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Two distinct α-L-fucosidases from *Bifidobacterium bifidum* are essential for the utilization of fucosylated milk oligosaccharides and glycoconjugates

Hisashi Ashida[1,2], Akiko Miyake[2], Masashi Kiyohara[2], Jun Wada[3], Erina Yoshida[3], Hidehiko Kumagai[3], Takane Katayama[3], and Kenji Yamamoto[2]

[2]Graduate School of Biostudies, Kyoto University, Kyoto 606-8502; and [3]Research Institute for Bioresources and Biotechnology, Ishikawa Prefectural University, Ishikawa 921-8836, Japan

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Bifidobacteria are predominant bacteria present in the intestines of breast-fed infants and offer important health benefits for the host. Human milk oligosaccharides are one of the most important growth factors for bifidobacteria and are frequently fucosylated at their non-reducing termini. Previously, we identified 1,2-α-L-fucosidase (AfcA) belonging to the novel glycoside hydrolase (GH) family 95, from *Bifidobacterium bifidum* JCM1254 (Katayama T, Sakuma A, Kimura T, Makimura Y, Hiratake J, Sakata K, Yamanoi T, Kumagai H, Yamamoto K. 2004. Molecular cloning and characterization of *Bifidobacterium bifidum* 1,2-α-L-fucosidase (AfcA), a novel inverting glycosidase (glycoside hydrolase family 95). *J Bacteriol.* 186:4885–4893). Here, we identified a gene encoding a novel 1,3–1,4-α-L-fucosidase from the same strain and termed it *afcB*. The *afcB* gene encodes a 1493-amino acid polypeptide containing an N-terminal signal sequence, a GH29 α-L-fucosidase domain, a carbohydrate binding module (CBM) 32 domain, a found-in-various-architectures (FIVAR) domain and a C-terminal transmembrane region, in this order. The recombinant enzyme was expressed in *Escherichia coli* and was characterized. The enzyme specifically released α1,3- and α1,4-linked fucosyl residues from 3-fucosyllactose, various Lewis blood group substances (a, b, x, and y types), and lacto-*N*-fucopentaose II and III. However, the enzyme did not act on glycoconjugates containing α1,2-fucosyl residue or on synthetic α-fucoside (*p*-nitrophenyl-α-L-fucoside). The *afcA* and *afcB* genes were introduced into the *B. longum* 105-A strain, which has no intrinsic α-L-fucosidase. The transformant carrying *afcA* could utilize 2′-fucosyllactose as the sole carbon source, whereas that carrying *afcB* was able to utilize 3-fucosyllactose and lacto-*N*-fucopentaose II. We suggest that AfcA and AfcB play essential roles in degrading α1,2- and α1,3/4-fucosylated milk oligosaccharides, respectively, and also glycoconjugates, in the gastrointestinal tracts.

Keywords: α-L-fucosidase/*Bifidobacterium bifidum*/GH29/Lewis blood group substance/milk oligosaccharide

Introduction

Bifidobacterium is a genus of Gram-positive anaerobic bacteria that naturally occur in the human intestinal tract. Bifidobacteria are considered to be beneficial commensal bacteria for human health because they prevent the growth of harmful bacteria by lowering the intestinal pH and stimulate the host's immune system to enhance anti-pathogenic and anti-carcinogenic activities. Therefore, they are believed to be the most promising probiotics, which are defined as live microorganisms that confer a health benefit on the host when administrated in adequate amounts.

In general, the intestines of breast-fed infants are rapidly colonized by bifidobacteria within a week after the birth, in comparison with those of the bottle-fed infants (Yoshioka et al. 1983; Harmsen et al. 2000). The selective growth of bifidobacteria observed in breast-fed newborns is attributed to oligosaccharides (excluding lactose) contained in human milk (human milk oligosaccharides, HMOs). Therefore, we believe that HMOs are natural prebiotics, which are defined as food ingredients that promote the growth and/or activity of probiotics. HMOs are present at a concentration of 10–20 g/L in human milk and are characterized by their highly complex structures: i.e., more than 130 types of HMOs have so far been isolated (Kunz et al. 2000; Urashima et al. 2001). HMOs comprise three basic units: lactose (Galβ1-4Glc), lacto-*N*-biose I (Galβ1-3GlcNAc; LNB), and *N*-acetyllactosamine (Galβ1-4GlcNAc; LacNAc). Lactose, at the reducing end, is elongated by the addition of either lacto-*N*-biose I or *N*-acetyllactosamine via a β1,3-linkage to form lacto-*N*-tetraose (Galβ1-3GlcNAcβ1-3Glaβ1-4Glc; type 1 chain) or lacto-*N*-neotetraose (Galβ1-4GlcNAcβ1-3Glaβ1-4Glc; type 2 chain). HMOs are further elongated and branched by the addition of lacto-*N*-biose I and/or *N*-acetyllactosamine via β1,3- and/or β1,6-linkages to form several complex core structures.

We have previously proposed that lacto-*N*-biose I, a building unit of type-1 HMOs, is a prebiotic component of HMOs (Kiyohara et al. 2009). We previously showed that bifidobacteria possess a unique pathway to metabolize lacto-*N*-tetraose, the core of type 1 HMOs, which is not found in cow milk but is one of the major components in HMOs (Urashima et al. 2009). Lacto-*N*-tetraose is first hydrolyzed into lacto-*N*-biose I and lactose by extracellular lacto-*N*-biosidase (Wada et al. 2008). Lacto-*N*-biose I is then incorporated into the bifidobacterial cells through a specific ABC-type transporter (Wada et al. 2007; Suzuki et al. 2008) and finally metabolized by intracellular enzymes such as galacto-*N*-biose (Galβ1-3GalNAc)/lacto-*N*-biose I phosphorylase, *N*-acetylhexosamine 1-kinase, UDP-glucose-hexose-1-phosphate uridylyltransferase, and UDP-galactose epimerase (Kitaoka et al. 2005; Nishimoto and Kitaoka 2007a, 2007b, 2008; Hidaka et al. 2009). Because β1,3-linked Gal at the nonreducing termini of lacto-*N*-tetraose and lacto-*N*-biose I is poorly hydrolyzed by typical enterobacterial exo-β-galactosidases, these oligosaccharides are selectively

[1]To whom correspondence should be addressed: Tel: +81-75-753-4298; Fax: +81-75-753-9228; e-mail: ashida@lif.kyoto-u.ac.jp

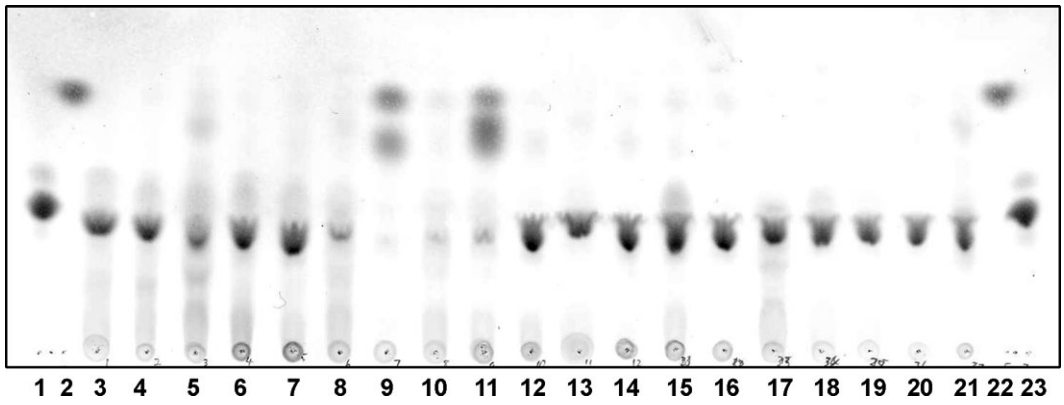

Fig. 1. Hydrolysis of 3-FL by incubation with cells of various bifidobacterial strains. Bifidobacterial cells cultured in the GAM medium were washed once with the 50 mM sodium phosphate buffer, pH 7.0. The cells (50 μg, wet weight) were incubated with 20 nmol 3-FL in 7 μL of the same buffer at 37°C for 12 h. The supernatants of the reaction mixtures were analyzed by TLC. Lanes 1 and 23, standard 3-FL; lanes 2 and 22, standard L-fucose; lane 3, *Bifidobacterium breve* 203; lane 4, *B. breve* clb; lane 5, *B. breve* JCM1192^T; lane 6, *B. pseudolongum* subsp. *pseudolongum* JCM1205^T; lane 7, *B. longum* subsp. *longum* JCM1217^T; lane 8, *B. longum* subsp. *infantis* JCM1222; lane 9, *B. bifidum* JCM1254; lane 10, *B. bifidum* JCM7004; lane 11, *B. bifidum* JCM1255^T; lane 12, *B. longum* 33R; lane 13, *B. longum* 705; lane 14, *B. longum* subsp. *longum* JCM7054; lane 15, *B. adolescentis* JCM1275^T; lane 16, *B. animalis* subsp. *lactis* JCM10602^T; lane 17, *B. gallicum* JCM8224^T; lane 18, *B. catenulatum* JCM1194^T; lane 19, *B. scardovii* JCM12489^T; lane 20, *B. angulatum* JCM7096^T; lane 21, *B. pseudocatenulatum* JCM1200^T.

assimilated by bifidobacteria. The core structures of HMOs, including lacto-*N*-tetraose and lactose, are frequently modified by the addition of fucose and sialic acid (Asakuma et al. 2007, 2008). HMOs have α1,2-, α1,3-, and α1,4-fucosyl residues at the nonreducing termini. Therefore, removal of these fucosyl residues may be essential for further degradation of HMOs. We previously identified a specific 1,2-α-L-fucosidase from *Bifidobacterium bifidum* and characterized the recombinant enzyme (Katayama et al. 2004, 2005, 2008; Nagae et al. 2007). However, the enzymes involved in releasing α1,3- and α1,4-linked fucosyl residues have not yet been identified.

Bifidobacteria can also utilize *O*-glycans on mucins secreted by gastrointestinal mucous cells as carbon sources. We found that endo-α-*N*-acetylgalactosaminidase acting on the core-1 structure (Galβ1-3GalNAcα1-Ser/Thr) of mucin-type *O*-glycans is widely distributed among various bifidobacterial strains (Fujita et al. 2005). The released disaccharide Galβ1–3GalNAc is metabolized through the same pathway as lacto-*N*-biose I, as described above. Mucin *O*-glycans are secreted in the intestinal tract as a blood group antigen, which reflects the blood type of the individual, such as ABH and Lewis. ABH blood group antigens essentially contain α1,2-linked fucose whereas Lewis antigens contain α1,3- and α1,4-linked fucoses in addition to α1,2-linked one. Here, we identified a novel bifidobacterial α-L-fucosidase termed AfcB, which acts on α1,3- and α1,4-linked fucoses that occur in both HMOs and Lewis blood groups on glycoconjugates. We subsequently demonstrated the distinct physiological significance of AfcA and AfcB for the utilization of fucosyloligosaccharides.

Results

Presence of a second α-L-*fucosidase in B. bifidum JCM1254*

We previously cloned and characterized the gene *afcA* encoding 1,2-α-L-fucosidase from *B. bifidum* JCM1254, which belongs to a novel glycoside hydrolase (GH) family 95 in the CAZy database (Katayama et al. 2004). When the *B. bifidum* JCM1254 cells were incubated with 3-fucosyllactose (3-FL), which is resistant to AfcA, the release of fucose was detected, indicating the presence of the other α-L-fucosidase(s). To investigate the distribution of the hydrolyzing activity of 3-FL in bifidobacteria, we incubated cells of various bifidobacterial strains grown in the GAM medium with 3-FL and analyzed the supernatants by thin layer chromatography (TLC). There was complete disappearance of 3-FL and concomitant appearance of L-fucose and lactose in the supernatants of *B. bifidum* JCM1254 and *B. bifidum* JCM1255 (Figure 1, lanes 9 and 11). In *B. bifidum* JCM7004, degraded products and 3-FL were not detected, suggesting that the degraded products or 3-FL were incorporated into the cells (lane 10). The remaining strains may not have 3-FL-degrading activity under this culture condition. This result suggests that 3-FL-degrading α-L-fucosidases seem to be distributed in limited strains among bifidobacteria.

The α-L-fucosidases are classified into two GH families, namely GH29 and GH95. The substrate specificity of the GH29 family is broad whereas that of the GH95 family is strict to α1,2-linked fucosyl residues. Thus, we searched for homologs of GH29 α-L-fucosidases in the genome of *B. bifidum* JCM1254, which we had previously sequenced. We found one candidate ORF containing a possible GH29 domain and designated it *afcB* (accession no. AB474964). The gene encodes a 1493-amino-acid polypeptide containing the following putative sequences/domains: an N-terminal signal sequence (amino acids 1–37), a GH29 α-fucosidase domain (amino acids 501–998), a carbohydrate binding module (CBM) 32 domain (amino acids 1133–1262), a found-in-various-architectures (FIVAR) domain (amino acids 1362–1413), and a C-terminal transmembrane region (amino acids 1469–1488) (Figure 2A). The presence of an N-terminal signal sequence and a C-terminal transmembrane region indicates that AfcB is a membrane-anchored protein with a large extracellular region that includes the GH29 domain. Although the membrane topology of AfcB is the same as AfcA, they are very different in terms of their accessory domains (Figure 2A). The GH29 domain of AfcB showed 35, 16, 11, and 11% identities in amino acid sequence with α-L-fucosidases from *Streptomyces* sp. 142 (accession no. AAD10477) (Sano et al. 1992), *Thermotoga maritima* MSB8 (AAD35394) (Tarling et al. 2003), *Sulfolobus solfataricus* P2 (AAK43159,

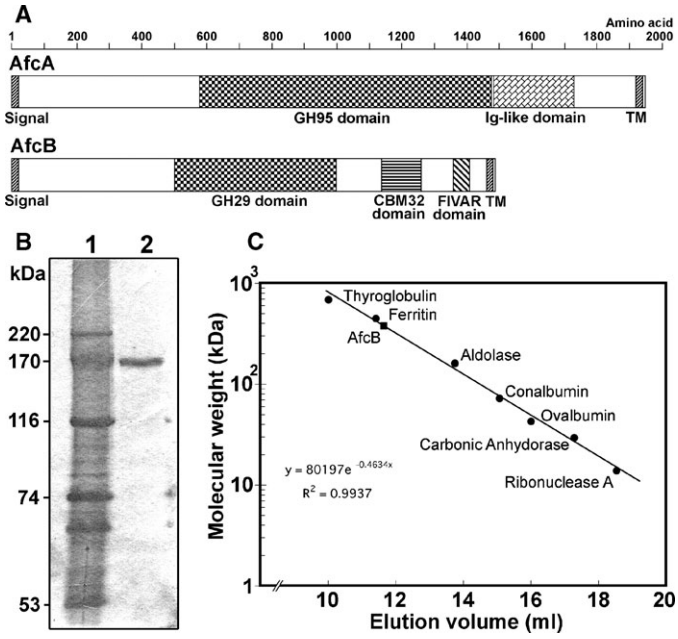

Fig. 2. Molecular cloning of AfcB. (**A**) Domain structures of AfcA and AfcB from *B. bifidum* JCM1254. (**B**) SDS–PAGE of the recombinant AfcB expressed in *E. coli*. Lane 1, molecular weight marker; lane 2, purified AfcB. (**C**) Molecular size estimation of AfcB by gel filtration using Superdex 200 10/300 GE. The standard markers (closed circles) are as follows: thyroglobulin, 669 kDa; ferritin, 440 kDa, aldolase, 158 kDa; conalbumin, 75 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa; ribonuclease A, 13.7 kDa. The molecular size of AfcB is indicated by the closed diamond.

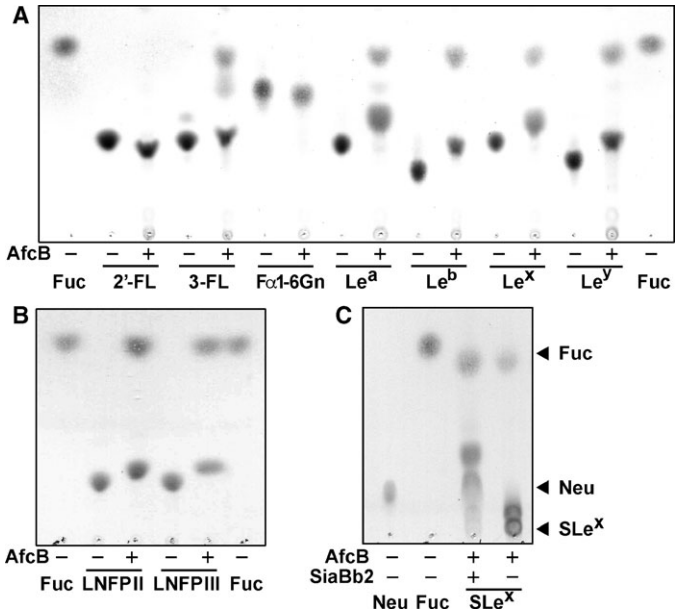

Fig. 3. Substrate specificity of AfcB. The substrates were incubated with recombinant AfcB and analyzed by TLC. (**A**) Action of AfcB on various fucosyl oligosaccharides. Fuc, standard L-fucose; 2′-FL, 2′-fucosyllactose; 3-FL, 3-fucosyllactose; Fα1-6Gn, Fucα1-6GlcNAc; Le^a, Lewis a trisaccharide; Le^b, Lewis b tetrasaccharide; Le^x, Lewis x trisaccharide; Le^y, Lewis y tetrasaccharide. (**B**) Action of AfcB on fucosylated HMO. LNFP II, lacto-*N*-fucopentaose II; LNFP III, lacto-*N*-fucopentaose III. (**C**) Action of AfcB on sialyl Lewis x tetrasaccharide in the absence or presence of sialidase. SiaBb2, a recombinant sialidase from *B. bifidum* JCM1254; Neu, Neu5Ac; SLe^x, sialyl Lewis x tetrasaccharide.

AAK43160) (Cobucci-Ponzano et al. 2003a), and humans (AAA35519) (Occhiodoro et al. 1989), respectively.

Cloning and expression of AfcB in Escherichia coli and its general properties

The DNA fragment of *afcB*, without the sequences encoding the N-terminal signal peptide and the C-terminal transmembrane region, was amplified by PCR using genomic DNA from *B. bifidum* JCM1254 as a template and ligated into a pET-23b(+) expression vector to produce N-terminally six histidine-tagged AfcB. The *E. coli* BL21(DE3) was transformed with pET-23b/*afcB*. Using 3-FL as a substrate, α-L-fucosidase activity was detected in the cell lysate of transformed *E. coli* cells cultured under isopropyl β-D-1-thiogalactopyranoside induction. The expressed protein was extracted from the cells and purified using a His-tag affinity column chromatography and gel filtration. The purified protein migrated as a single protein band of 160 kDa on reducing SDS–PAGE (Figure 2B). By gel-filtration using Superdex 200 10/300 GE, the molecular weight was estimated to be around 350 kDa, suggesting that AfcB is a homodimeric enzyme (Figure 2C). Although we could not determine the precise K_m value for 3-FL because of the linear increase in the initial velocity for hydrolysis up to 20 mM, an adequate k_{cat}/K_m value was obtained for this enzyme and was 460 ± 47 M^{-1} s^{-1} for 3-FL.

The general properties of AfcB were determined using 3-FL as a substrate. The optimum pH and temperature were pH 5.5–6.0 and 45°C, respectively. The enzyme was stable at a pH range from 3.5 to 8.0, and below 50°C at pH 6.0. The divalent cations Zn^{2+}, Mg^{2+}, Ca^{2+}, and Mn^{2+} did not affect enzyme activity, whereas Co^{2+} reduced the activity to 40% and Cu^{2+} completely inactivated the enzyme.

Substrate specificity of AfcB

To examine the substrate specificity, we added an excess amount of the recombinant enzyme to various oligosaccharides containing α-linked fucose(s) and analyzed the reaction mixtures using TLC (Figure 3A and B). Fucose was released from 3-FL, Lewis blood group substances such as Lewis a, b, x, and y oligosaccharides, lacto-*N*-fucopentaose II, and lacto-*N*-fucopentaose III, but not from 2′-FL or Fucα1-6GlcNAc. The α1,2-linked fucose in Lewis b and y was not hydrolyzed according to the mobility of the hydrolyzed products: i.e., trisaccharide and fucose were detected from both tetrasaccharides. These results indicate that the enzyme specifically hydrolyzed α1,3- and α1,4-fucosidic linkages but not α1,2- and α1,6-linkages. Furthermore, the enzyme acted on neither the synthetic substrate *p*NP-α-L-fucoside (*p*NP-Fuc) nor the other *p*NP-monosacchairdes tested. To quantify the relative activity of the enzyme toward various substrates, the initial velocities of released L-fucose were measured by L-fucose dehydrogenase-coupled assay method at a concentration of 1.0 mM substrates (Table I). Lewis a, Lewis x, Lewis y, lacto-*N*-fucopentaose II, and lacto-*N*-fucopentaose III were more rapidly hydrolyzed than 3-FL. A comparison of the relative activities toward 3-FL and Lewis x trisaccharide suggests that the enzyme prefers GlcNAc rather than Glc as a sugar to which Fuc is α1,3-linked. The activities toward α1,3- and α1,4-linkages of fucosyl residues were not clearly different (compare Lewis a and Lewis x; lacto-*N*-fucopentaose II and

Table I. Substrate specificity of AfcB

Substrate[a]	Abbreviation	Structure	Relative activity (%)
2′-Fucosyllactose	2′-FL	Fucα1-2Galβ1$\searrow_{4}$Glc	nd[b]
3-Fucosyllactose	3-FL	Galβ1$\searrow_{4}$Glc Fucα1$\swarrow_{3}$	100
6-Fucosyl-*N*-acetylglucosamine	Fα1-6Gn	Fucα1-6GlcNAc	nd[b]
Lewis a trisaccharide	Le[a]	Fucα1$\searrow_{4}$GlcNAc Galβ1$\swarrow_{3}$	166
Lewis b tetrasaccharide	Le[b]	Fucα1$\searrow_{4}$GlcNAc Fucα1-2Galβ1$\swarrow_{3}$	14
Lewis x trisaccharide	Le[x]	Galβ1$\searrow_{4}$GlcNAc Fucα1$\swarrow_{3}$	160
Lewis y tetrasaccharide	Le[y]	Fucα1-2Galβ1$\searrow_{4}$GlcNAc Fucα1$\swarrow_{3}$	128
Lacto-*N*-fucopentaose II	LNFP II	Fucα1$\searrow_{4}$ Galβ1$\swarrow_{3}$GlcNAcβ1-3Galβ1-4Glc	186
Lacto-*N*-fucopentaose III	LNFP III	Galβ1$\searrow_{4}$ Fucα1$\swarrow_{3}$GlcNAcβ1-3Galβ1-4Glc	159
Sialyl Lewis x tetrasaccharide	SLe[x]	Neu5Acα2-3Galβ1$\searrow_{4}$GlcNAc Fucα1$\swarrow_{3}$	Trace
p-Nitrophenyl-α-L-fucoside	*p*NP-Fuc	Fucα1-*p*NP	nd[b]

[a]Substrates were used at a concentration of 1.0 mM.
[b]Not detected.

lacto-*N*-fucopentaose III). The attachment of α1,2-linked Fuc at the branched β-Gal residue reduced the hydrolytic activity (compare Lewis a and Lewis b; Lewis x and Lewis y); a particularly marked reduction occurred for the Lewis b tetrasaccharide. Sialyl Lewis x tetrasaccharide, which has an α2,3-linked Neu5Ac at the branched β-Gal of Lewis x, was also very slowly hydrolyzed by AfcB, although the release of fucose was accelerated by the addition of a recombinant bifidobacterial sialidase (SiaBb2 from *B. bifidum* JCM1254, unpublished results), suggesting sequential actions of sialidase and AfcB α-L-fucosidase in the degradation of sialyl Lewis x, in this order (Figure 3C). Since the α1,3- and α1,4-fucoses are also found in the sugar chains of glycoproteins, we further examined the substrate specificity toward various pyridylamino (PA)-oligosaccharides derived from *N*-glycans. The hydrolyses were monitored by reversed-phase HPLC (data not shown). The α1,3-linked fucosyl residue attached to GlcNAc in the *N*-acetyllactosamine-type triantennary structure (PA-sugar chain #005, Takara Bio Inc.) was readily hydrolyzed to the product identical to de-fucosylated one (#002) by AfcB, and the rate of hydrolysis was almost equal to that on the same linkage of lacto-*N*-fucopentaose III (#045). The α1,6-linked fucosyl residue on the core of the triantennary *N*-acetyllactosamine type (#010) was resistant as expected. Similarly, nonfucosylated PA-oligosaccharides such as disialylated biantennary *N*-acetyllactosamine type (#023), agalacto biantennary *N*-acetyllactosamine type (#012), and M6B oligomannose type (#018) could not be substrates.

AfcA and AfcB are critical for bifidobacteria to utilize α1,2- and α1,3/4-fucosylated milk oligosaccharides, respectively

To evaluate the physiological significance of α-L-fucosidases, we introduced the *afcA* or *afcB* gene of *B. bifidum* JCM1254 into an α-L-fucosidase-negative strain *B. longum* 105-A, which can assimilate lactose but not L-fucose. The transformants were anaerobically incubated in the medium containing either 2′-FL, 3-FL, or lacto-*N*-fucopentaose II, which have α1,2-, α1,3-, or α1,4-fucosyl residues, respectively, and the culture supernatants were analyzed by TLC (Figure 4). The cells expressing AfcA liberated fucose from 2′-FL, and they became able to utilize the lactose moiety as a carbon source, whereas cells expressing AfcB could not. In contrast, the AfcA-expressing cells degraded neither 3-FL nor lacto-*N*-fucopentaose II, but the cells expressing AfcB were able to assimilate these oligosaccharides after releasing fucose residues via AfcB activity. A faint spot corresponding to lacto-*N*-biose I appeared upon incubation of lacto-*N*-fucopentaose II with AfcB-positive cells, which strongly suggests the presence of lacto-*N*-biosidase in

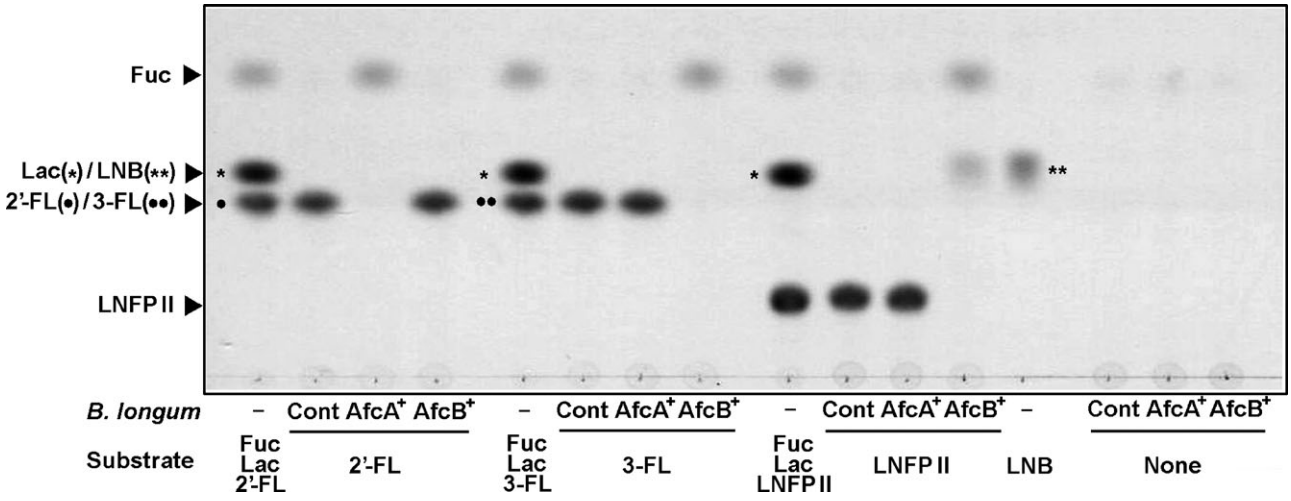

Fig. 4. Degradation of fucosylated HMOs by *Bifidobacterium longum* expressing AfcA and AfcB. The α-L-fucosidase-negative *B. longum* strain was transformed with an empty vector (Cont), an *afcA*-carrying plasmid (AfcA$^+$), or an *afcB*-carrying plasmid (AfcB$^+$) and was anaerobically incubated for 3 h in the presence of 4 mM 2′-fucosyllactose (2′-FL), 3-fucosyllactose (3-FL), or lacto-*N*-fucopentaose II (LNFP II). The culture supernatants were analyzed by TLC. The positions of standard mono- and oligosaccharides are indicated on the left side.

B. longum 105-A cells. Unlike α-L-fucosidase-expressing cells, the control cells with an empty vector could not degrade or incorporate the fucosyloligosaccharides. Thus, this is the first evidence that AfcA and AfcB are essential for the utilization of fucosylated HMOs.

Discussion

In this report, we identified the gene encoding an enterobacterial 1,3–1,4-α-L-fucosidase, a member of the GH29 family, for the first time. The GH29 family, which is exclusively composed of α-L-fucosidases, seems to be divided into two subfamilies based on phylogenetic analysis (Figure 5). One contains animal, archaeal, and bacterial enzymes, and the other consists of only plant and bacterial ones, including AfcB. Among the members of the former subfamily, α-L-fucosidases from a bacterium *Thermotoga maritima* (Tarling et al. 2003; Sulzenbacher et al. 2004), an archaea *Sulfolobus solfataricus* (Cobucci-Ponzano et al. 2003a, 2003b, 2005), and humans (Liu et al. 2009) were biochemically (for all enzymes) and structurally (only for the *Thermotoga* enzyme) characterized and found to be retaining glycosidases. The human lysosomal α-L-fucosidase hydrolyzes all α-fucosyl linkages (α1,2, α1,3, α1,4, and α1,6) and synthetic α-fucosides such as *p*NP-Fuc. Two microbial enzymes from *Thermotoga* and *Sulfolobus* also act on synthetic α-fucosides but have not been tested with natural oligosaccharides. By contrast, among the latter subfamily, only the enzymes from a soil actinomycete, *Streptomyces* sp. (Sano et al. 1992) and a plant *Arabidopsis thaliana* (Zeleny et al. 2006) have so far been molecularly cloned and characterized, and were found to act on α1,3- and α1,4-fucosidic linkages but not on *p*NP-Fuc. AfcB belongs to this subfamily and its substrate specificity is very similar to that of the *Streptomyces* and plant enzymes. Thus, the members of the former subfamily seem to have broader substrate specificity, whereas the members of the latter subfamily may have strict specificity toward α1,3- and α1,4-linkages. This may be caused by structural differences in the catalytic regions. Catalytic nucleophile and general acid/base residues in the active sites were identified in the former subfamily, whereas those in the latter subfamily have not yet been identified. Multiple alignments revealed that the sequences around the nucleophile are moderately aligned in all previously known enzymes, but those around the acid/base are poorly aligned.

Another unique feature of AfcB is its membrane topology and multidomain structure. As shown by in silico analyses and also by ectopic expression studies, AfcB is an extracellular membrane-bound enzyme and possesses two accessory domains, CBM32 (also called F5/8 type C domain) and FIVAR, that are located near the membrane and may function as sugar-binding domains. The CBM32 domain is found in a variety of microbial glycosidases and may recognize Gal or *N*-acetyllactosamine (Abbott et al. 2008). The FIVAR domain is also a putative sugar-binding domain that is frequently found in membrane-bound or cell wall-associated proteins in microbes but remain poorly characterized (Chitayat et al. 2008). These domains in AfcB may function with increasing affinity toward substrates. They are also found in other bifidobacterial glycosidases previously characterized by our group; EngBF (endo-α-*N*-acetylgalactosaminidase) contains one CBM32 and three FIVAR domains (Fujita et al. 2005), and LnbB (lacto-*N*-biosidase) contains a CBM32 domain (Wada et al. 2008). In contrast to AfcB, the other bacterial α-L-fucosidases do not have apparent accessory domains. The presence of such domains may be necessary in bifidobacterial glycosidases.

Bifidobacterium bifidum is one of the frequently found bacteria in the intestine of breast-fed infants. Our strain *B. bifidum* JCM1254 shows high adaptation for the utilization of HMOs and possess a complete degradation pathway for all HMOs. Here, we have characterized AfcA and AfcB, which can release all terminal α-fucosyl residues in HMOs. We have further identified two sialidases in this strain (unpublished results). The cooperative action of these glycosidases unmasks the modified HMOs to produce the core structures, which are easily assimilated by bifidobacteria. The type I chain, one of the major core structures in HMOs, is specifically degraded by lacto-*N*-biosidase (LnbB)

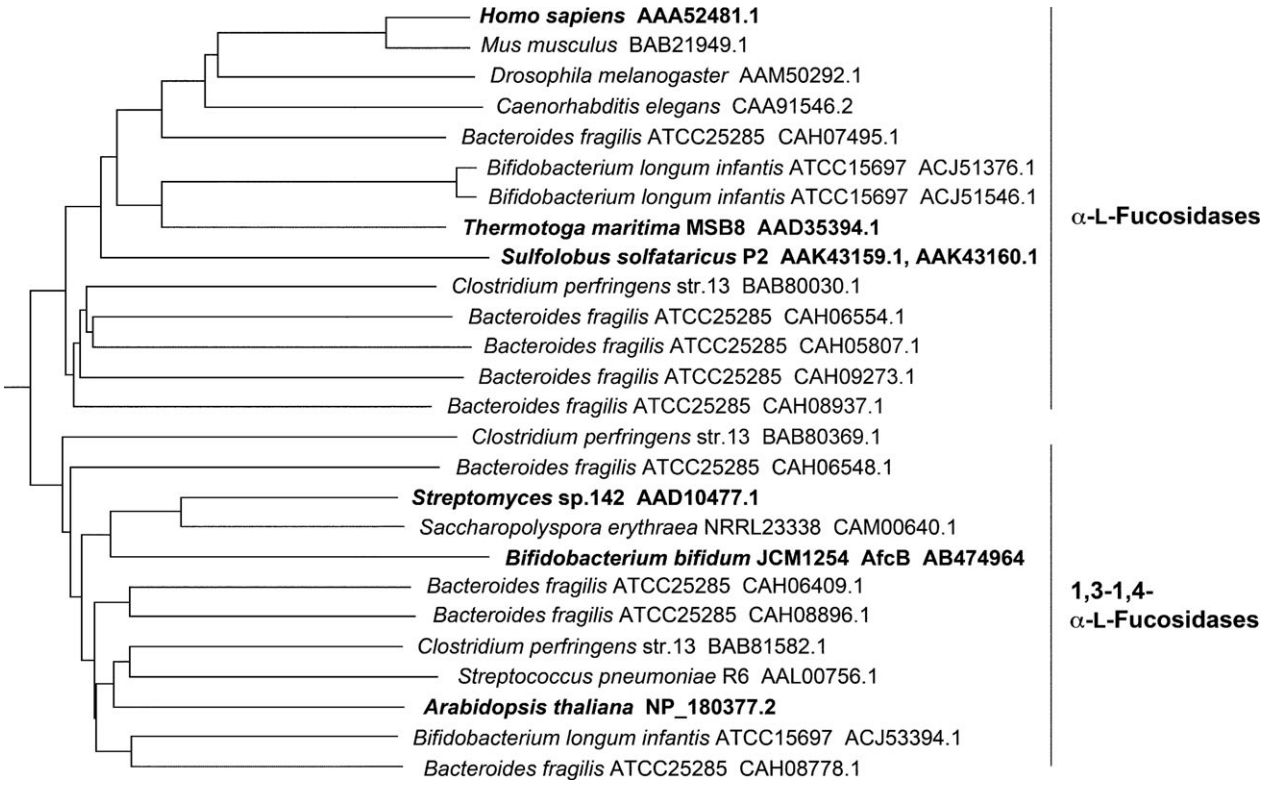

Fig. 5. The phylogenetic tree of GH29 α-L-fucosidases. The tree was constructed using the ClustalW program with a neighbor-joining method. Established α-L-fucosidases are shown in bold letters, putative ones in normal letters.

(Wada et al. 2008) and incorporated into the cells, as described in *Introduction*. On the other hand, the type II chain may be sequentially degraded from the nonreducing terminus: i.e., the genes encoding the membrane-bound β-galactosidases and β-*N*-acetylhexosaminidases were found in the genome of this strain (unpublished results).

The complete genome sequence of *B. longum* ssp. *infantis* ATCC15697 was recently reported (Sela et al. 2008). There are four putative α-L-fucosidases: three in GH29 (Figure 5) and one in GH95. Interestingly, they all seem to be intracellular enzymes because the N-terminal signal sequence is lacking, unlike AfcA and AfcB. Therefore, this subspecies may possess a different strategy for HMO utilization because it may incorporate oligosaccharides in the fucosylated form and then de-fucosylate them within the cells for assimilation. Our results of TLC analysis support this notion because *B. longum* ssp. *infantis* JCM1222 appeared to consume 3-FL but not to liberate L-fucose (Figure 1, lane 8). With the exception of *B. longum* ssp. *infantis*, most bifidobacteria probably require extracellular α-L-fucosidases to utilize fucosylated oligosaccharides as carbon sources. The presence of both AfcA and AfcB could benefit the organisms to make their habitat in intestines.

Material and methods

Bacterial strains and culture

The bifidobacterial strains were obtained from the Japan Collection of Microorganisms (JCM, RIKEN Bioresource Center, Japan). The bacteria were cultured in GAM broth (Nissui Pharmaceutical, Japan) for 16 h at 37°C under anaerobic conditions using Anaeropack (Mitsubishi Chemical, Japan).

Genome sequence of B. bifidum JCM1254

Draft sequencing of the genome of *B. bifidum* JCM1254 was performed using a Genome Sequencer 20 System (Roche Applied Science). The details will be reported elsewhere.

Cloning and expression of afcB in E. coli

To construct the AfcB expression vector, a DNA fragment encoding amino acids 38–1468 (without an N-terminal signal peptide and C-terminal transmembrane region) was amplified by high-fidelity PCR using genomic DNA from *B. bifidum* JCM1254 as a template and the following primers: forward 5′-ctagctagcatggcggatccgatggaatacct and reverse 5′-ccgctcgagcattgtggaggcgccggtctttg, digested with *NheI* and *XhoI*, and ligated into pET23b(+). The nucleotide sequence was confirmed by sequencing. The *E. coli* BL21(DE3) strain was transformed with this plasmid and cultured in the Luria-Bertani liquid medium containing 100 μg/mL ampicillin at 25°C until the optical density at 600 nm reached 0.5. Then, to induce the expression, isopropyl β-D-1-thiogalactopyranoside was added to the culture at a final concentration of 0.5 mM and cultured for 5 h at 25°C.

Purification of the recombinant AfcB

The transformed *E. coli* cells grown under the induced condition were lysed by BugBuster Protein Extraction Reagent (Novagen). After centrifugation, the supernatant was applied to a

HisTrap HP (1 mL, GE Healthcare), and the adsorbed proteins were eluted by a stepwise imidazole concentration gradient in a 50 mM sodium phosphate buffer, pH 7.0, containing 250 mM NaCl. The active fraction (1 mL) was applied onto a Superdex 200 10/300 GL (GE healthcare) gel filtration column equipped with ÄKTA explorer (GE Healthcare). Elution was carried out using a 50 mM sodium phosphate buffer, pH 7.0, containing 150 mM NaCl. Active fractions were collected, concentrated, and desalted using an Amicon Ultra 30K (Millipore).

Enzyme assay

Oligosaccharide substrates, except for sialyl Lewis x tetrasaccharide, were purchased from Dextra Laboratories; sialyl Lewis x tetrasaccharide was from Sigma. Standard assays for 1,3-1,4-α-L-fucosidase were carried out using 3-FL as a substrate. To detect hydrolysis, the enzyme products were separated by silica-gel TLC (Merck 5553) with 1-butanol:acetic acid:water (2:1:1, by volume) or chloroform:methanol:water (7:6:2, by volume) as developing solvents and visualized using diphenylamine-aniline-phosphoric acid (Anderson et al. 2000). The amount of released fucose was determined by a fucose dehydrogenase-coupled method (Cohenford et al. 1989; Katayama et al. 2004). The k_{cat}/K_m value for 3-FL was determined at the concentration of 1.0 mM. PA-oligosaccharides were purchased from Takara Bio Inc. (Tokyo, Japan). Fifty pmoles of the substrate were incubated with the enzyme (0.25–25 mU) at 37°C for an appropriate time in the reaction mixture (50 μL) containing 100 mM sodium phosphate (pH 6.0). The reactions were stopped by heating, and the products were analyzed by reversed-phase high-performance liquid chromatography (HPLC). Elution and detection were carried out as described (Wada et al. 2008).

Expression of AfcA and AfcB in B. longum

The *afcA* and *afcB* genes were introduced into *B. longum* 105-A using an *E. coli-Bifidobacterium* shuttle vector (Matsumura et al. 1997). The *afcA* gene was amplified by PCR using genomic DNA from *B. bifidum* JCM1254 as a template and a primer pair (5′-aacggtatccagggactctctga and 5′-caagccaccgtcccccgccgggg), and then inserted into the *Sma*I site of *E. coli* low-copy number plasmid pMW118 (Nippon Gene, Japan). After confirming the sequence, the plasmid was digested by *Hin*dIII and ligated with a 5.6-kb *Hin*dIII fragment containing the *B. longum* replicon and the spectinomycin resistance gene (Sp^R) derived from pBLES100 (Matsumura et al. 1997). The shuttle vector carrying *afcB* was constructed similarly. The *afcB* gene was amplified using the primer pair 5′-gctgaacatgaccggtgccggag and 5′-tgccggccaccagcaccgtggag. The *Sph*I site was used to insert the *B. longum ori* region and the Sp^R gene. The resulting plasmids were used to transform the α-L-fucosidase-negative strain *B. longum* 105-A.

The recombinant strains were anaerobically grown in 1 ml of GAM medium, harvested by centrifugation, and then washed with the same volume of basal medium consisting of 0.02% yeast extract, 1% peptone, 0.5% sodium acetate, 0.2% diammonium citrate, 0.02% magnesium sulfate, and 0.2% dipotassium phosphate. A small aliquot (50 μL) was withdrawn and incubated anaerobically for 3 h in the presence of 2′-FL, 3-FL or lacto-*N*-fucopentaose II.

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Conflict of interest statement

None declared.

Abbreviations

CBM, carbohydrate binding module; FIVAR, found-in-various-architectures; FL, fucosyllactose; GH, glycoside hydrolase; HMO, human milk oligosaccharide; PA, pyridylamino; *p*NP, *para*-nitrophenyl; TLC, thin layer chromatography.

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