**α-N-Acetylgalactosaminidase from Infant-associated Bifidobacteria Belonging to Novel Glycoside Hydrolyase Family 129 Is Implicated in Alternative Mucin Degradation Pathway**

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**Background:** The degradation pathway of the intestinal mucin by bifidobacteria is poorly understood.

**Results:** A novel α-N-acetylgalactosaminidase, NagBb, was identified from *Bifidobacterium bifidum* JCM 1254.

**Conclusion:** NagBb might be involved in intracellular degradation of Tn antigen (GalNAc1-Ser/Thr).

**Significance:** NagBb represents a novel glycoside hydrolase family 129 in the CAZY database.

Bifidobacteria inhabit the lower intestine of mammals including humans where the mucin gel layer forms a space for commensal bacteria. We previously identified that infant-associated bifidobacteria possess an extracellular membrane-bound endo-α-N-acetylgalactosaminidase (EngBF) that may be involved in degradation and assimilation of mucin-type oligosaccharides. However, EngBF is highly specific for core-1-type O-glycan (Galβ1–3GalNAcα1-Ser/Thr), also called T antigen, which is mainly attached onto gastroduodenal mucins. By contrast, core-1-type O-glycans (GlcNAcβ1–3GalNAcα1-Ser/Thr) are predominantly found on the mucins in the intestines. Here, we identified a novel α-N-acetylgalactosaminidase (NagBb) from *Bifidobacterium bifidum* JCM 1254 that hydrolyzes the Tn antigen (GalNAcα1-Ser/Thr). Sialyl and galactosyl core-3 (Galβ1–3/4GlcNAcβ1–3( Neu5Acα2–6)GalNAcα1-Ser/Thr), a major tetrascarhide structure on MUC2 mucin primarily secreted from goblet cells in human sigmoid colon, can be serially hydrolyzed into Tn antigen by newly identified bifidobacterial extracellular glycosidases such as α-sialidase (SiaBb2), lacto-N-biosidase (LnbB), β-galactosidase (BbgIII), and β-N-acetyllactosaminidases (BbhI and BbhII). Because NagBb is an intracellular enzyme without an N-terminal secretion signal sequence, it is likely involved in intracellular degradation and assimilation of Tn antigen-containing polypeptides, which might be incorporated through unknown transporters. Thus, bifidobacteria possess two distinct pathways for assimilation of O-glycans on gastroduodenal and intestinal mucins. NagBb homologs are conserved in infant-associated bifidobacteria, suggesting a significant role for their adaptation within the infant gut, and they were found to form a new glycoside hydrolase family 129.

Bifidobacteria naturally occur in human intestines and are predominant in those of newborn infants. They give various beneficial effects to the host such as stimulation of the immune response, prevention of the growth of pathogenic enterobacteria, and suppression of inflammatory and allergic responses; therefore, they are recognized as probiotics (1). Because they mainly reside in the lower intestines where the sugars are highly limited, they possess various glycosidases to hydrolyze digestible oligosaccharides and glycoconjugates. Endo-α-N-acetylgalactosaminidase (EC 3.2.1.97) acts on the O-glycosidic linkage of GalNAcα1-O-Ser/Thr in the core structures of mucin-type oligosaccharides to release oligosaccharides from glycoproteins. Several pathogenic and non-pathogenic bacteria possess enzymes with this type of activity (2–5). Previously, we identified the gene *engBF* encoding endo-α-N-acetylgalactosaminidase from *Bifidobacterium longum* subsp. *longum* JCM 1217 and subsequently found that the homologous genes are conserved in various infant-associated bifidobacteria (6). The *EngBF* and its homologs in bacteria were classified into a new glycoside hydrolase (GH) family, 101, in the CAZY database (7). EngBF showed relatively strict specificity toward the core-1-type O-glycan (Galβ1–3GalNAcα1-Ser/Thr) compared with the other GH101 enzymes (8–10). The β1,3-linked Gal in the core-1 structure is known to be highly resistant to common β-galactosidases from general intestinal bacteria. However,
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bifidobacteria can release the core-1 disaccharide from mucin glycoproteins through the action of the extracellular membrane-bound EngBF, incorporate it into the cytosol through a specific ATP-binding cassette-type transporter on the plasma membrane (11–13), and finally phosphorylate it by a specific phosphorylase in the cytosol (14). These findings suggest that bifidobacteria selectively grow in the intestines by assimilating mucin-type oligosaccharides utilizing a unique degradation pathway. In fact, the isomeric disaccharide Gal\(\beta\)1–3GlcNAc, a building unit of the type-1 chain in human milk oligosaccharides, which can be recognized by the same transporter and phosphorylase, specifically enhanced the growth of bifidobacteria in vitro (15).

Mucins are secreted from specific mucin-producing epithelial cells in mucosal tissues and form a gel layer to protect the epithelia from pathogenic bacteria and viruses, digestive enzymes, and acidic digestive juice. In the gastrointestinal tract, the stomach and duodenum mainly secrete MUC5AC and MUC6, whereas the small and large intestines secrete MUC2, MUC3, and MUC4. It has been reported that MUC2 is the major mucin secreted from goblet cells in human colon (16) and that a swollen layer of MUC2 supplies the space for commensal bacteria (17, 18). The structures of O-glycans on mucin are quite different depending on the source of mucosal tissues. Gastric and duodenal mucins generally contain the core-1 and core-2 structures. Recent studies revealed that MUC2 in the sigmoid colon mainly contains the core-3 structure (GlcNAc\(\beta\)1–3GalNAc\(\alpha\)-Ser/Thr) (19). As mentioned above, EngBF acts on the core-1 structure with a 300 times higher rate than core-3; thus, we speculate that EngBF is involved in degradation of gastroduodenal mucins, which are released and transported from the stomach and duodenum.

To better understand the degradation and assimilation of the intestinal mucin by bifidobacteria, we searched the genome of \textit{Bifidobacterium bifidum} JCM 1254 using the sequence of EngBF as a query. As a result, we found an uncharacterized gene encoding a putative intracellular protein showing very slight similarity from EngBF. Heterologous expression of this protein revealed that it showed glycosidase activity with substrate specificity distinct from EngBF. Interestingly, its putative homologs were conserved in infant-associated bifidobacteria, and they were found to form a novel GH family.

**EXPERIMENTAL PROCEDURES**

**Culture and Genome Sequence of \textit{B. bifidum} JCM 1254—\textit{B. bifidum} JCM 1254 was cultured on Gifu Anaerobic Medium (GAM) broth (Nissui Pharmaceutical, Japan) for 16 h at 37 °C under anaerobic conditions using Anaeropack-Anaero (Mitsubishi Gas Chemical, Japan). Genomic DNA was extracted from bacterial cells using standard methods. Draft sequencing of the genome of \textit{B. bifidum} JCM 1254 was performed using a Genome Sequencer 20 System (Roche Applied Science). The full-length DNA fragment encoding the 634-amino acid polypeptide of NagBb was amplified by high fidelity PCR using PrimeSTAR HS DNA polymerase (Takara Bio, Japan), genomic DNA from \textit{B. bifidum} JCM 1254 as a template, and the following primers: forward, 5′-ggatctcgatgtcgaattcactgcc-3′; and reverse, 5′-acctgtagttacctgtcgtgctc-3′. The PCR product was digested with EcoRI and XhoI and ligated into the corresponding sites of pET23b (+) (Novagen, Germany) to express C-terminal His\(\alpha\)-tagged protein. The \textit{Escherichia coli} BL21(DE3)\(\Delta lacZ\) strain that lacks \(\beta\)-galactosidase activity was transformed with the constructed plasmid and cultured in Luria-Bertani liquid medium containing 100 \(\mu\)g/ml ampicillin at 25 °C.**
diphenylamine/aniline/phosphoric acid (25). For the TLC assay, the following natural substrates were also used: GalNAcα1-1-Ser, Neu5Acα2–6GalNAcα1-1-Ser, blood group A trisaccharide GalNAcα1–3(Fucα1–2)Gal, GalNAcα1–3Galβ1–4Glc (Dextra Laboratories, UK), and GalNAcα1-UDP (Nacalai Tesque). Quantification of released GalNAc was performed using the LaChrom Elite HPLC system (Hitachi, Japan) equipped with a Cosmosil Sugar-D column (4.6 × 250 mm; Nacalai Tesque). Elution was carried out at 30 °C using acetonitrile/water (4:1, v/v) as a solvent at a flow rate of 1 ml/min, and the absorbance was monitored at 214 nm.

Stereochemical Analysis—GalNAcα1-1-pNP (2 ms) was incubated with NagBb in 20 mM sodium acetate buffer (pH 5.0) at 37 °C. The reaction mixtures were immediately analyzed by normal-phase HPLC using a TSKgel Amide 80 column (4.6 × 250 mm; Tosoh, Japan). Elution was carried out at 25 °C using acetonitrile/water (3:1, v/v) as a solvent at a flow rate of 1.5 ml/min, and the absorbance was monitored at 214 nm.

Transglycosylation Reaction—NagBb (35 ng/μl) was incubated with 3 mM GalNAcα1-1-pNP as a donor in the presence of 100 mM 5er as an acceptor in 50 mM sodium acetate buffer (pH 5.0) at 37 °C. The reaction mixture was analyzed by the Prominence amino acid analyzer equipped with a postcolumn fluorescence labeling system using o-phthalaldehyde and a Shim-pack Amino-Na column (Shimadzu, Japan) as described previously (26).

RESULTS

Cloning of Novel α-N-Acetylgalactosaminidase from B. bifidum—We searched the genome of B. bifidum JCM 1254 using the amino acid sequence of GH101 endo-α-N-acetylgalactosaminidase (EngBF) as a query. An open reading frame encoding a 634-amino acid protein was found that showed a slight similarity to EngBF, i.e. 15% overall identity with the GH101 conserved domain (amino acids 345–1035) of EngBF and a relatively higher (~25%) identity with the central regions (supplemental Fig. S1). No other domain or motif was found in this putative protein. In addition, an N-terminal secretion signal sequence and C-terminal transmembrane region were not found in contrast to EngBF, suggesting that this is an intracellular soluble protein. We cloned this gene and expressed it in E. coli as the C-terminal His6-tagged protein. Affinity-purified protein migrated as a single band of ~70 kDa on reducing SDS-PAGE coincident with the calculated molecular mass of this protein (supplemental Fig. S2). The activity assay using various pNP-monosaccharides as substrates revealed that the recombinant protein exhibited exo-α-N-acetylgalactosaminidase activity toward GalNAcα1-1-pNP (specific activity, 456 milliunits/mg of protein) but did not show any other exoglycosidase activities for the following substrates: GalNAcβ1–1-pNP, GlcNAcβ1–1-pNP, GlcNAcα1–1-pNP, Galα1–1-pNP, Galβ1–1-pNP, Glcα1–1-pNP, Glcβ1–1-pNP, Fucα1–1-pNP, Fucβ1–1-pNP, Manα1–1-pNP, Manβ1–1-pNP, Araα1–1-pNP, Araβ1–1-pNP, Xylα1–1-pNP, and Xylβ1–1-pNP. Also the enzyme did not hydrolyze the following pNP-disaccharide substrates: Glcα1–4Fucβ1–1-pNP, Glcβ1–4Fucβ1–1-pNP, GlcNAcβ1–4GlcNAcα1–1-pNP, Galβ1–3GlcNAcβ1–1-pNP, and Galβ1–3GalNAcβ1–1-pNP. However, the enzyme weakly hydrolyzed Galβ1–3GalNAcα1–1-pNP, a standard substrate for EngBF (specific activity, 17.1 milliunits/mg of protein). These results suggest that this enzyme is a novel α-N-acetylgalactosaminidase that shows a major exo activity with a minor endo activity. Thus, we named this gene nagBb (DDBJ accession number AB636148).

To confirm the expression of NagBb in the bifidobacterial cells, we incubated GalNAcα1-1-pNP with the cell-free extracts of B. bifidum JCM 1254 cultured in the presence of several types of sugars. The activities were detected in all extracts; however, the activities were ~2-fold higher in the lysates from the cells cultured in the presence of GalNAc or Galβ1–3GalNAc than in the absence of GalNAc (Table 1). Because EngBF (8) and GH36 α-galactosidase MelA/Aga2 (27, 28) never act on GalNAcα1-1-pNP, this result suggests that the nagBb gene is constitutively expressed and that its expression is enhanced in the presence of GalNAc. No activity was detected in the culture supernatants and the non-disrupted cell suspensions, indicating that NagBb is expressed intracellularly as predicted by its amino acid sequence.

Substrate Specificity of NagBb—To characterize the detailed substrate specificity of NagBb, we incubated the recombinant enzyme expressed in E. coli with various natural substrates containing α-linked GalNAc and analyzed the reaction mixtures by TLC (Fig. 1). NagBb completely hydrolyzed GalNAcα1-Ser, the minimum structure of the Tn antigen. The enzyme acted very slowly on GalNAcα1-1-UDP and GalNAcα1–3Galβ1–4Glc but not at all on Neu5Acα2–6GalNAcα1-Ser (sialyl Tn antigen) and GalNAcα1–3(Fucα1–2)Gal (blood group A trisaccharide). The Km and kcat values for GalNAcα1-Ser were estimated to be 2.2 mM and 47.6 s−1, respectively, but those for other substrates could not be determined accurately due to high Km values and low solubility. Therefore, only kcat/Km values were estimated (Table 2). The kcat/Km value for GalNAcα1-Ser was ~10 times higher than that for GalNAcα1-1-pNP.

Next, we investigated endo-type activities of NagBb using synthetic pNP-substrates with various core structures of mucin-type O-glycans. Hydrolyses were monitored by measuring released para-nitrophenol (Table 2) and also by detecting released oligosaccharides (Fig. 1). As mentioned above, NagBb hydrolyzed core-1 structure (Galβ1–3GalNAcα1-1-pNP) 27 times slower than GalNAcα1-1-pNP. In addition, NagBb very slowly hydrolyzed core-3 (GlcNAcβ1–3GalNAcα1-1-pNP)- and core-8 (Galα1–3GalNAcα1-1-pNP)-type structures but not other core structures. Core-7 (GalNAcα1–6GalNAcα1-1-pNP) may be sequentially hydrolyzed from the non-reducing termini.
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FIGURE 1. Substrate specificity of NagBb. Substrates (250 μM for pNP-glycosides; 3 mM for the others) were incubated with recombinant NagBb (14 ng/μl for pNP-glycosides; 50 ng/μl for the others) and analyzed by TLC. Gn and GNB, standard GalNAc and Galβ1-3GalNAc; Tn, GalNAcα1-Ser; sTn, Neu5Acα2–6GalNAcα1-Ser; UDP-Gn, GalNAcα1-UDP; A tri, GalNAcα1–3(Fucα1–2)Gal; Gn-Lac, GalNAcα1–3Galβ1–4Glc; Core-1–Core-8, pNP derivatives of each core glycan; Gn-pNP, GalNAcα1-pNP. Plus and minus, presence and absence of NagBb; asterisk, contaminant in the core-4-pNP sample.

TABLE 2
Kinetic parameters of NagBb from B. bifidum JCM 1254

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Alternative name</th>
<th>Hydrolysis</th>
<th>para-nitrophenol releasing activity</th>
<th>(k_{\text{cat}}/K_m)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(\text{milliunits/mg})</td>
<td>(s^{-1})</td>
</tr>
<tr>
<td>GalNAcα1-Ser</td>
<td>Tn antigen</td>
<td>+</td>
<td>2.2 ± 0.11</td>
<td>47.6 ± 1.66</td>
</tr>
<tr>
<td>Neu5Acα2–6GalNAcα1-Ser</td>
<td>Sialyl Tn antigen</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>GalNAcα1–3Galβ1–4Glc</td>
<td></td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>GalNAcα1–3(Fucα1–2)Gal</td>
<td></td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>GalNAcα1-pNP</td>
<td></td>
<td>+</td>
<td>456 ± 5.5</td>
<td>ND</td>
</tr>
<tr>
<td>GalNAcα1-DMT</td>
<td></td>
<td>+</td>
<td>17.1 ± 0.21</td>
<td>16.7 ± 0.35</td>
</tr>
<tr>
<td>Galβ1–3(GlcNAcβ1–6)GalNAcα1-pNP</td>
<td>Core-2, T antigen</td>
<td>–</td>
<td>17.1 ± 0.79</td>
<td>ND</td>
</tr>
<tr>
<td>GlcNAcβ1–3(GlcNAcα1-pNP)</td>
<td>Core-3</td>
<td>–</td>
<td>5.8 ± 0.40</td>
<td>ND</td>
</tr>
<tr>
<td>GlcNAcβ1–3(GlcNAcα1–6)GalNAcα1-pNP</td>
<td>Core-4</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>GalNAcα1–3(GlcNAcα1-pNP)</td>
<td>Core-5</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>GlcNAcβ1–6GalNAcα1-pNP</td>
<td>Core-6</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>GalNAcα1–6GalNAcα1-pNP</td>
<td>Core-7</td>
<td>+ *</td>
<td>0.6 ± 0.19</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Kinetic parameters were determined by quantifying the released GalNAc using an HPLC system. Data represent mean ± S.D. (n = 3).
* Not determined.
* Sequentially hydrolyzed from non-reducing terminus by exo activity.

nus by exo-α-N-acetylgalactosaminidase activity because the disaccharide was never detected. From these results, we concluded that the natural substrate for NagBb occurring in the intestines is essentially the Tn antigen.

General Properties of NagBb—General enzyme properties of NagBb were determined using GalNAcα1-pNP as a substrate. The enzyme was stable over the pH range of 3.0–11.0 and most active at pH 5.0 ± 0.5 (supplemental Fig. S3, A and B). The optimal temperature for activity was found to be 55 °C, and the enzyme was stable up to 45 °C (supplemental Fig. S3, C and D). These pH and thermal profiles of NagBb provide sufficient enzyme property for functioning in human intestines. The enzyme activity was inhibited in the presence of several metal ions at 5 mM such as Mn2+ (reduced to 75%), Zn2+ (30%), and Cu2+ (7%) but was not affected by Mg2+, Ca2+, Co2+, Ni2+, and EDTA (supplemental Fig. S4A). However, the enzyme was stabilized by the addition of Mn2+ (supplemental Fig. S4B) as observed for EngBF, an enzyme that has been shown to contain four Mn2+ ions in the crystal (29).

Catalytic Mechanism and Critical Residues of NagBb—To determine the stereochemical course of the hydrolysis catalyzed by NagBb, GalNAcα1-pNP was incubated with NagBb, and the anomeric configuration of the released GalNAc was analyzed by normal-phase HPLC (Fig. 2). Generally, an α-anomeric sugar is eluted from a normal-phase column slightly faster than the corresponding β-anomer (30, 31). After a 2-min reaction, the released GalNAc was analyzed by normal-phase HPLC (Fig. 2). The stereochemical analysis of hydrolysis catalyzed by NagBb confirmed that the natural substrate for NagBb occurring in the intestines is essentially the Tn antigen.

FIGURE 2. Stereochemical analysis of hydrolysis catalyzed by NagBb. GalNAcα1-pNP (2 mM) was incubated with NagBb in 20 mM sodium acetate buffer (pH 5.0) at 37 °C for the indicated time period. The reaction mixtures were immediately analyzed by normal-phase HPLC.
enzyme reaction, almost exclusively GalNAcαOH was detected (α/β ratio, 94:6). Then the ratio of the α- and β-anomers of GalNAc gradually changed to reach an equilibrium (α/β ratio, 55:45) due to the slow mutarotation of GalNAcαOH. This data confirmed that NagBb catalyzes the hydrolysis reaction via the retaining mechanism.

We previously identified the catalytic residues of EngBF by docking analysis of its three-dimensional structure with Galβ1–3GalNAc (29). Alignment of NagBb and EngBF revealed that several critical residues are aligned: i.e. Asp-435 in NagBb corresponds to the catalytic nucleophile Asp-789 in EngBF, and Asp-330 in NagBb corresponds to “fixer” Asp-682, the third essential residue in EngBF (supplemental Fig. S1). Asp-682 in EngBF is an important residue forming hydrogen bonds with O4 and O6 of GalNAc and O6 of Gal. However, the catalytic acid/base residue corresponding to Glu-822 in EngBF was not found in NagBb. To predict the three-dimensional structure of NagBb, we analyzed its sequence by the remote homology-based fold recognition method using the Phyre server (32). Amino acid residues 172–630 in NagBb was modeled using GH13 homology-based fold recognition method using the Phyre version 2 server (32). Amino acid residues 172–630 in NagBb could be modeled using GH13 α-amylose 1 (TVAl) from Thermocactinomyces vulgaris R-47 (Protein Data Bank code 1J11) (33), whose active center is similar to that of EngBF, as a template. Asp-435 and Asp-330 in NagBb are located at positions close to the previously identified nucleophile and fixer, respectively, of TVAl and EngBF (supplemental Fig. S5). Both D435A and D330A NagBb mutations completely lost their catalytic activity toward GalNAcα1-pNP similarly to what was observed for the corresponding EngBF mutants, suggesting that these residues function as nucleophile and fixer, respectively.

Transglycosylation Reaction—Some of the GH101 enzymes including EngBF catalyze a transglycosylation reaction in which the released Galβ1–3GalNAc is transferred to the hydroxyl group of a suitable acceptor via the same anomeric linkage because they are retaining glycosidases (5, 34, 35). To test whether NagBb catalyzes a transglycosylation reaction, we incubated 3 mM GalNAcα1-pNP and 35 ng/μl NagBb in the presence of 100 mM Ser as an acceptor in 50 mM sodium acetate buffer (pH 5.0) at 37 °C. The reaction mixture was analyzed by the Prominence amino acid analyzer equipped with a postcolumn fluorescence labeling system using o-phthalaldehyde and a Shim-pack Amino-Na column. A, heat-inactivated enzyme; B, active enzyme; C, standard GalNAcα1-Ser.

![Figure 3: Transglycosylation activity of NagBb.](https://example.com/figure3.png)

**FIGURE 3.** Transglycosylation activity of NagBb. NagBb (35 ng/μl) was incubated with 3 mM GalNAcα1-pNP as a donor in the presence of 100 mM Ser as an acceptor in 50 mM sodium acetate buffer (pH 5.0) at 37 °C. The reaction mixture was analyzed by the Prominence amino acid analyzer equipped with a postcolumn fluorescence labeling system using o-phthalaldehyde and a Shim-pack Amino-Na column. A, heat-inactivated enzyme; B, active enzyme; C, standard GalNAcα1-Ser.
groups can be clearly divided. Thus, we propose that NagBb and its homologs should be assigned to a new family GH 129.

**DISCUSSION**

Mucins are secreted from mucosal tissues and protect epithelia from various outer environmental agents including bacteria. In the intestines, goblet cells secrete mucins that are mainly the MUC2 type. When MUC2 is secreted, it folds tightly and forms a dense inner gel layer, which functions as a barrier against intestinal bacteria (17). By contrast, MUC2 in the tight layer is converted into an expanded outer layer due to proteolytic cleavage; this layer is the habitat of commensal flora (18). Recently, O-glycan structures of MUC2 from the sigmoid colon of normal patients were determined (19). The structures were mainly based on the core-3-containing tetrasaccharide (Galβ1→3GlcNAcβ1→3(Neu5Acα2→6)GalNAcα1→Ser/Thr) and were further elongated by addition of GlcNAc, Gal, sulfated Gal, Neu5Ac, and Fuc. We previously identified several extracellular membrane-bound glycosidases from *B. bifidum* JCM 1254 that are involved in degradation of human milk oligosaccharides. These glycosidases may also function in degradation of O-glycans on MUC2 (Fig. 5). Namely, two α-1-fucosidases, GH95 AfcA specific for the α1,2-linkage (39, 40) and GH29 AfcB specific for the α1,3- and α1,4-linkages (41), can remove Fuc at the non-reducing termini except for any that are α1,6-linked. GH33 α-sialidase SiaBb2 can remove both α2,3- and α2,6-linked sialic acids (42). The type-1 chain (Galβ1→3GlcNAcβ1-) is likely eliminated by GH20 lacto-N-biosidase LnbB (43), and the released lacto-N-biose I (Galβ1→3GlcNAc) is incorporated into the cytosol via a Galβ1→3GlcNAc GlcNAc/glactoN-biose transporter (11, 12). The type-2 chain (Galβ1→4GlcNAcβ1-) is sequentially degraded by GH2 β-galactosidase BbgIII acting on N-acetyllactosamine (Galβ1→4GlcNAc) and GH20 β-N-acetyhexosaminidases BbhI and BbhII specific for GlcNAcβ1→3Galβ1-R (44). Through cooperation of these glycosidases, which were shown to be expressed constitutively in
the normal condition, the Tn antigen could be uncovered on MUC2. Before or after the generation of the Tn structure, core proteins may be cleaved by some kind of protease and then incorporated into the cytosol of bifidobacteria and degraded by NagBb. The transporter responsible for Tn antigen-containing peptides is currently unknown. In the vicinity of the nagBb locus, there are putative ATP-binding cassette transporter genes, one of which may be a candidate for the transporter of Tn antigen-containing peptides. NagBb homologs are conserved in infant-associated bifidobacteria, i.e., B. longum subsp. longum, B. longum subsp. infantis, and B. breve. Thus, this novel degradation pathway for core-3 O-glycan is likely important for growth and adaptation of bifidobacteria in the gut of newborn infants. Because the NagBb homolog has never been found in pathogenic and opportunistic infectious bacteria, this novel pathway could be a promising target for the development of a highly selective bifidogenic factor, a so-called “prebiotic.” GalNAcα1 Ser or α GalNAc-containing glycosides might be better prebiotics, and the transglycosylation activity of NagBb may be exploited for their productions.

Exo-α-N-acetylgalactosaminidases (α-N-acetylgalactosaminidases; EC 3.2.1.49) have been found in GH27, GH36, and GH109 families. GH27 contains eukaryotic lysosomal α-N-acetylgalactosaminidases, which have generally broad specificity. A fungal enzyme, NagA, from Acremonium sp., a member of GH27, acts on both blood group A substance and Tn antigen (45). AagA from C. perfringens is the only α-N-acetylgalactosaminidase in GH36 and acts on blood group A substance (46). Both GH27 and GH36 contain many α-galactosidases and share a common catalytic mechanism (47). GH109 exclusively contains α-N-acetylgalactosaminidases that display an unusual mechanism involving NAD+ and have high activity toward blood group A substance (48). So far as we know, the enzymes belonging to these three GH families do not show any endotype activity. NagBb does not show significant sequence similarity with these enzymes, and its substrate specificity is quite different; therefore, we are identifying it as a novel GH129 exo-/endo-α-N-acetylgalactosaminidase.

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