Bifidobacterial α-galactosidase with unique carbohydrate-binding module specifically acts on blood group B antigen

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Bifidobacterium bifidum is one of the most frequently found bifidobacteria in the intestines of newborn infants. We previously reported that B. bifidum possesses unique metabolic pathways for O-linked glycans on gastrointestinal mucin (Yoshida E, Sakurama H, Kiyohara M, Nakajima M, Kitaoka M, Ashida H, Hirose J, Katayama T, Yamamoto K, Kumagai H. 2012. Bifidobacterium longum subsp. infantis uses two different β-galactosidases for selectively degrading type-1 and type-2 human milk oligosaccharides. Glycobiology, 22:361–368). The nonreducing termini of O-linked glycans on mucin are frequently covered with histo-blood group antigens. Here, we identified a gene agabb from B. bifidum JCM 1254, which encodes glycoside hydrolase (GH) family 110 α-galactosidase. AgAbB is a 1289-amino acid polypeptide containing a N-terminal signal sequence, a GH110 domain, a carbohydrate-binding module (CBM) 51 domain, a bacterial Ig-like (Btg) 2 domain and a C-terminal transmembrane region, in this order. The recombinant enzyme expressed in Escherichia coli hydrolyzed α1,3-linked Gal in branched blood group B antigen [Galα1-3(Fucα1-2Galβ1-R), but not in a linear xenotransplantation antigen (Galα1-3Galβ1-R)]. The enzyme also acted on group B human salivary mucin and erythrocytes. We also revealed that CBM51 specifically bound blood group B antigen using both isothermal titration calorimetry and a solid-phase binding assay, and it enhanced the affinity of the enzyme toward substrates with multivalent B antigens. We suggest that this enzyme plays an important role in degrading B antigens to acquire nutrients from mucin oligosaccharides in the gastrointestinal tracts.

Keywords: Bifidobacterium bifidum/blood group antigen/α-galactosidase/glycosidase/probiotics

Introduction

Bifidobacterium bifidum, the type species of the genus, is one of the most frequently found bifidobacteria in the intestines of newborn infants (Benno et al. 1984; Turroni et al. 2012). This bacterium also resides in the intestines of adults, although the population diminishes over time (Turroni et al. 2012). Bifidobacterium bifidum, as well as other bifidobacteria such as Bifidobacterium longum, Bifidobacterium breve and Bifidobacterium animalis lactis, gives a health benefit to the hosts, hence they are recognized and commercially used as probiotics. Bifidobacteria lower the intestinal pH to prevent the growth of harmful bacteria by producing lactic acid and acetic acid from sugars via a unique phosphoketolase-dependent hetero lactic acid fermentation (Suzuki et al. 2010). In addition, they stimulate host’s immune system to enhance anti-pathogenic and anti-carcinogenic activities; on the other hand, they control unwanted immune responses to reduce chronic inflammation and allergies (Picard et al. 2005; Trebichavsky et al. 2009). Since they mainly reside in the lower intestines where the sugars are highly limited, they possess various glycosidases to hydrolyze indigestible oligosaccharides and glycoconjugates. We previously reported that B. bifidum possesses unique metabolic pathways for free oligosaccharides in milk and O-linked glycans on gastrointestinal mucin (Katayama et al. 2004; Wada et al. 2008; Ashida et al. 2009; Miwa et al. 2010; Asakuma et al. 2011; Kiyohara et al. 2011). It is worth noting that this species expresses two distinct enzymes acting on the core structures in mucin O-glycans: glycoside hydrolase (GH) family 101 endo-α-N-acetylglactosaminidase specific for core 1 structure, also known as T-antigen (Galβ1-3GalNAcβ1-Ser/Thr) (Fujita et al. 2005; Ashida et al. 2008), and GH129 α-N-acetylglactosaminidase specific for Tn-antigen (GalNAcβ1-Ser/Thr) (Kiyohara et al. 2012). Whole genomic analysis also revealed that B. bifidum is highly adapted to acquire the nutrients from O-glycans in mucin by producing related glycosidases (Turroni et al. 2010).

The nonreducing termini of O-glycans in gastrointestinal mucin are usually covered with various histo-blood glyco-antigens, which confer resistance to digestive enzymes of general commensal bacteria. ABH blood group antigens are the well-known cell surface antigens on red blood cells (RBCs) and other somatic cells. ABH antigens are also
expressed as secreted glycoproteins including gastrointestinal mucin in humans with secretor phenotype. An H antigen consists of the basic disaccharide α1-2 fucosylgalactose (Fucα1-2Gal), and either N-acetylgalactosamine (GalNAc) or galactose (Gal) is bound to the Gal residue of the H antigen via α1-3 linkage to form A [GalNAcα1-3(Fucα1-2)Gal] or B [Galα1-3(Fucα1-2)Gal] antigen (Yamamoto et al. 1990). We previously identified GH95 1,2-α-fucosidase from *B. bifidum* JCM 1254, which specifically acts on the H antigen but not entirely on A and B antigens. Therefore, to degrade oligosaccharides with A and B antigens at nonreducing termini, prior processing of α1,3-linked GalNAc and Gal is required. To better understand the degradation and assimilation of the gastrointestinal mucin oligosaccharides by bifidobacteria, we screened A and B antigen-degrading bifidobacteria and found that only *B. bifidum* was capable of releasing Gal from the B antigen. We subsequently found a candidate gene from *B. bifidum* JCM 1254 encoding an α-galactosidase belonging to GH110 that was recently established in the carbohydrate-active enzymes (CAZy) database (Liu et al. 2007). The enzyme contained a carbohydrate-binding module (CBM) 51 domain, which is unique among previously reported GH110 enzymes. In this study, we characterized the hydrolytic specificity of the GH110 domain and binding specificity of the CBM51 domain using recombinant enzymes/proteins, and showed a synergistic effect on degradation of multivalent substrates. We also demonstrated that the recombinant enzyme efficiently converted group B RBCs to O RBCs that are universal for transfusion.

**Results**

**Presence of blood group B antigen-degrading α-galactosidase in *B. bifidum***

In order to test whether bifidobacteria possess abilities for degrading blood group A and B antigens, we incubated various bifidobacterial cells with either blood group A or B trisaccharide, and analyzed the products by TLC. No bifidobacterial strain tested was able to degrade A trisaccharide, but *B. bifidum* JCM 1254 and *B. bifidum* JCM 1255 (type strain) released Gal from B trisaccharide (Figure 1). However, *B. bifidum* JCM 7004 and the other bifidobacterial species could not readily hydrolyze it.

We searched the genome of *B. bifidum* JCM 1254, which was previously sequenced, and found a candidate gene encoding a putative GH110 α-galactosidase and named it *agabb* (accession number AB735681). GH110 was recently established in the CAZy database, in which a few bacterial enzymes were experimentally characterized as α-galactosidases specific for blood group B antigen and the xenotransplantation antigen Galα1-3Galβ1-R (Liu et al. 2007, 2008). The gene *agabb* consists of a 3870 bp open-reading frame with an unusual initiation codon GTG, and encodes a polypeptide with 1289 amino acids (aa) containing the following putative sequences/domains: an N-terminal signal sequence (aa 1–23), a GH110 domain (aa 30–600), a CBM 51 domain (aa 943–1095), a bacterial Ig-like (Big) 2 domain (aa 1103–1184) and a C-terminal transmembrane region (aa 1256–1283) (Figure 2A). The presence of an N-terminal signal sequence and a C-terminal transmembrane region indicates that an AgaBb is a membrane-anchored protein with a large extracellular region that includes the GH110 domain.

The substrate specificity and general properties of AgaBb

A DNA fragment of *agabb* lacking the sequences encoding the N-terminal signal peptide and the C-terminal transmembrane region was amplified by high-fidelity PCR and ligated into a pET23b(+) expression vector to produce C-terminal 6× His-tagged AgaBb. *Escherichia coli* BL21(DE3) transformed with pET23b/agabb was cultured, and AgaBb expression was induced with isopropyl β-D-thiogalactopyranoside (IPTG). The 6×His-tagged protein was purified from cell lysates using immobilized Ni²⁺ affinity chromatography and gel filtration. The purified protein migrated as a single protein band of 130 kDa on reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), which coincides with the calculated molecular mass (132,498 Da) (Figure 2B). By
gel filtration using Superdex 200 10/300 GE, the molecular weight of the native enzyme was estimated to be around 270 kDa (data not shown), suggesting that AgaBb is a homodimeric enzyme. To examine the enzyme activity of AgaBb, first we incubated the purified recombinant enzyme with various p-nitrophenyl (pNP)-monosaccharides, but all glycosides including pNP-α-Gal were resistant (data not shown). Next, we incubated the enzyme with various oligosaccharides containing α-linked Gal and analyzed the reaction products by TLC (Figure 2C). Gal was released only from blood group B trisaccharide. Interestingly, the same α1-3 galactosyl linkage in linear B-2 trisaccharide (Galα1-3Galβ1-4GlcNAc) and α1-3 galabiose (Galα1-3Gal) were not hydrolyzed at all. The other galactosyl linkages such as α1-2, α1-4 and α1-6 were also completely resistant. These results indicate that AgaBb strictly recognizes not only the α1-3 linked Gal but also α1-2 linked Fuc in the trisaccharide antigen. The general properties of AgaBb were determined using blood group B trisaccharide as the substrate (Supplementary data, Figure S1). The optimum pH and temperature were pH 6.0–6.5 and 30°C, respectively. The divalent cations, Ca2+, Mg2+ and Ni2+, did not affect enzyme activity at 5 mM concentration, whereas 5 mM Cu2+ reduced the activity to 30%. The $K_m$ and $k_{cat}$ values were estimated from the Hanes–Woolf plot as 1.8 mM and 6.4 × 10^{-2} s^{-1}, respectively.

**AgaBb acts on blood group B antigens on glycoconjugates**

Blood group B antigens exist in the nonreducing termini of glycan chains on both glycoproteins and glycolipids, but are hardly found in free glycans such as milk oligosaccharides. To test whether AgaBb acts on B antigens on glycoproteins, we used human salivary mucin as a substrate. The crude mucin samples from four volunteers who belong to group A, B, O and AB with secretor phenotype were incubated with the enzyme. Released Gal was detected in the supernatants of reaction mixtures including the group B and AB mucin samples by TLC analysis but not in those including the groups A and O mucin samples (Figure 3A).

Next, we investigated the enzymatic activity toward B antigens on cell surface glycoconjugates. ABH blood group antigens on red blood cells (RBCs) are found in O-glycans and N-glycans of membrane glycoproteins and also in glycosphingolipids (Clausen and Hakomori 1989). We treated the blood group B RBCs with AgaBb at 37°C in phosphate-buffered saline and hemagglutination was compared with blood group O RBCs using an anti-B monoclonal antibody and anti-H *Ulex* lectin. Agglutination tests were carried out by mixing RBCs with serially diluted antibody and lectin (Figure 3B and C). In both tests, AgaBb-treated B-RBCs behaved indistinguishably from O-RBCs. This result shows that AgaBb removed all cell surface B antigens, and that it may be a useful tool to make universally transfusible O-RBCs from B-RBCs.

**CBM51 contributes to enhance the affinity of AgaBb toward mucin substrate**

All GH110 enzymes previously reported are single-domain enzymes (Liu et al. 2007, 2008). Therefore, we focused on the function of the CBM51 domain in AgaBb. The members of CBM51 were found in several glycosidasases from bacteria, and among them only three proteins such as *Clostridium perfringens* (Cp) GH95-CBM51, GH98-CBM51 and *Streptococcus pneumoniae* (Sp) GH98-CBM51 were characterized (Gregg et al. 2008; Higgins et al. 2011). CpGH95-CBM51 was shown to bind mainly β-linked Gal residues, whereas CpGH98-CBM1 and SpGH98-CBM51 specifically bind blood group A and B antigens. However, functions of CBM51 in these glycosidase activities were not investigated.

First, we examined which types of mucin bound to the CBM51 in AgaBb. Catalytically inactivated AgaBb was prepared by replacing conserved acidic aa residues in GH110 domain to prevent degradation of B antigen during the binding assay. Four mutants with single point mutation, AgaBb-D328N, D351N, D352N and E551Q, completely lost activity toward B trisaccharide (data not shown), suggesting that two of the four are likely the catalytic base and acid residues. Salivary mucin samples of group A, B and O were dotted onto PVDF membrane, and then catalytically inactivated AgaBb-D328N was overlaid. After washing the membrane thoroughly, AgaBb-D328N was detected to bind to only group B mucin using an anti-His antibody (Figure 4A) while CBM51-deleted AgaBb-D328N (aa 23–701) did not. This result suggests that CBM51 in AgaBb specifically recognizes and binds B antigen.

Next, five CBM51 mutants were generated using both wild-type AgaBb and AgaBb-D328N as templates, by replacing putative important residues such as D962, H997, H1031, W1082 and H1085, based on an alignment with CpGH95-CBM51 and CpGH98-CBM51 (Supplementary data, Figure S2). Their binding abilities toward group B mucin were quantified by an ELISA-like 96-well plate assay using AgaBb-D328N-based mutants, and also the enzymatic activities toward B trisaccharide and group B mucin were measured using enzymatically
active mutants (Figure 4B). Three CBM51 mutants such as D962A, H997A and H1085A almost completely lost their binding activity toward group B mucin, but H1031A and W1082A retained activity to some extent. The enzymatic activities of the corresponding active forms toward group B mucin were strongly correlated with their binding activities. Interestingly, however, the hydrolysis activities for B trisaccharide were not affected by mutations in CBM51. This result suggests that CBM51 in AgaBb enhances the enzymatic activity toward multivalent B antigens like mucin.

Isothermal titration calorimetry
In order to determine sugar-binding specificity of AgaBb-CBM51 in detail, isothermal titration calorimetry (ITC) was employed. We replaced the GH110 domain of AgaBb with trigger factor (TF), a chaperone in E. coli, to eliminate the effect of the GH110 domain. TF-tagged CBM51 was expressed under low-temperature condition (15°C). The purified protein was filled in the reaction cell, and titrated with the following oligosaccharide solutions: blood group B trisaccharide, blood group A trisaccharide, α1-3 galabiose (Galα1-3Gal) and blood group H disaccharide (Fucα1-2Gal). The addition of blood group B trisaccharide (Figure 5A), but not the others (Figure 5B–D), resulted in substantial heat of binding, indicating that CBM51 specifically binds blood group B trisaccharide. These results confirmed that AgaBb-CBM51 strictly recognizes both α1-3-linked Gal and α1-2-linked Fuc in B antigen. The binding curve obtained for blood group B trisaccharide could be fitted, assuming a one-site binding model (Figure 5A, bottom panel). The curve could not be fitted into a two-site binding model. The derived thermodynamic values such as $K_a$, $\Delta H$, $\Delta S$ and $\Delta G$ for the experiment involving blood group B trisaccharide binding to TF-tagged CBM51 are summarized (Table I). The binding affinity of CBM51 for blood group B trisaccharide is approximately in the millimolar range. The binding process is enthalpically driven, and the entropy loss opposes binding.

Discussion
In this report, we identified the GH110 α-galactosidase from bifidobacteria for the first time. The GH110 family that is exclusively composed of α-galactosidases could be divided into two subfamilies based on their substrate specificities (Liu et al. 2008). Subfamily GH110a contains enzymes with a strict specificity for B antigen, whereas GH110b contains those with broad specificity for α1,3-linked Gal and also synthetic aryl α-galactoside. AgaBb turned out to belong to the former subfamily because it is strictly specific to B antigen.
Phylogenetic analysis also supports the subfamily classification (Figure 6). The GH110a domain of AgaBb appears to be related to the same Gram-positive actinobacterial enzymes from Streptomyces avermitilis (SaGH110a, CAJ33349) and Streptomyces griseoplanus (SgGH110a, CAJ90659), whose amino acid identities are 46 and 43%, respectively. Intestinal opportunistic pathogens, Bacteroides thetaiotaomicron (Bt) and Bacteroides fragilis (Bf) possess both GH110a and GH110b enzymes. BtGH110a (CAJ33352) and BfGH110a (CAJ09922) show 37 and 33% amino acid identities with AgaBb. Since the latter BtGH110b (CAJ33353) and BfGH110b (CAJ33351) act on a linear Galα1-3Gal structure that is a xenotransplantation antigen expressed in mammals other than humans and primates, the enzymes may contribute to the association of Bacteroides in animal intestines. On the other hand, B. bifidum possesses only GH110a AgaBb, which suggests the selective adaptation of the bifidobacterial species to the group B humans and primates. In humans, ABH antigens are frequently found on the terminus of the type 1 chain, whose building unit lacto-N-biose I (Galβ1-3GlcNAc) is one of the most effective endogenous bifidogenic factors (Kiyohara et al. 2009). Once ABH antigens are removed, lacto-N-biose I is sequentially released from type 1 chain by an extracellular lacto-N-biosidase (Wada et al. 2008), then incorporated by lacto-N-biose I transporter (Suzuki et al. 2008) and metabolized by an intracellular lacto-N-biose I phosphorylase (Kitaoaka et al. 2005). H antigen-degrading GH95 1,2-α-L-fucosidase is also common in infant-associated bifidobacteria such as B. breve, B. bifidum and B. longum infantis (Katayama et al. 2004). However, as far as we know, no bifidobacterial strain can degrade blood group A antigen, and only B. bifidum degrades B antigen. In fact, the cells of B. bifidum JCM 1254 released Gal, Fuc and other sugars from group B and O salivary mucin, but only a trace from group A mucin according to TLC analysis (data not shown).

Importantly, other bifidobacterial species released sugars from only group O mucin but not from group A and B salivary mucin, suggesting that AgaBb is critical for degrading mucin oligosaccharides from group B humans. Evolutionarily, the B and O alleles in humans are believed to have derived independently from the ancestral A allele by point mutations in α1,3-N-acetylgalactosaminyltransferase (Saitou and Yamamoto 1997). Dominant association of bifidobacteria to group B and O newborn infants reduces the mortality of infectious diseases in intestines and might be a driving force of increasing frequency of B and O alleles in humans. Very recently, the diversity and amount of bifidobacteria in the human intestine was reported to be considerably reduced in nonsecretor individuals defined by a mutation in the FUT2 gene (Wacklin et al. 2011). An older report documented that in vitro culture of enteric bacteria from B secretors produced greater levels of blood group B-degrading activity (Hoskins and Boulding 1976), suggesting the association of

| Table 1. Thermodynamic parameters for binding of blood group B trisaccharide to TF-tagged CBM51 at 30°C as determined by isothermal titration calorimetry |
|-------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| $K_a$ ($\times 10^3/M$) | $\Delta G^0$ (kcal/mol) | $\Delta H^0$ (kcal/mol) | $T\Delta S^0$ (kcal/mol) | $\Delta S^0$ (cal/mol/K) |
| 10.7 ± 2.9 | −5.6 ± 0.19 | −6.7 ± 0.88 | −1.1 ± 0.69 | −3.6 ± 2.2 |

Fig. 5. Isothermal titration calorimetry of CBM51. Binding specificity of CBM51 domain was analyzed by ITC. TF-tagged CBM51 was titrated by blood group B trisaccharide (A), blood group A trisaccharide (B), linear B-2 trisaccharide (Galα1-3Galβ1-4GlcNAc) (C) and blood group H disaccharide (Fucα1-2Gal) (D). TF only was titrated with blood group B trisaccharide (E). Upper panel of A and panels B–E, raw thermograms; bottom panel of A, binding isotherm.
B-degrading bacteria within human intestine. Thus, the relative frequency of AgaBb-expressing *B. bifidum* in B secretors should be studied.

Another unique feature of AgaBb is its multidomain structure. AgaBb is a C-terminally membrane-anchored extracellular enzyme containing two accessory domains such as CBM51 and Big2, which are located near the membrane. AgaBb-CBM51 was found to specifically bind B antigens but not A and H antigens, as demonstrated by ITC and solid-phase binding assay. The members of the CBM51 family are distributed throughout a wide variety of bacteria, and phylogenetically classified into six subfamilies tentatively (Gregg et al. 2008). Among them, only two in the CBM51a subfamily and one in the CBM51b subfamily have so far been characterized (Gregg et al. 2008; Higgins et al. 2011). CBM51a domains were found in GH98 andendo-β-galactosidase that is specific to blood group A and B antigens and releases A and H trisaccharides (Anderson et al. 2005). *Cp*GH98-CBM51a and *Sp*CBM51a were shown to bind both A and B trisaccharides by glycan microarray and crystal analyses. On the other hand, CBM51b found in *Cp*GH951.2-α-1-fucosidase was reported to have broad binding specificity toward mainly β-linked Gal and also other structures. Although AgaBb-CBM51 seems to be related to the CBM51a subfamily judged from the binding specificity, it shows rather higher sequence identity to the CBM51b subfamily: namely, the identities are 42% to *Cp*GH95-CBM51b (aa 900–1050, ABG82552), 36% to *Cp*GH98-CBM51a (aa 32–211, AAC84225) and 39% to *Sp*GH98-CBM51a (aa 65–233, EDK74349). Three residues such as D962, H997 and H1085 in AgaBb-CBM51 were identified to be essential for binding, but they are not completely aligned with these known members. Phylogenetic affiliation is also unclear, because there is no highly conserved motif or region. Thus, the molecular recognition mechanism of B antigen-specific AgaBb-CBM51 may be different from previously reported CBM51 domains and should be an interesting issue in the future. Recently, thermodynamic parameters for binding of blood group A and B antigens to CBM51a of *Sp*3GH98 from *S. pneumoniae* SP3-BS71 have been determined using ITC (Higgins et al. 2011). In our study, we investigated the binding specificity of AgaBb-CBM51 and determined thermodynamic parameters for binding of blood group B trisaccharide to the module. The association constant (*K*<sub>a</sub>) found for the binding of blood group B trisaccharide to AgaBb-CBM51 is seven times lower than that observed for the binding of group B tetrasaccharide derivative (Galα1-3(Fucα1-2)Galβ1-4GlcNAcβ1-(CH<sub>2</sub>)<sub>6</sub>C) to *Sp*CBM51-1. The reducing end GlcNAc residue of the tetrasaccharide is involved in the fixation of distal trisaccharide conformation. Therefore, the decreased *K*<sub>a</sub> of AgaBb-CBM51 might be due to the lack of this GlcNAc moiety in group B trisaccharide, although direct interaction between the GlcNAc residue and *Sp*CBM51-1 was absent in *Sp*CBM51-1/group B tetrasaccharide derivative complex structure.

The function of CBM domains has been mainly elucidated in polysaccharide-degrading glycosidases such as amylases, chitinases and cellulases (Shoseyov et al. 2006; Guillen et al. 2010). According to the previous studies, CBMs in such enzymes enhance the hydrolytic activity toward insoluble crystallized substrates. In contrast, those in glycosidases acting on glycoconjugates have been poorly investigated (Fujita et al. 2011). In this report, we showed AgaBb-CBM51 enhanced the catalytic activity toward multivalent mucin substrates but not toward monomeric B trisaccharide. Probably the presence of CBM51 could lower the *K*<sub>m</sub> value of AgaBb for mucin, although the value was not determined because of the difficulty of the molecular weight estimation of the mucin.

The enzymatic removal of blood group antigens to prepare universal RBCs for transfusion was a pioneering vision originally proposed about 30 years ago (Goldstein et al. 1982). Several α-galactosidases in GH27 and GH36 were investigated for this purpose, but they had problems of low pH optima, broad specificity and poor kinetic properties with the branched B antigen (Olsson et al. 2004). Recently discovered GH110 enzymes are expected to overcome these problems (Liu et al. 2007; Olsson and Clausen 2008). Newly identified AgaBb from the safe commensal bifidobacteria is inferred to have advantage acting on group B RBCs due to its unique CBM51 domain.
Materials and methods

Bacterial strains and culture

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

Genome sequence of B. bifidum JCM 1254

Draft sequencing of the genome of B. bifidum JCM 1254 was performed using a Genome Sequencer 20 System (Roche Applied Science, IN). The details will be reported elsewhere.

Cloning and expression of AgaBb in E. coli

To construct the AgaBb expression vector, a DNA fragment encoding aa 24–1255 (without an N-terminal signal peptide and C-terminal transmembrane region) was amplified by high-fidelity PCR with PrimeSTAR Max DNA Polymerase (Takara Bio, Japan) using genomic DNA from B. bifidum JCM 1254 as a template and the primers (AgaBb-F and AgaBb-R, Supplementary data, Table S1), digested with EcoRI and XhoI, and ligated into pET23b(+) . The nucleotide sequence was confirmed by sequencing. Escherichia coli BL21 (DE3) was transformed with pET23b/agabb and cultured in Luria–Bertani liquid medium containing 100 μg/mL ampicillin at 37°C until the optical density at 600 nm reached 0.5. Then, to induce expression, IPTG was added to the culture at a final concentration of 0.5 mM, and the cells were further cultured for 2 h at 37°C.

Purification of the recombinant AgaBb

After the 2 h induction, cells were harvested and lysed by BugBuster Protein Extraction Reagent (Novagen, Germany). After centrifugation, the supernatant was applied to a HisTrap HP column (1 mL, GE Healthcare, UK), 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 with an ÄKTA Explorer system (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 (Merck Millipore, MA).

Mutants of AgaBb

To construct various expression vectors, high-fidelity PrimeSTAR Max DNA Polymerase (Takara Bio) and the primers listed in Supplementary data, Table S1 were used for PCR. For point-mutated AgaBb expression vectors, PCR was performed using pET23b/agabb as a template. For AgaBb-D328N expression vector, a DNA fragment was amplified using pET23b/agabb (D328N) as a template and the primers ΔCBM51-F and ΔCBM51-R, digested with XhoI, and self-ligated. Transformation and expression were performed under the same conditions as the wild-type AgaBb. For TF-tagged CBM51 expression vector, a DNA fragment was amplified using pET23b/agabb as a template and the primers CBM51-F and CBM51-R, digested with BamHI and HindIII, and ligated into pCold TF (Takara Bio). Escherichia coli BL21 (DE3) was transformed with the plasmid and cultured in Luria–Bertani liquid medium containing 100 μg/mL ampicillin at 37°C until the optical density at 600 nm reached 0.5. Then, the culture was cooled at 15°C for 30 min and incubated for 24 h at 15°C after the addition of 0.5 mM IPTG.

Enzyme assay

Blood group B trisaccharide (Dextra Laboratories, UK) and crude salivary mucin prepared from human saliva by 75% ethanol precipitation were used as substrates for enzyme assay. Substrate was incubated with the enzyme at 37°C for an appropriate time in 50 mM sodium phosphate buffer (pH 6.0). The reaction products were separated by silica-gel TLC (Merek 5553, Germany) with 1-butanol:acetic acid:water (2:1:1, by volume) as developing solvent and visualized using diphenylamine–aniline–phosphoric acid (Anderson et al. 2000). Released Gal was quantified by the galactose dehydrogenase-coupled method (Miwa et al. 2010; Yoshida et al. 2012) after stopping the reaction by heating.

Hemagglutination test

Group B and O RBCs were collected by centrifugation, washed once with PBS, and resuspended in PBS to make a 2% suspension. AgaBb (20 pmol/mL) was added to the suspension and incubated at 37°C for 10 min. After the enzymatic reaction, the suspension is mixed with mouse anti-B monoclonal antibody (clone 5362B, Funakoshi, Japan) or Ulex europaeus agglutinin (UEA)-1 (Seikagaku Biobusiness, Japan) in a V-shape 96-well plate. Anti-B antibody and anti-H lectin were serially diluted twice from 0.125 to 0.25 mg/mL, respectively. After letting the plate stand for 1 h at room temperature, the agglutination was analyzed.

Dot-blot overlay assay

Protein concentration was measured using the BCA protein assay reagent (Thermo Scientific, IL). Salivary mucin was blotted onto a PVDF membrane, followed by blocking with 10% skim milk (Wako Pure Chemical Industries, Japan) and 0.05% Tween 20 in PBS. The membrane was then treated with 1 μM purified AgaBb-D328N or AgaBb-D328NΔCBM51 in 50 mM sodium phosphate (pH 6.0). After washing the membrane, subsequent detection was carried out by immunoblotting using a rabbit anti-His-tag polyclonal primary antibody (1/1000, MBL, Japan), and horseradish peroxidase-conjugated anti-rabbit IgG (1/5000, Santa Cruz Biotechnology, CA) as the secondary antibody. Detection was carried out using West Pico Chemiluminescent Kit (Thermo Scientific) and LAS Image Analyzer (Fuji Film, Japan).

ELISA-like assay

Crude salivary mucin from group B human (50 μg/mL) was dispensed in a 96-well plate and let stand for 1 h at room temperature to adsorb the proteins. After removing the solution, the plate was blocked as for the dot-blot assay. The primary and secondary antibodies were the same as for the dot-blot.
assay. Measurements were carried out by Powerscan HT (DS Parma Biomedical, Japan) at 415 nm using 2,2'azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) and H₂O₂ as substrates.

Isothermal titration calorimetry

ITC experiments were carried out using MicroCal iTC₂₀₀ (GE Healthcare). Proteins were concentrated to ~9.0 mg/mL using Amicon Ultra Centrifugal Filters 30K (Merck Millipore) and dialyzed extensively against 50 mM sodium phosphate buffer (pH 7.0). Buffer saved from the dialysis was used to dissolve sugar ligands. All solutions were filtered before use. Each ligand (1 mM) was titrated into the protein solution (68 μM TF-tagged CBM51 or 218 M TF) filling in the reaction cell under stirring (300 rpm) at 30°C.

Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Conflict of interest

None declared.

Abbreviations

aa, amino acids; Bf, Bacteroides fragilis; Bt, Bacteroides thetaiotaomicron; CAZY, carbohydrate-active enzymes; CBM, carbohydrate-binding module; Big, bacterial Ig-like; Cp, Clostridium perfringens; Fucα1-2Gal, α-1,2 fucosylgalactose; GH, glycoside hydrolase; IPTG, isopropyl β-D-thiogalactopyranoside; ITC, isothermal titration calorimetry; pNP, p-nitrophenyl; RBCs, red blood cells; Sp, Streptococcus pneumoniae; TF, trigger factor; UEA, Ulex europaeus agglutinin.

References


