available by Fukuda \textit{et al.} in 2011 (27). Interestingly, we found that this strain possesses no gene homologous to the previously identified LNBase (glycoside hydrolase family 20, GH20) from \textit{B. bifidum} (17). Although the \textit{B. longum} JCM1217 genome encodes a GH20 enzyme, the protein obviously lacks the sequence motifs to distinguish LNBase from \textit{\beta-N-acetylgalactosaminidase} (another GH20 member) (28). Actually, the GH20 enzyme of \textit{B. longum} JCM1217 was recently found to be \textit{\beta-N-acetylgalactosaminidase} active on lacto-\textit{N-triose II} (GlcNAc\textsubscript{1–3}Gal\textsubscript{1–4Glc}) and on chitin oligosaccharides but not on LNT (29). These findings prompted us to identify a potentially novel LNBase gene encoded in the genome of \textit{B. longum}.

Here, we found that the locus tags BLLJ\textsubscript{1505} and BLLJ\textsubscript{1506}, neither of which has been functionally annotated, constitute LNBase. The enzyme body is the product of BLLJ\textsubscript{1505}, whereas the product of BLLJ\textsubscript{1506} acts as a designated chaperone for the BLLJ\textsubscript{1505} protein (LNBase). The substrate specificity of this novel LNBase was found to be quite different from that of GH20 LNBase. Interestingly, it liberated GlcNAc\textsubscript{1–3}GlcNAc from the \textit{p}-nitrophenyl sugar. The disaccharide structure is present in the \textit{O}-glycans of \textit{\alpha-dystroglycan} (30). BLLJ\textsubscript{1505} and BLLJ\textsubscript{1506} homologs were found in the genomes of \textit{B. longum}, \textit{B. bifidum}, and a few gut microbes, suggesting that this LNBase has uniquely evolved in particular species.

**EXPERIMENTAL PROCEDURES**

\textit{Chemicals—LNT, lacto-\textit{N-neo}-tetrose (LN\textit{Nt}, Gal\textsubscript{1–4GlcNAc\textsubscript{1–3}Gal\textsubscript{1–4Glc}}, LNFP I, LNFP II (Gal\textsubscript{1–3} (Fuc\textsubscript{1–4})GlcNAc\textsubscript{3}Gal\textsubscript{1–4Glc}), sialylacto-\textit{N}-tetrose (LST) a (Neu5Ac\textsubscript{2–6}Gal\textsubscript{1–3}GlcNAc\textsubscript{1–3}Gal\textsubscript{1–4Glc}), and LST b (Gal\textsubscript{1–3}Neu5Ac\textsubscript{2–6}GlcNAc\textsubscript{1–3}Gal\textsubscript{1–4Glc}) were purchased from IsoSep (Tullinge, Sweden). Lacto-\textit{N}-hexaose (LNH, Gal\textsubscript{1–3}GlcNAc\textsubscript{1–3}Gal\textsubscript{1–3}GlcNAc\textsubscript{1–3}Gal\textsubscript{1–4Glc} was obtained from Dextra Laboratories (Reading, UK). LNBase was partly purified using Aminex HP26 (Bio-Rad) and MonoQ (Bio-Rad) columns and concentrated using an AVESTA Centriplus 800 filter. LNT, LNH, and lacto-\textit{N}-triose II (GlcNAc\textsubscript{1–3}Gal\textsubscript{1–4Glc}) were prepared as
**Novel Lacto-N-biosidase from B. longum**

Described previously (18, 31), p-Nitrophenyl (pNP)-β-lacto-N-bioside (LNB-β-pNP) was obtained from Sigma-Aldrich or synthesized essentially as described previously (32). Briefly, hepta-O-acetyl-LNB was prepared by treating LNB with acetic anhydride and pyridine. Following chlorination with hydrogen chloride in acetic acid, p-nitrophenol was glycosylated in dimethylformamide. O-Deacetylation was carried out using sodium methoxide in methanol. The synthesized LNB-β-pNP was used for screening a genomic library of B. longum subsp. longum JCM1217 (described below). GalNAcβ1-3GlcNAcβ-pNP and GalNAcβ1-4GlcNAc (LacdiNAc)β-pNP were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Other reagents of analytical grade were obtained from commercial sources. The oligonucleotides used in the plasmid and strain constructions are listed in supplemental Table S1.

**Bacterial Strains and Media**—The strains used in this study were B. longum subsp. longum JCM1217T and 105-A (33) and Escherichia coli DH5α and BL21ΔλacZ (DE3)/pRARE2 (23). Bifidobacteria were grown in GAM broth (Nissui Pharmaceutical, Tokyo, Japan) or basal medium (17) at 37 °C under anaerobic conditions. Chromosomal and spectinomycin were added at final concentrations of 4 and 30 μg/ml, respectively, as required. E. coli strains were grown in LB broth, and when necessary, ampicillin, chloramphenicol, and spectinomycin were added at concentrations of 100, 30, and 30 μg/ml, respectively.

**Construction of a Genomic Library of B. longum JCM1217**—The genome of strain JCM1217 was extracted as described previously (13) and partially digested with Sau3AI. The 8–10-kbp DNA fragments were recovered and ligated with the BamHI-digested pBR322. The resulting reaction mixture was used to transform E. coli DH5α to obtain the genomic library, which was then screened for LNB-β-pNP hydrolase. Note that E. coli DH5α exhibits neither β,1-3-galactosidase nor LNBase activity.

**Gene Disruption and Complementation Analysis of B. longum**—A targeted gene disruption in B. longum was carried out as follows. The region corresponding to nucleotide numbers 1794468–1795807 (see Fig. 1) was amplified by high fidelity PCR using PrimeStar MAX DNA polymerase (Takara Shuzo, Shiga, Japan). BamHI sites were attached at both ends (supplemental Table S1). The amplified fragment (a partial lnbX gene) was ligated with the 1.9-kbp BamHI fragment (carrying ColE1 ori and the spectinomycin resistance gene) of pBS423 (34) to generate a suicide plasmid. The resulting plasmid was introduced into B. longum 105-A by electroporation and integrated into the genome by a single crossover event at the lnbX locus. Spectinomycin-resistant colonies were isolated, and the disruption of the lnbX gene was confirmed by genomic PCR analysis (data not shown).

Complementation analysis was performed using E. coli-bifidobacteria shuttle vector pTK2064, in which ColE1 ori (high copy) of plasmid pBS423 (34) was replaced with pSC101 ori (low copy) (supplemental Table S1). Exchanging the ori ensured stable maintenance of the target genes (~6.3 kbp, GC content 66%) in cells. The lnbX and lnbXY genes (1792583–1798023 and 1792583–1798862 nt, respectively, in Fig. 1) were amplified by high fidelity PCR and inserted into the Ndel site of pTK2064 by the In-Fusion methodology (Clontech) to generate pTK2193 (lnbX+) and pTK2174 (lnbX+ Y+), respectively. A plasmid (pTK2241) carrying the lnbY gene under the control of the lnbX promoter was constructed by inverse PCR using pTK2174 as a template, in which nucleotides 1793160–1797962 (Fig. 1) were eliminated (supplemental Table S1). All of the PCR-amplified fragments were sequenced to ensure that no base changes other than those designed had occurred.

**Expression and Purification of Recombinant Proteins**—Recombinant LnbX and LnbY were expressed in E. coli and purified as both non-tagged and hexahistidine (His)-tagged forms. The expression vectors contained the DNAs coding for amino acid residues 31–1573 of LnbX and amino acid residues 30–280 of LnbY, following genetic removal of the signal peptide and membrane anchor of LnbX and the signal peptide of LnbY. The non-tagged and C-terminal His-tagged LnbX proteins were expressed using pET3a and pET23b (Novagen, Darmstadt, Germany), respectively (supplemental Table S1). The PCR-generated fragments were inserted into the Ndel-BamHI and Ndel-NotI sites of pET3a and pET23b, respectively, by the In-Fusion methodology. To express the non-tagged and N-terminal His-tagged LnbY, the amplified genes were first inserted into the Ndel-BamHI site of pET3a, and the resulting T7 expression cassettes were moved to a ColE1-compatible plasmid pCDF (Novagen) (supplemental Table S1). The entire fragments used for later manipulation were sequenced.

The non-tagged LNBase (LnbX) was purified from the cell-free extract of E. coli cells co-expressing the lnbX and lnbY genes. The cells were grown in 2 liters of LB medium, and when the optical density at 600 nm reached 0.5, isopropyl-β-D-thiogalactopyranoside was added to induce protein expression. The cells were harvested, suspended in 20 mM Tris-HCl buffer (pH 8.0), and disrupted by sonication. The supernatant was applied to a DEAE-Sepharose fast flow column (GE Healthcare), and proteins were eluted by a linear gradient of 1 M NaCl. Each fraction was tested for LNB-β-pNP-hydrolyzing activity. Active fractions were combined, dialyzed to 50 mM sodium phosphate buffer (pH 7.0) containing 0.8 M ammonium sulfate, and applied to a butyl-Sepharose 4 column (GE Healthcare). Elution was performed by decreasing the concentration of ammonium sulfate to 0 M. The protein was further purified by Mono Q 5/50 GL (0–1 M NaCl in 20 mM Tris-HCl buffer (pH 8.0)) (GE Healthcare), Poros HP (0.8–0 M ammonium sulfate in 50 mM sodium phosphate buffer (pH 7.0)) (PerSeptive Biosystems), and Superdex 200 10/300 GL (20 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl) (GE Healthcare) column chromatography. The purified protein was concentrated using an Amicon Ultra 100K concentrator (Millipore, MA). His-tagged LnbX was similarly expressed and purified by nickel-nitritolactric acid-agarose (Qiagen, Hilden, Germany), Mono Q 5/50 GL, and Superdex 200 10/300 GL column chromatography.

Non-tagged LnbY was purified similarly, using the same columns. The purity of LnbY was assessed by SDS-polyacrylamide gel electrophoresis of each fraction. His-tagged LnbY was purified by nickel-nitritolactric acid-agarose and Superdex 75 10/300 GL column chromatography. The purified protein was concentrated using an Amicon Ultra 10K concentrator.

Protein concentrations were determined using a Bradford protein assay kit (Bio-Rad). Bovine serum albumin was used as a standard.
Inductively Coupled Plasma Emission Spectroscopy—Prior to the analysis, the purified, non-tagged LnbX and LnbY were extensively dialyzed against 5 mM Tris- HCl (pH 8.0) in the presence and absence of 0.2 mM EDTA. The content and concentration of metal ions in the proteins were determined by inductively coupled plasma emission spectroscopy using ICP-8100 (Shimadzu, Kyoto, Japan), with dialysis buffer as a control. The standard was ICP multielement standard solution IV.

Edman Degradation Analysis—The N-terminal amino acid sequence of the non-tagged LnbX was determined by Edman degradation using a PPSQ-33A protein sequencer (Shimadzu).

Enzyme Assay—The standard reaction mixture contained 50 mM MES buffer (pH 5.4). pNP-sugars and oligosaccharides were used as the substrates. The reaction was initiated by adding the enzyme, and the reaction mixture was incubated at 25 °C for an appropriate time, in which the linearity of the reaction rate was observed. The enzyme concentrations used in the kinetic analysis were 0.90 nM (LNB-β-pNP), 1.8 nM (GalNAcβ1–3GlcNAcβ–pNP and LNT), and 140 nM (LNFP I). The substrate concentrations were varied from 0.3 to 2 times the respective K_m values. The reaction was terminated by adding 1 M sodium carbonate (for pNP-sugars) or by heating (for oligosaccharides). The amount of liberated p-nitrophenol was determined from the absorbance at 400 nm. Oligosaccharide hydrolysis was monitored by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using a Dionex ICS 3000 system, equipped with a CarboPac PA-1 column (Dionex, Sunnyvale, CA). The elution was performed under constant flow (0.25 ml/min) with a linear gradient of 0-330 mM sodium acetate in 125 mM NaOH at 30 °C for 20 min. Standard curve was constructed from known concentrations of Lac. The reaction products of the LNFP I hydrolysis were treated with 1,2-aminobenzanthrol. The reaction products of the enzyme, and the reaction mixture was incubated at 30 °C for an appropriate time, in which the linearity of the reaction rate was observed. The enzyme concentrations used in the kinetic analysis were 0.90 nM (LNB-β-pNP), and the enzyme (7 milliunits) was incubated at 30 °C in a total volume of 60 μl. The mixture was sampled at specified times and immediately injected into a high performance liquid chromatography (HPLC) system equipped with TSKgel Amide-80 (4.6 × 250 mm) (Tohos, Tokyo, Japan). Elution was carried out using a solvent system of acetonitrile/water of 65:35 at a flow rate of 1 ml/min and was monitored at 214 nm.

Electrospray Ionization-MS—For oligosaccharide analysis, mass spectra were obtained on an LCMS-2020 system (Shimadzu) in positive ion mode. The samples were dissolved in 0.1% formic acid/acetonitrile (1:1 by volume) and injected at 3 μl/min with a microsyringe pump.

Western Blot Analysis Using Anti-His Tag Antibodies—Cell-free extracts (0.6 mg) of E. coli cells expressing His-tagged LnbX in the presence and absence of co-expression of non-tagged LnbY were separated by size exclusion chromatography (Superdex 200 10/300 GL). Each fraction (0.5 ml) was assayed for protein concentration, LNBase activity, and LnbX content. LnbX was detected using anti-His tag antibodies conjugated with horseradish peroxidase (Qiagen), following SDS-polyacrylamide gel electrophoresis and membrane transfer (Immobilon-P, Millipore). Chemiluminescence was detected using ECL Western blotting detection reagents (GE Healthcare Life Sciences) and a LAS-3000 imaging system (Fujifilm, Tokyo, Japan).

RESULTS

Identification of BLLJ_1505 and BLLJ_1506 as the Constituents of LNBase from B. longum—A genomic library of B. longum JCM1217 was constructed using E. coli DH5a, and the transformants were screened for their ability to liberate p-nitrophenol from LNB-β-pNP. Five of 1920 transformants tested positive for the activity. Sequence analysis revealed that the DNA fragment in the plasmid isolated from the clones covers a particular genomic locus. The shortest fragment comprises genomic nucleotides 1792199–1800198 (Fig. 1). This region includes two complete open reading frames (ORFs) designated BLLJ_1505 (1599 aa) and BLLJ_1506 (280 aa). Neither ORF had been functionally annotated; nor did they share
sequence similarities with any characterized proteins. The insert also contained two truncated ORFs (BLLJ_1504 and BLLJ_1507), which were close homologs of bacterial γ-glutamylcysteine synthetase and 2-hydroxyglutaryl-CoA dehydratase activator, respectively. Pfam analysis indicated a right-handed β helix region (β helix, PF13229) at amino acid residues 158–315 of BLLJ_1505 and an uncharacterized sugar-binding domain (FIVAR, PF07554) at residues 1435–1487, whereas BLLJ_1506 lacks any functional motifs (35). The SignalP 4.1 and PSORT servers predicted the presence of a signal peptide (1–29 aa) and a membrane anchor (1577–1593 aa) in BLLJ_1505 and a signal peptide (1–29 aa) in BLLJ_1506 (36). BLLJ_1505 and BLLJ_1506 were separated by 3 bp, and an inverted repeat was found immediately downstream of BLLJ_1506 (1798848–1798889 nt). Homologs of these proteins in the database are introduced below.

We first disrupted locus tag BLLJ_1505 by a homologous recombination event. This manipulation was performed on B. longum strain 105-A (LNBase+) rather than JCM1217, because the latter is not amenable to standard genetic manipulation (33). Sequence identity of 99% was observed between the genes from both strains (data not shown). Disruption of the corresponding gene in strain 105-A resulted in complete loss of LNT-hydrolyzing ability, as revealed by TLC analysis (Fig. 2, lane 3). Activity was not restored by introducing BLLJ_1505 via a shuttle vector (Fig. 2, lane 5). BLLJ_1506 alone similarly failed to rescue the LNBase− phenotype (Fig. 2, lane 6); however, when both BLLJ_1505 and BLLJ_1506 were introduced, the activity resumed (Fig. 2, lane 7). These results indicate that LNBase of B. longum JCM1217 consists of BLLJ_1505 and BLLJ_1506 (hereafter referred to as LnbX and LnbY, respectively).

Besides B. longum, both LnbX and LnbY were required for the active LNBase expression in E. coli. In this experiment, the signal peptide and membrane anchor of LnbX and the signal peptide of LnbY were genetically removed, and the corresponding genes were expressed in separate plasmids (see “Experimental Procedures”). The cell-free extract of the E. coli strain carrying both LnbX and LnbY showed LNB-β-pNP-hydrolyzing activity (9.5 units/mg), whereas strains expressing either LnbX or LnbY did not (Table 1). Interestingly, mixing the cell-free extracts of E. coli strains individually expressing LnbX and LnbY induced low level activity (0.38 unit/mg). When added prior to mixing, EDTA completely quenched the activity, whereas after mixing, it produced no effect on activity (0.34 unit/mg). Similarly, EDTA did not alter the activity in the cell-free extract of E. coli cells co-expressing LnbX and LnbY (9.5 versus 9.6 units/mg).

### Table 1: Summary of purification of recombinant non-tagged LNBase (LnbX)

| Purification step | Total protein | Total units | Activity | -Fold Yield |
|------------------|--------------|-------------|----------|-------------|
| Cell-free extract | mg | units | units/mg | % |
| DEAE-Sepharose fast flow | 262 | 5120 | 19.5 | 1.9 | 85 |
| Butyl-Sepharose 4 | 113 | 3410 | 30.2 | 3.0 | 57 |
| Mono Q 5/50 GL | 61.8 | 2150 | 34.8 | 3.4 | 36 |
| Poros HP 4.6/100 | 30.9 | 1130 | 36.6 | 3.6 | 19 |
| Superdex 200 10/300 GL | 18.0 | 670 | 37.2 | 3.6 | 11 |

* LNBase was purified from the cell-free extracts of E. coli cells co-expressing non-tagged LnbX and LnbY proteins. Purification was carried out by monitoring LNB-β-pNP-hydrolyzing activity, as described under “Experimental Procedures.” See also Fig. 3.
sis with an apparent molecular mass of 161 kDa, which agrees with the calculated mass of LnbX (164 kDa) (Fig. 3a). Edman degradation analysis revealed the N-terminal amino acid sequence as MQSAT, consistent with the designed construct for expressing LnbX (31–1573 residues). The addition of His tag to the C terminus of the protein did not alter the enzymatic properties of LnbX (discussed below), and the tagged protein was easily purified by Ni²⁺ affinity chromatography. Moreover, LnbX migrated to a virtually identical position in the SDS-polyacrylamide gel electrophoresis regardless of whether LnbY was co-expressed or not (see Fig. 7a). The addition of purified LnbY to purified LnbX (correctly folded form, described below) produced no effect on LNBase activity (data not shown). These results indicated that the active LNBase molecule from B. longum is a single gene product (that of LnbX). The native molecular mass of LNBase (LnbX) deduced from size exclusion chromatography was 356 kDa, indicating that the enzyme forms a dimer in solution (Fig. 3b).

Inductively coupled plasma emission spectroscopy revealed that LnbX contains 1 ± 0.1 molecule of magnesium, 3 ± 0.6 molecules of calcium, and 0.4 ± 0.1 molecule of zinc. Magnesium and calcium ions are probably tightly bound to or buried in the protein because the addition of EDTA to the dialysis buffer did not alter the metal contents; however, zinc ion was removed from the protein by adding EDTA. Recall that EDTA does not affect the specific activity of the purified enzyme.

The optimal pH and temperature of the enzyme for LNB-β-pNP hydrolysis was 5.4 (MES buffer) and 60 °C, respectively. The enzyme was stable in the pH range 4.5–9.5 and below 45 °C for 30 min. The purified recombinant enzyme maintained its activity for at least 3 weeks (data not shown).

We also purified non-tagged LnbY (Fig. 3a). The molecular mass of recombinant LnbY (30–280 aa) was estimated to be 28.2 kDa (calculated, 27.4 kDa) on SDS-polyacrylamide gel electrophoresis and 48.5 kDa on gel filtration analysis, indicating a dimer formation (Fig. 3b). LnbY appeared to contain no metal ions.

**Substrate Specificity**—Substrate specificity was determined using C-terminal His-tagged LnbX. The specific activity on LNB-β-pNP was essentially the same for the His-tagged (34.1 units/mg) and non-tagged (37.2 units/mg) enzymes. Hydrolytic activity was evaluated by overnight incubation of the enzyme (6 milliunits toward LNB-β-pNP) with various pNP-sugars (1 mM) and oligosaccharides (5 mM) (Table 3 and Fig. 4). The activity of GH20 LNBase from B. bifidum (LnbB) was also evaluated for comparison (17, 28). LnbX hydrolyzed both LNB-β-pNP and GalNAcβ1–3GlcNAcβ-pNP, whereas LnbB was inactive toward GalNAcβ1–3GlcNAcβ-pNP (Table 3). LnbX was able to act on LNT, LNFP I, and LST α (data not shown). Neither enzyme acted only on LNT, LNFP I, and LST α by LnbX at the Galβ1–3GlcNAc linkage was confirmed by electrospray ionization-MS analysis. The reaction products of LNT hydrolysis yielded molecular ion peaks corresponding to sodium adducts of Lac (calculated for [M + Na]+, 365.11; observed, 365.05) and Lnb (calculated for [M + Na]+, 406.13; observed, 406.05) (Fig. 5a), whereas the LNFP I hydrolysis gave rise to sodium adducts of Lac (observed, 365.05) and 2′-fucosyl-LNB (calculated for [M + Na]+, 552.19; observed, 552.20) (Fig. 5b). The spot corresponding to a trisaccharide in the TLC analysis of the LST a hydrolysate yielded molecular ion peaks matching sodium adduct (calculated for [M + H + 2Na]+, 719.21; observed, 719.15) of 3′-sialyl-LNB (Fig. 5c). No corresponding peaks appeared in the control experiments without enzyme (data not shown).

Both LnbX and LnbB were inactive on LNpT, lacto-N-triose II, LNFP II, and LST b (Fig. 4, b, c, f, and h). Neither enzyme hydrolyzed pNP-monosaccharides (l-ArAα-pNP, Galβ-pNP, GalNAcβ-pNP, Glcβ-pNP, GlcNAcβ-pNP, GlcUAβ-pNP, Manβ-pNP, Xylβ-pNP), Lacβ-pNP, or GalNAcβ1–4GlcNAc (LacdiNAc)β-pNP.

The activity of LnbX toward LNB-β-pNP (1 mM) was not inhibited by LacNAc (5 mM). The rate of hydrolysis of GalNAcβ1–3GlcNAcβ-pNP (1 mM) by LnbX was not affected in the presence of 5 mM LacdiNAcβ-pNP (data not shown). Kinetic analysis revealed that the catalytic efficiency of LnbX toward LNB-β-pNP and LNT is 5- and 4-fold higher, respec-
**Novel Lacto-N-biosidase from B. longum**

| Substrate | Hydrolysis* | LnbX | K<sub>m</sub> | k<sub>cat</sub> | k<sub>cat</sub>/K<sub>m</sub> | LnbB<sup>a</sup> | K<sub>m</sub> | k<sub>cat</sub> | k<sub>cat</sub>/K<sub>m</sub> |
|-----------|-------------|------|-------------|------------|----------------|------------|-------------|------------|----------------|-------------|
| LNB-β-pNP | Galβ1–3GalNAcβ-p-pNP | ++ + | 0.119 ± 0.022 | 96.1 ± 4.1 | 806 ± 123 | 0.099 ± 0.011 | 15.4 ± 0.7 | 156 ± 11 |
| GalNAcGlcNAcβ-β-pNP | Galβ1–3GalNAcβ-β-p-pNP | ++ + | 0.186 ± 0.010 | 39.4 ± 0.6 | 211 ± 8 |
| Lacto-N-tetraose | Galβ1–3GalNAcβ1–3Galβ1–4Glc | ++ + | 0.401 ± 0.015 | 113 ± 2 | 282 ± 7 | 0.626 ± 0.029 | 42.1 ± 0.9 | 67.2 ± 1.9 |
| Lacto-N-neotetraose | Galβ1–4GlcNAcβ1–3Galβ1–4Glc | - - |
| Lacto-N-triote | GlcNAcβ1–3Galβ1–4Glc | - - |
| Lacto-N-hexaoe | Galβ1–3GalNAcβ1–3(Galβ1–4GlcNAcβ1–6)Galβ1–4Glc | ++ ++ | ND<sup>b</sup> | ND | ND | ND | ND | ND |
| Lacto-N-fucopentaeose I | Fucα1–2Galβ1–3GlcNAcβ1–3Galβ1–4Glc | ++ + | 14.6 ± 4.3 | 13.5 ± 1.5 | 0.923 ± 0.179 |
| Lacto-N-fucopentaeose II | Galβ1–3(Fucα1–4)GlcNAcβ1–3Galβ1–4Glc | - - |
| Sialylacto-N-tetraose a | Neu5Acα2–3Galβ1–3GalNAcβ1–3Galβ1–4Glc | + - | ND | ND | ND | ND |
| Sialylacto-N-tetraose b | Galβ1–3Neu5Acα2–3Galβ1–3GalNAcβ1–3Galβ1–4Glc | - - |

*The reactions were carried out in 50 mM MES buffer (pH 5.4) (for LnbX) and citrate-phosphate buffer (pH 4.5) (for LnbB) containing 1 mM (for pNP sugars) and 5 mM (for oligosaccharides) substrates. Mixtures were incubated overnight at 25 °C in the presence and absence of the enzyme (6 milliliters toward pNP-LNB). The reaction products were analyzed by thin layer chromatography (see Fig. 4). Neither LnbX nor LnbB acted on pNP-monosaccharides (L-Ara, n-pNP, Galβ, GalNAcβ, Glcβ, GlcNAβ, GlcUβ, Manα, Xyβ, Lacβ-pNP, and LacdNAcβ-pNP). ++, complete hydrolysis (100%); ++, significant hydrolysis (>50%); +, partial hydrolysis (<50%); −, no hydrolysis.

<sup>a</sup> The kinetic parameters of LnbX are taken from our recent report (28).

<sup>b</sup> ND, not determined.

- tively, than that of LnbB (806 versus 156 mm<sup>-1</sup>s<sup>-1</sup> for LNB-β-pNP; 282 versus 67.2 mm<sup>-1</sup>s<sup>-1</sup> for LNT) (Table 3). This difference is mostly due to the higher k<sub>cat</sub> values of LnbX (96.1 s<sup>-1</sup> for LNB-β-pNP and 113 s<sup>-1</sup> for LNT) than those of LnbB (15.4 s<sup>-1</sup> for LNB-β-pNP and 42.1 s<sup>-1</sup> for LNT) toward these substrates. The kinetic analysis also revealed that activity of LnbX on GalNAcβ1–3GalNAcβ-pNP (k<sub>cat</sub>/K<sub>m</sub> value of 211 mm<sup>-1</sup>s<sup>-1</sup>) is significant and comparable with that on LNB-β-pNP. Relative to LNT, LnbX showed 40-fold higher K<sub>m</sub> value (14.6 versus 0.401 mm) and 8-fold lower k<sub>cat</sub> value (13.5 versus 113 s<sup>-1</sup>) for LNFP I, implying that the enzyme is less effective toward LNFP I (nonetheless significant; see Fig. 4e). The activity of the enzyme on LST a was too low to determine the kinetic parameters.

**Stereochemistry of Hydrolysis**—Stereochimical course of the reaction was determined by monitoring the anomeric configuration of LNB released from the pNP-substrate (Fig. 6). A normal phase HPLC was employed for this purpose. LNB appeared with a ratio of α-anomer to β-anomer of 25:75 and 35:65 after 1- and 10-min incubation, respectively. The peak area for LNB-β-pNP decreased, whereas those of p-nitrophenol and LNB increased as the reaction proceeded (10 and 30 min), and the α/β-ratio of LNB had almost reached the equilibration of mutarotation (60/40) at 120 min. These results showed that LnbX is a retaining enzyme.

**Role of LnbY in the Folding Process of LnbX**—To examine the folding state of LnbX, we performed gel filtration analysis using the cell-free extracts of E. coli cells expressing His-tagged LnbX with and without LnbY (non-tagged), followed by SDS-polyacrylamide gel electrophoresis and Western blot analysis using anti-His tag antibodies (Fig. 7, a and b). The protein concentration and specific activity (from 7.5- to 13-mL elution volume) were also determined for each fraction. The quantity of His-tagged LnbX obtained in a soluble fraction was consistent and independent of LnbY co-expression (Fig. 7a). LnbX eluted in a void volume in the absence of LnbY, whereas it eluted at 10.5–11.0 mL (molecular mass 399–316 kDa) in the presence of LnbY. No LNBase activity was detected in any fractions of cell-free extract of the strain expressing LnbX alone, whereas the activity exactly coincided with the LnbX protein peak of the cell-free extract of the strain co-expressing LnbX in the chromatography.

**In vitro refolding experiment of LnbX** was also performed, in which the refolding process was represented by the enzymatic activity on LNB-β-pNP (see “Experimental Procedures”). When the denatured LnbX was incubated and dialyzed alone, no detectable activity was observed throughout the incubation periods (Fig. 7c). No precipitation occurred during dialysis to reflect that the protein formed a soluble aggregate in the cell-free extracts of E. coli cells expressing LnbX alone (Fig. 7b, left). In contrast, when the refolding solution contained LnbY (non-denatured), activity gradually recovered. Efficient refolding occurred when Ca<sup>2+</sup> and Mg<sup>2+</sup> were supplemented together with LnbY. As mentioned above, both metal ions are contained in the native enzyme. Magnesium ions were found to be more important than calcium ions in the refolding process. When Mg<sup>2+</sup> alone was added, the activity was over 90% of that obtained in the presence of the two metal ions, whereas Ca<sup>2+</sup>...
alone failed to attain 70% activity (data not shown). Refolding also depended on the concentration of the metal ions; 0.1 mM was found to optimize refolding within the tested range (0.1 mM, 0.1 mM, 1 mM, and 5 mM; data not shown). Interestingly, the metal ions by themselves could slightly stimulate the refolding of denatured LnbX, and very low but non-negligible activity was detected as the incubation continued (Fig. 7c). Refolding efficiency varied with the molar ratio of LnbY added to denatured LnbX. When the ratios were increased to 5 and 20 from equimolar, LNB-β-pNP-hydrolyzing activity was elevated by 1.2- and 1.5-fold, respectively; however, a ratio of 50 caused a decline in the activity (Fig. 7d). The highest activity attained in the refolding experiments (6.3 units/mg) was one-sixth that of the native recombinant enzyme (37.2 units/mg), indicating a renaturation efficiency of about 17%.

**DISCUSSION**

The present study revealed a novel LNBase in *B. longum* that completely differs from hitherto identified GH families with respect to amino acid sequence, substrate specificity, maturation process, and possibly structure.

**Physicochemical Properties**—Initially, we considered that LNBase is a heterodimer comprising the LnbX and LnbY subunits because LNBP-β-pNP-hydrolyzing activity occurred when the cell-free extracts of *E. coli* strains individually expressing LnbX and LnbY were mixed and incubated (Table 1). However, the subsequent experiments revealed that the purified LNBase consists solely of the *lnbX* gene product. The C terminus of the protein could be truncated to 1431 amino acid residues (immediately upstream of the FIVAR domain; Fig. 1) without loss of activity (data not shown).

**Sequence Features**—In the complementation analysis using *lnbX*-deficient *B. longum*, plasmid-mediated introduction of the *lnbX* gene failed to restore LNBase activity, although the genome of the deficient strain contained an intact *lnbY*. Introduction of both *lnbX* and *lnbY* genes by plasmid recovery restored the LNBase phenotype of the parental strain (Fig. 2). These results indicate that the *lnbX* and *lnbY* genes constitute an operon, and the failure of the phenotypic change in the strain carrying plasmid-borne *lnbX* is due to a polar effect. The two genes were separated by a mere 3 bp, and a promoter-like sequence was not found upstream of the ORF of *lnbY*; instead, a 42-bp inverted repeat was found immediately downstream of *lnbY*.

In the genomes of *B. longum* JCM1217 and *B. longum* BBMN68, the *lnbX* gene is located between the γ-glutamylcysteine synthetase and 2-hydroxyglutaryl-CoA dehydratase activator genes (99% identity in nucleotide sequence over the region covering the four genes) (supplemental Fig. S2). In
the genomes of *B. longum* strains DJ010A, F8, JDM301, KACC91563, and NCC2705 (possibly LNBase/H11002/H9253), the glutamyl-cysteine synthetase gene is located just downstream of the 2-hydroxyglutaryl-CoA dehydratase activator gene (KEGG database). We currently lack knowledge of how strains JCM1217 and BBMN68 acquired the *lnbXY* gene at this locus. No insertion sequence was found at either end of the gene (IS Finder). Interestingly, however, the genomes of *B. bifidum* strains BGN4, PRL2010, and S17 contain *lnbX* and *lnbY* homologs with 50–60% amino acid sequence identity, which are also located near the γ-glutamylcysteine synthetase and 2-hydroxyglutaryl-CoA dehydratase activator genes (supplemental Fig. S2). In these genomes, the *lnbX* and *lnbY* homologs are separated by the glutamylcysteine synthetase gene, and their sense strands are opposed. Note that in *B. bifidum*, either the LnbX homolog or the LnbY homolog lacks a signal peptide; therefore, although both proteins are expressed in the cells, they cannot associate to assist the folding of LnbX.

The LnbX homologs have been found in the human gut microbial genomes of *Ruminococcus lactaris* ATCC29176 (RUMLAC_00599), *Clostridium nexile* DSM1787 (clonex_02958), and *Clostridium cellatum* DSM1785 (HMPRF0216_02974). The amino acid identity between these homologs and *B. longum* LnbX is about 40%. Among these bacteria, *R. lactaris* ATCC29176 alone possesses the LnbY homolog (27% identity, RUMLAC_00598), but the protein lacks a signal peptide. These results suggest that the gene(s) has specifically evolved within the gut niche. Given the length and sequence diversity among the few known occurrences, the gene(s) might still be evolving. Further elucidation requires characterization of each of these homologs.

**Substrate Specificity**—Besides sequence, the substrate specificity of LBase from *B. longum* significantly differs from that of GH20 LBase from *B. bifidum*. Whereas GH20 LBase (including one from *Streptomyces* sp.) acts only on unmodified LNB structures (Fig. 4) (17, 37), the novel LBase from *B. longum* was able to act on LNFP I and LST a, which have Fuc and Neu5Ac residues at the O2 and O3 positions of the distal Gal residue, respectively. Considering the high activity of LnbX on LNT, followed by LNFP I and LST a, and that the enzyme is inactive on GlcNAc-pNP, binding of the substrate at subsite −2 might be indispensable for the enzyme to exert its activity. The subsite −2 is slightly widened at the O2 position of −2 Gal, enabling LnbX to efficiently hydrolyze GalNAcβ1−3-GlcNAcβ-pNP (Table 3). GalNAcβ1−3GlcNAcβ disaccharide

![FIGURE 5. Electrospray ionization-MS analysis of the reaction products catalyzed by LnbX. The reactions were carried out in 50 mM MES (pH 5.4) buffer containing 5 mM LNT (a), LNFP I (b), and LST a (c) in the presence of the enzyme. When LNT and LNFP I were used as substrates, the reaction products were deionized by Amberlite MB-3 (Millipore) and lyophilized prior to the analysis. In LST a hydrolysis, the reaction products were first separated by thin layer chromatography, and the spot corresponding to a trisaccharide was extracted and purified by Sep-Pak C18 Plus (Waters, MA). Control experiments were performed without the enzyme (data not shown).](image-url)
structure has been found in the O-mannosyl glycans of α-dystroglycan, and β1–3GalNAc transferase with acceptor specificity toward GlcNAc (B3GALNT2) has been identified in humans and mice (38). Recently, Stevens et al. (39) reported that mutations in B3GALNT2 cause congenital muscular dystrophy-dystroglycanopathy, characterized by brain and eye anomalies. GalNAcβ1–3GlcNAc is a linkage isomer of LacdiNAc (N,N′-diacetyllactosediamine, GaNAcβ1–4GlcNAc) found in the N-glycans of glycoproteins such as lutropin (a hormone produced by gonadotroph cells) and tenascin-R (an extracellular matrix exclusively expressed in the central nervous system) as well as in glycoproteins from parasites (40, 41). The LacdiNAc structure in the leukemia inhibitory factor receptor is known to be specialized for C1-galactosylation, because the activity of other glycosyltransferases is normal in Cosmc−/−Jurkat cells. Interestingly, both C1-Gal-T and Cosmc are secretory proteins and form homodimers and thus share a functional similarity with LnbX and LnbY. Another functional homolog is seen in the synthetic pathway of the glycosylphosphatidylinositol anchor. PGI-X has been identified as an important component for active expression of PIG-M, the catalytic subunit of glycosylphosphatidylinositol mannosyltransferase 1 (44). Interestingly, these chaperone proteins are of similar size (Cosmc (318 aa), PGI-X (252 aa), and LnbY (280 aa)) despite their sequence dissimilarity.

**Physiology in HMO Assimilation**—By identifying the lnbXY gene, we unequivocally revealed that *B. longum* JCM1217 extracellularly decomposes LNT by this novel LNBase. The produced LNB and Lac should be imported into the bifidobacterial cells by the GNB/LNB transporter and the Lac transporter, respectively, for further degradation (19, 20, 21) ([supplemental Fig. S1](#)). Among the infant gut-associated bifidobacteria, *B. bifidum* and some strains of *B. longum* specifically degrade type-1 HMOs by LNBase (LnbB and LnbX, respectively). *B. longum* LNBase showed lower $K_m$ and higher $k_{cat}$ values for LNT than *B. bifidum* LNBase. Therefore, assuming that the LnbX homolog of *B. bifidum* does not function within cells (as described above), *B. longum* can exploit LNT over *B. bifidum*. This might be an important survival strategy for gut-
dwellling *B. longum* because this subspecies essentially assimilates LNT only among HMOs due to the lack of /H9251-L-fucosidase (12). *B. longum* might employ its highly active LNBase to sequester LNTs formed from LNFP I and lacto-N-difucohexaose I (the main HMO species) by *B. bifidum* 1,2-, and 1,3/4/H9251-L-fucosidases in the gut (13, 15). In this sense, it is interesting that *B. bifidum* grown in HMO medium ignores the degradation products of HMOs in the spent medium even during the exponential growth phase (12). *B. infantis* probably circumvents the interception by ingesting all HMOs as intact forms and digesting them intracellularly (23, 24, 25).

**Conclusions**—Hitherto uncharacterized protein sequences of unknown function, namely BLIJ1_1505 and BLIJ1_1506, are found to constitute LNBase, an enzyme exhibiting a unique substrate specificity and maturation process. These findings are important not only for comprehensive understanding of HMO assimilation by infant gut bifidobacteria but also in...
functional glycomics research because B. longum LNase is the first reported enzyme to liberate GalNAcβ1→3GlcNAc.

Acknowledgment—We thank Yuko Mori for technical assistance.

REFERENCES

1. Mitsuoka, T. (1988) Intestinal flora and host. Asian Med. J. 31, 400–409
2. Penders, J., Thuis, C., Vink, C., Stelma, F. F., Snijders, B., Kummeling, L. van den Brandt, P. A., and Stobberingh, E. E. (2006) Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics 118, 511–521
3. Kunz, C., Rudloff, S., Baier, W., Klein, N., and Strobel, S. (2000) Oligosaccharides in human milk. Structural, functional, and metabolic aspects. Annu. Rev. Nutr. 20, 699–722
4. Bode, L. (2006) Recent advances on structure, metabolism, and function of human milk oligosaccharides. J. Nutr. 136, 2127–2130
5. Newburg, D. S., and Neuberger, S. H. (1995) Carbohydrates in milk. in Handbook of Milk Composition (Jensen, R. G., ed) pp. 273–349, Academic Press, Inc., New York
6. Kobata, A. (2013) Exo- and endoglycosidases revisited. Proc. Jpn. Acad. Ser. B Phys. Biol. Sci. 89, 97–117
7. Brand-Miller, J. C., McVeagh, P., McNeil, Y., and Messer, M. (1998) Digestion of human milk oligosaccharides by healthy infants evaluated by the lactulose hydrogen breath test. J. Pediatr. 133, 95–98
8. Gnoth, M. J., Kunz, C., Kinne-Saffran, E., and Rudloff, S. (2000) Human milk oligosaccharides are minimally digested in vitro. J. Nutr. 130, 3014–3020
9. Urashima, T., Kitaoka, M., Terabayashi, T., Fukuda, K., Ohnishi, M., and Kobata, A. (2011) Milk oligosaccharides. in Oligosaccharides: Sources, properties and applications (Gordon, N. G., ed) pp. 1–58, Nova Science Publishers, New York
10. Kobata, A. (2010) Structures and application of oligosaccharides in human milk. Proc. Jpn. Acad. Ser. B Phys. Biol. Sci. 86, 731–747
11. Sel, D. A., Chapman, J., Adeuya, A., Kim, J. H., Chen, F., Whitehead, T. R., Lapidus, A., Roukhsar, D. S., Lebrilla, C. B., German, J. B., Price, N. P., Richardson, P. M., and Mills, D. A. (2008) The genome sequence of Bifidobacterium longum subsp. infantis reveals adaptations for milk utilization within the infant microbiome. Proc. Natl. Acad. Sci. U.S.A. 105, 18964–18969
12. Asakuma, S., Hatakeyama, E., Urashima, T., Yoshida, E., Katayama, T., Yamamoto, K., Kumagai, H., Ashida, H., Hirose, J., and Kitaoka, M. (2011) Physiology of consumption of human milk oligosaccharides by infant gut-associated bifidobacteria. J. Biol. Chem. 286, 34583–34592
13. Katayama, T., Sakum, A., Kimura, T., Makimura, Y., Hattori, J., Kikuchi, J., Morita, H., Hattori, M., and Ohno, H. (2011) Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature 469, 543–547
14. Ito, T., Katayama, T., Hattie, M., Sakum, A., Wada, J., Suzuki, R., Ashida, H., Wakoagi, T., Yamamoto, K., Stubbs, K. A., and Fushinobu, S. (2013) Crystal structures of a glycoside hydrolase family 20 lacto-N-biosidase from Bifidobacterium bifidum. J. Biol. Chem. 288, 11795–11806
15. Honda, Y., Nishimoto, M., Katayama, T., and Kitaoka, M. (2013) Characterization of the cytosolic β-N-acetylchitobiosidase from Bifidobacterium bifidum subsp. longum. J. Appl. Glycosci., in press
16. Inaba, T., Ohgushi, T., Iga, Y., and Hasegawa, E. (1984) Synthesis of 4-methylcoumarin-7-γ-fucopyra-N-acetylated chitosetraoside, a novel synthetic substrate for the fluorometric assay of lysozyme. Chem. Pharm. Bull. 32, 1597–1603
17. Matsumura, H., Takeuchi, A., and Yamamoto, K. (2011) An exo-α-fucosidase from bifidobacteria involved in the degradation of sialyloligosaccharides in human milk and intestinal glycoconjugates. Glycobiology 18, 1010–1017
18. Miwa, M., Horimoto, T., Yikohara, M., Katayama, T., Kitaoka, M., Ashida, H., and Yamamoto, K. (2011) An exo-α-fucosidase from bifidobacteria involved in the degradation of sialyloligosaccharides in human milk and intestinal glycoconjugates. Glycobiology 21, 437–447
19. Wada, J., Ando, T., Yikohara, M., Ashida, H., Kitaoka, M., Yamaguchi, M., Kumagai, H., Katayama, T., and Yamamoto, K. (2008) Bifidobacterium bifidum lacto-N-biosidase, a critical enzyme for the degradation of human milk oligosaccharides with a type-1 structure. Appl. Environ. Microbiol. 74, 3996–4004
20. Miyata, T., Kiyohara, M., Katayama, T., Kitaoka, M., Ashida, H., and Yamamoto, K. (2010) Cooperation of β-galactosidase and β-N-acetylchitobiosidase from bifidobacteria in assimilation of human milk oligosaccharides with type 2 structure. Glycobiology 20, 1402–1409
21. Nishimoto, M., and Kitaoka, M. (2007) Identification of N-acetylgalactosamine 1-kinase in the complete lacto-N-biose I/lactagallo-N-biose metabolic pathway in Bifidobacterium longum. Appl. Environ. Microbiol. 73, 6444–6449
22. Suzuki, R., Wada, J., Kiyohara, M., Sasakobi, T., Shou, H., Sugimoto, H., Tanaka, A., Kumagai, H., Ashida, H., Kitaoka, M., and Yamamoto, K. (2008) Structural and thermodynamic analyses of solute-binding protein from Bifidobacterium longum specific for core 1 disaccharide and lacto-N-biose I. J. Biol. Chem. 283, 13165–13173
23. Xiao, J.-Z., Takahashi, S., Nishimoto, M., Odamaki, T., Yasahima, T., Iwatsuki, K., Kitaoka, M. (2010) Distribution of in vitro fermentation ability of lacto-N-biose I, a major building block of human milk oligosaccharides, in bifidobacteria strains. Appl. Environ. Microbiol. 76, 54–59
24. Yoshida, E., Sakurama, H., Yikohara, M., Nakajima, M., Kitaoka, M., Ashida, H., Hirose, J., Katayama, T., Yamamoto K, and Kumagai H. (2012) Bifidobacterium bifidum subsp. infantis uses two different β-galactosidases for selectively degrading type-1 and type-2 human milk oligosaccharides. Glycobiology 22, 361–368
25. Ward, R. E., Nishonuevo, M., Mills, D. A., Lebrilla, C. B., and German, J. B. (2007) In vitro fermentability of human milk oligosaccharides by several strains of bifidobacteria. Mol. Nutr. Food Res. 51, 1398–1405
26. Fukuda, S., Toh, H., Hase, K., Oshima, K., Nakashima, Y., Yoshimura, K., Tohe, T., Clarke, J. M., Topping, D. L., Suzuki, T., Taylor, D. D., Itoh, K., Kikuchi, J., Morita, H., Hattori, M., and Ohno, H. (2011) Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature 469, 543–547
27. Inaba, T., Ohgushi, T., Iga, Y., and Hasegawa, E. (1984) Synthesis of 4-methylcoumarin–γ-fucopyra-N-acetylated chitosetraoside, a novel synthetic substrate for the fluorometric assay of lysozyme. Chem. Pharm. Bull. 32, 1597–1603
28. Matsumura, H., Takeuchi, A., and Yamamoto, K. (1997) Construction of Escherichia coli–Bifidobacterium longum shuttle vector transforming B. longum A-t and A-8. Biosci. Biotechnol. Biochem. 61, 1211–1212
29. Hirayama, Y., Sakamaki, M., Fushinobu, H., Murayama, H., Kano, Y., Fukuya, S., and Yokota, A. (2012) Development of a double-crossover markerless gene deletion system in Bifidobacterium longum. Functional analysis of the α-galactosidase gene for raffinose assimilation. Appl. Environ. Microbiol. 78, 4984–4994
30. Punta, M., Coggill, P. C., Eberhardt, R. Y., Mistry, J., Tate, J., Bourne, C., Pang, N., Forslund, K., Ceric, G., Clements, J., Heger, A., Holm, L., Sonnhammer, E. L., Eddy, S. R., Bateman, A., and Finn, R. D. (2012) The Pfam
proteins families database. *Nucleic Acids Res.* **40**, D290–D301
36. Petersen, T. N., Brunak, S., von Heijne, G., and Nielsen, H. (2011) SignalP 4.0. Discriminating signal peptides from transmembrane regions. *Nat. Methods* **8**, 785–786
37. Sano, M., Hayakawa, K., and Kato, I. (1992) An enzyme releasing lacto-**N**-biose from oligosaccharides. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 8512–8516
38. Hiruma, T., Togayachi, A., Okamura, K., Sato, T., Kikuchi, N., Kwon, Y.-D., Nakamura, A., Fujimura, K., Gotoh, M., Tachibana, K., Ishizuka, Y., Noce, T., Nakanishi, H., and Narimatsu, H. (2004) A novel human β1,3-**N**-Acetylgalactosaminyltransferase that synthesizes a unique carbohydrate structure, GalNAcβ1–3GlcNAc. *J. Biol. Chem.* **279**, 14087–14095
39. Stevens, E., Carss, K. J., Cirak, S., Foley, A. R., Torelli, S., Willer, T., Tambunan, D. E., Yau, S., Brodd, L., Sewry, C. A., Feng, L., Haliloglu, G., Orhan, D., Dobyns, W. B., Enns, G. M., Manning, M., Krause, A., Salih, M. A., Walsh, C. A., Hurles, M., Campbell, K. P., Manzini, M. C., UK10K Consortium, Stemple, D., Lin, Y. Y., Muntoni, F. (2013) Mutations in B3GALNT2 cause congenital muscular dystrophy and hypoglycosylation of α-dystroglycan. *Am. J. Hum. Genet.* **92**, 354–365
40. Woodworth, A., Fiete, D., and Baenziger, J. U. (2002) Spatial and temporal regulation of tenascin-R glycosylation in the cerebellum. *J. Biol. Chem.* **277**, 50941–50947
41. Wuhrer, M., Koeleman, C. A., Deelder, A. M., and Hokke, C. H. (2006) Repeats of LactoN-acetyl and fucosylated LactoN-acetylen N-glycans of the human parasite *Schistosoma mansoni*. *FEBS J.* **273**, 347–361
42. Sasaki, N., Shinomi, M., Hirano, K., Ui-Tei, K., and Nishihara S. (2011) LactoN-acetyl (GalNAcβ1–4GlcNAc) contributes to self-renewal of mouse embryonic stem cells by regulating leukemia inhibitory factor/STAT3 signaling. *Stem Cells* **29**, 641–650
43. Ju, T., and Cummings, R. D. (2002) A unique molecular chaperon Cosmc required for activity of the mammalian core 1 β3-galactosyltransferase. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 16613–16618
44. Ashida, H., Hong, Y., Murakami, Y., Shishioh, N., Sugimoto, N., Kim, Y. U., Maeda, Y., and Kinoshita, T. (2005) Mammalian PIG-X and yeast Pbn1p are the essential components of glycosylphosphatidylinositol-mannosyltransferase I. *Mol. Biol. Cell* **16**, 1439–1448
45. Anderson, K., Li, S. C., and Li, Y. T. (2000) Diphenylamine-aniline-phosphoric acid reagent, a versatile spray reagent for revealing glycoconjugates on thin-layer chromatography plates. *Anal. Biochem.* **287**, 337–339