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1,3-1,4-α-L-Fucosynthase That Specifically Introduces Lewis a/x Antigens into Type-1/2 Chains*[S]

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Background: Regiospecific installation of α-L-fucosyl residue into glycoconjugates is quite difficult.
Results: A glycosynthase mutant of 1,3-1,4-α-L-fucosidase specifically synthesized Lewis a/x trisaccharides using Galβ1–3/4GlcNAc as acceptors.
Conclusion: Structural studies provide a rationale to explain the unusually strict substrate specificity exhibited by the enzyme.
Significance: A new enzymatic route for specifically introducing Lewis a/x epitopes into type-1/2 chains becomes available.

α-L-Fucosyl residues attached at the non-reducing ends of glycoconjugates constitute histo-blood group antigens Lewis (Le) and ABO and play fundamental roles in various biological processes. Therefore, establishing a method for synthesizing the antigens is important for functional glycomics studies. However, regiospecific synthesis of glycosyl linkages, especially α-L-fucosyl linkages, is quite difficult to control both by chemists and enzymologists. Here, we generated an α-L-fucosynthase that specifically introduces Le^a and Le^x antigens into the type-1 and type-2 chains, respectively; *i.e.* the enzyme specifically accepts the disaccharide structures (Galβ1–3/4GlcNAc) at the non-reducing ends and attaches a Fuc residue via an α-(1,4/3)-linkage to the GlcNAc. X-ray crystallographic studies revealed the structural basis of this strict regio- and acceptor specificity, which includes the induced fit movement of the catalytically important residues, and the difference between the active site structures of 1,3-1,4-α-L-fucosidase (EC 3.2.1.111) and α-L-fucosidase (EC 3.2.1.51) in glycoside hydrolase family 29. The glycosynthase developed in this study should serve as a potentially powerful tool to specifically introduce the $Le^{a/x}$ epitopes onto labile glycoconjugates including glycoproteins. Mining glycosidases with strict specificity may represent the most efficient route to the specific synthesis of glycosidic bonds.

α-L-Fucosyl residues attached at the non-reducing ends of glycoconjugates constitute histo-blood group antigens Lewis (Le)[2] and ABO. The blood group antigens are involved in various important biological processes, and especially Lewis a and x epitopes (Galβ1–3/4(Fucα1–4/3)GlcNAc-; $Le^{a/x}$) play fundamental roles in mammalian cell-to-cell communications at the developmental stages and at the sites of inflammation (1–5). In mouse embryogenesis, the stage-specific synthesis of Le^x antigen has been observed, and this transient expression is thought to be involved in embryo compaction (2). Regulated expression of Le^x epitope is also found in the brain and postulated to be important for the development of the central nervous system. In addition, recent studies show that Le antigens modulate host-pathogen interactions (6, 7). *Helicobacter pylori* shows phase-variable expression of Le antigens in its lipopolysaccharides (LPS), and the antigens act as adhesion pedestals between the organism and the host epithelial cells. The bacterium also utilizes the Le^x epitope of LPS to suppress immune responses through the dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (8). The $Le^{a/x}$ antigen-containing oligosaccharides are therefore indispensable tools for functional glycomics studies and for pharmaceutical applications.

The synthesis of oligosaccharides with defined structures requires the precise control of regio- and stereospecificity at the glycosidic bond. In this context, enzymatic synthesis has several advantages over chemical synthesis because it enables the perfect control of anomeric configurations and provides relatively high regiospecificity without the requirement of laborious protection/deprotection steps. Enzymatic synthesis of oligosaccharides usually utilizes glycosyltransferases and glycosidases (9, 10). Glycosyltransferases generally have strict regio- and acceptor specificity and are therefore good catalysts for defined oligosaccharide syntheses. Efficient preparations of Le^x and H antigen oligosaccharides using α-1,3- and α-1,2-fucosyltransferases from *H. pylori* have been reported (11–13). However, as

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The atomic coordinates and structure factors (codes 3UES and 3UET) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).
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[2] The abbreviations used are: Le, Lewis; AfcA, 1,2-α-L-fucosidase; AfcB, 1,3-1,4-α-L-fucosidase; CAD, charged aerosol detector; DFJ, deoxyfuconojirimycin; FL, fucosyllactose; FucF, β-L-fucopyranosyl fluoride; GH, glycoside hydrolase; GlcGlcNAc, 2-acetamide-2-deoxy-4-*O*-(β-glucosyl)-glucose; LacNAc, *N*-acetyllactosamine; LNB, lacto-*N*-biose I; LNFP, lacto-*N*-fucopentaose; LNT, lacto-*N*-tetraose; *Bb*, *B. bifidum*; Lac, lactose; *Bi*, *B. longum* subsp. *infantis*; *p*NP-Fuc, *para*-nitrophenyl-α-L-fucopyranoside; EG, ethylene glycol; *Tm*, *T. maritima*; *Ss*, *S. solfataricus*.

described in the literature (11), the use of glycosyltransferases requires an expensive sugar nucleotide or a complex system for nucleotide recycling. Moreover, the expression of glycosyltransferases is generally difficult to handle. In contrast, glycosidase-catalyzed transglycosylation has been efficiently used as a method for oligosaccharide synthesis because of the simplicity and versatility of the reactions (9, 10). Notably, a new class of enzymes, glycosynthases, has been developed in the last decade (14–17). Glycosynthase is a mutant glycosidase that is devoid of hydrolysis but is able to perform the transglycosylation reaction when a suitably activated donor (generally glycosyl fluoride and in a few cases glycosyl azide) (18, 19) is used as a substrate. Use of glycosidases and their mutants has thus become a promising option for the synthesis of oligosaccharides (10, 20, 21). However, as the regioselectivity and acceptor specificity of glycosidase-catalyzed transglycosylation are usually not that high, the reaction products are frequently obtained as mixtures of several oligosaccharides with varied linkages and sometimes varied lengths. These drawbacks become apparent in the synthesis of fucosyl oligosaccharides using α-L-fucosidases (22–25) and α-L-fucosynthases (18) (details are described later) that belong to glycoside hydrolase family 29 (GH29) (26). Such a result prevents the use of these enzymes in the synthesis of oligosaccharides with defined structures. Thus, in view of the requirement of strict glycosidic bond formation, it is crucial to find glycosidases that provide strict specificity, both in linkage and in leaving groups that become acceptors for transglycosylation.

In previous studies, we have isolated two α-L-fucosidases from *Bifidobacterium bifidum* and revealed that the enzymes have strict substrate specificities. One is 1,2-α-L-fucosidase (*Bb*AfcA) that belongs to GH95 (27, 28), and the other is 1,3-1,4-α-L-fucosidase (*Bb*AfcB) belonging to GH29 (29). The high substrate specificities of the two α-L-fucosidases prompted us to examine the possible use of these enzymes in the defined synthesis of fucosyl oligosaccharides. In a recent study, we introduced the glycosynthase technology into *Bb*AfcA (an inverting enzyme) and succeeded in generating 1,2-α-L-fucosynthase, which synthesized 2′-fucosyllactose (Fucα1–2Galβ1–4Glc; 2′-FL) exclusively when β-L-fucopyranosyl fluoride (FucF) and lactose (Lac) were used as a donor and an acceptor, respectively (30). No by-products were formed in that reaction.

In the present study, to expand the possibility of glycosynthase technology, we converted *Bb*AfcB to a 1,3-1,4-α-L-fucosynthase and characterized the enzyme. The results indicated that the synthase should serve as a valuable tool to specifically introduce Le^a and Le^x epitopes into the type-1 (Galβ1-3GlcNAc; lacto-*N*-biose I (LNB)) and type-2 (Galβ1–4GlcNAc; *N*-acetyllactosamine (LacNAc)) chains, respectively. Moreover, the crystal structures of AfcB from *Bifidobacterium longum* subsp. *infantis* (*Bi*AfcB; Blon_2336), a paralogue of *Bb*AfcB, in complex with an inhibitor, deoxyfuconojirimycin (DFJ), and with lacto-*N*-fucopentaose II (Galβ1–3(Fucα1–4)GlcNAcβ1–3Galβ1–4Glc; LNFP II) (comprising the Le^a antigen), were determined. The structures revealed how the enzyme exerts its strict regio- and acceptor specificity through an induced fit motion of catalytically important residues.

EXPERIMENTAL PROCEDURES

Chemicals—2′-FL, LacNAc, Fucα1–6GlcNAc disaccharide, and Le^a and Le^x trisaccharides were purchased from Dextra Laboratories (Reading, UK). Lacto-*N*-tetraose (Galβ1–3GlcNAcβ1-3Galβ1–4Glc; LNT) and lactodifucotetraose (Fucα1–2Galβ1–4(Fucα1–3)Glc) were from Isosep (Tullinge, Sweden). LNFP II and Fucα1–3/4GlcNAc disaccharides were from Carbosynth (Compton, UK). *N*,*N*′-Diacetylchitobiose, DFJ, and *para*-nitrophenyl-α-L-fucopyranoside (*p*NP-Fuc) were from Seikagaku Kogyo (Tokyo, Japan), Toronto Research Chemicals (North York, Ontario, Canada), and Sigma, respectively. LNB (31), galacto-*N*-biose (Galβ1–3GalNAc) (32), 2-acetamide-2-deoxy-4-*O*-(β-glucosyl)-glucose (Glcβ1–4GlcNAc; GlcGlcNAc) (33), and FucF (30) were synthesized as described previously. 3-Fucosyllactose (Galβ1–4(Fucα1–3)Glc; 3-FL) was purchased from Dextra Laboratories and further purified using Bio-Gel P2 gel filtration chromatography (Bio-Rad). L-Fucose dehydrogenase was purchased from Kikkoman (Noda, Japan). Other reagents of analytical grade were obtained from commercial sources.

Construction of BbAfcB Mutants—The mutants of *Bb*AfcB (D703A, D703C, D703G, D703S, W742A, E746A, D763A, D766A, D778A, and D807A) were constructed using the QuikChange site-directed mutagenesis method (Stratagene) with the plasmid pET23b-*afcB* as the template (29). The following primers and their complementary primers were used: 5′-gaggtctggttcgcgggtgcccaaggc-3′ (D703A), 5′-aggtctggttctgcggtgcccaaggc-3′ (D703C), 5′-gaggtctggttcggcggtgcccaaggc-3′ (D703G), 5′-gaggtctggttcagcggtgcccaaggc-3′ (D703S), 5′-acgatgcccgagcggtgggcaacg-3′ (W742A), 5′-ggtgggcaacgcggacggctggg-3′ (E746A), 5′-ggcatacaacgccggcgtggaca-3′ (D763A), 5′-cgacggcgtggccaaggtgtcgc-3′ (D766A), 5′-gatggcccccgccggtaagcttg-3′ (D778A), and 5′-ggccgaagtcgccgccaagaacc-3′ (D807A). The entire sequence used for later manipulation was sequenced to check that no base change other than those designed had occurred. The resulting plasmids were used to transform *Escherichia coli* BL21 Δ*lacZ* (DE3) (29).

Expression and Purification of BbAfcB Variants—The recombinant strains were cultured in Luria-Bertani medium containing 100 μg/ml ampicillin at 18 °C until the optical density at 600 nm reached 0.5. Isopropyl β-D-thiogalactopyranoside was then added to a final concentration of 0.1 mM to induce protein expression. Following further incubation for 15 h, the cells were harvested and disrupted by sonication. After centrifugation, the supernatant was applied to a nickel-nitrilotriacetic acid-agarose column (Qiagen, Hilden, Germany), and the protein was eluted according to the manufacturer's instructions. The fractions were combined, concentrated using Amicon Ultra 50K (Millipore), and loaded onto a Superdex 200 10/300 GL gel filtration column (GE Healthcare). The elution was carried out using 20 mM Tris-HCl (pH 8.0) containing 150 mM NaCl. The purified protein was extensively dialyzed against a 50 mM HEPES buffer (pH 7.0). Protein concentrations were determined using a BCA protein assay kit (Thermo Scientific) with bovine serum albumin as a standard.

Enzyme Assay—The hydrolytic activities of *Bb*AfcB variants were determined using 3-FL and *p*NP-Fuc as the substrates. The reaction mixture contained 100 mM MOPS buffer (pH 6.5),

1 mM substrate, and the enzyme (for 3-FL hydrolysis: 14 nM wild-type (WT) and D766A, 12 μM Asp-703 mutants, 17 μM W742A and E746A, 44 nM D763A and D778A, and 890 nM D807A; for *p*NP-Fuc hydrolysis: 17 μM WT and mutants). The reaction was carried out for an appropriate time in which the linearity of the reaction rate was observed at 30 °C (*i.e.* in the 3-FL hydrolysis: ~15 min for WT, W742A, D807A, D763A, D766A, and D778A mutants; ~880 min for Asp-703 mutants; and ~240 min for E746A mutant; in the *p*NP-Fuc hydrolysis: ~150 min for WT and all mutants except for W742A and ~500 min for W742A). The reaction was terminated by heating (for 3-FL hydrolysis) or by the addition of 1 M sodium carbonate (for *p*NP-Fuc hydrolysis). The amount of released Fuc was determined using a fucose dehydrogenase-coupled method (34). The amount of the liberated *p*NP was determined by measuring the absorbance at 405 nm. One unit of enzyme activity was determined as the amount of enzyme required to produce 1 μmol of Fuc or *p*NP under the specified conditions.

The glycosynthase activities of *Bb*AfcB mutants were examined by incubating the reaction mixtures (50 μl) containing 100 mM MOPS buffer (pH 7.0), 20 mM FucF (donor), 100 mM LNB/LacNAc (acceptor), and a 17 μM concentration of the enzymes. The reaction was performed for 10 min at 30 °C and terminated by adding 5 μl of 30% trichloroacetic acid. The reaction products were analyzed by high-performance liquid chromatography (HPLC) equipped with a sugar-D column (4.6 × 250 mm; Nacalai Tesque, Kyoto, Japan). The elution was performed under a constant flow (1.0 ml/min) of 75 or 78% acetonitrile at 40 °C and monitored at 214 nm or using a charged aerosol detector (CAD) (Corona CAD, Thermo Scientific). The amounts of the reaction products were estimated by a standard curve created using known concentrations of Le^a and Le^x trisaccharides.

The optimal pH for the glycosynthase reaction was determined using the *Bb*AfcB D703S mutant. The buffers (100 mM) used were as follows: citrate-NaOH (pH 4.0–5.0), MES (pH 5.0–6.5), and MOPS (pH 6.5–8.0). The acceptor specificity of the *Bb*AfcB D703S mutant was examined using 13 different substrates. The reaction was carried out in 100 mM MES buffer (pH 5.0) containing 40 mM FucF and a 100 mM concentration of each acceptor for 40 min at 30 °C in the presence and absence of 17 μM D703S. The reaction products were analyzed by HPLC-CAD.

Synthesis, Purification, and Structural Analysis of Reaction Products—For Le^a synthesis, the *Bb*AfcB D703S mutant (17 μM) was incubated in the reaction mixture (300 μl) consisting of 100 mM MES buffer (pH 5.0), 40 mM FucF (donor), and 200 mM LNB (acceptor) at 30 °C for 40 min. Le^x trisaccharide and LNFP II were synthesized in a similar manner except that 100 mM LacNAc and LNT were used as the acceptors, respectively. The reaction products were deionized with Amberlite MB-3, lyophilized, and purified using HPLC equipped with a sugar-D column. The elution was performed as described above. Peak fractions were collected, lyophilized, and further purified by a TSKgel ODS-80TS column (4.6 × 250 mm; Tosoh, Japan) to remove the remaining acceptors (LNB/LacNAc/LNT). The elution was carried out using water at a flow rate of 0.5 ml/min and monitored by a refractive index detector (RID-10A, Shimadzu, Kyoto, Japan). ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 500 spectrometer. Electrospray ionization-mass spectrometry (MS) was carried out in positive mode using a Bruker APEX II 70e Fourier transformed ion-cyclotron resonance mass spectrometer.

X-ray Crystallography of BiAfcB—The gene (locus tag Blon_2336) was amplified by PCR using the genomic DNA of *B. longum* subsp. *infantis* ATCC15697 and a primer pair (5′-gcatatgaacaatcctgcagatgc-3′ and 5′-ctcgagtcagatgcgcacggcagcc-3′). The amplified fragment was inserted into the NdeI and XhoI sites of pET-30b to generate a non-tagged protein (Novagen). The resulting plasmid, pET30b-*BiafcB*, was used to transform *E. coli* BL21 Δ*lacZ* (DE3). The transformants were cultured in Luria-Bertani medium containing 30 μg/ml kanamycin at 18 °C to an optical density at 600 nm of 0.5. Isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM to induce protein expression. Following further incubation for 20 h, the harvested cells were disrupted by sonication. The protein was purified using Mono Q 5/50 GL column chromatography (GE Healthcare) followed by a Superdex 200 10/300 GL gel filtration chromatography step. The *Bi*AfcB D172A/E217A double mutant was constructed using the QuikChange site-directed mutagenesis method with the plasmid pET30b-*BiafcB* as the template. The following primers and their complementary primers were used: 5′-ccgtctggcttgctggcgccaatgg-3′ (D172A) and 5′-tgggccgggaacgcagccgggcatgtg-3′ (E217A). Purification of the mutant protein was performed using a procedure similar to that described for the WT protein. The proteins were crystallized using the hanging drop-vapor diffusion method at 20 °C. The crystal of *Bi*AfcB complexed with DFJ and ethylene glycol (WT-DFJ-EG) was obtained by mixing 1 μl of a protein solution (10 mg/ml in 10 mM HEPES buffer (pH 7.0) containing 40 mM DFJ) with 1 μl of a reservoir solution consisting of 0.1 M citrate buffer (pH 4.0) and 20% (w/v) polyethylene glycol (PEG) 6000. The crystal of *Bi*AfcB D172A/E217A complexed with LNFP II (D172A/E217A-LNFP II) was obtained by mixing 1 μl of a protein solution (10 mg/ml in 10 mM HEPES buffer (pH 7.0) containing 100 mM LNFP II) with 1 μl of a reservoir solution consisting of 0.1 M sodium citrate buffer (pH 5.5) and 20% PEG 3000. After cryoprotection with 20% ethylene glycol, the crystals were flash cooled in a nitrogen stream at 100 K. Diffraction data were collected using beamline NW12A at the Photon Factory-Advanced Ring, KEK, Tsukuba (λ = 1.0 Å). Diffraction images were processed using HKL2000 (35). Molecular replacement was performed with MOLREP (36) using the ligand-free *Bi*AfcB structure (Protein Data Bank code 3MO4) (37) as a search model. Model rebuilding and refinement were performed using Coot (38) and Refmac5 (39). Figures were prepared using PyMOL (Schrödinger, LLC).

Automated Docking—Automated docking analysis was performed as described previously (40). Ligand model preparation and automated docking were performed using the Sweet2 server (41) and AUTODOCK 4.0 (42), respectively. Rotatable ligand bonds (eight in Le^x trisaccharide) were defined using the AutoDockTools interface. Water molecules in the D172A/E217A-LNFP II complex structure were removed. After adding polar hydrogens, Gasteiger charges were calculated for the

ligand and protein. Grid maps were prepared with $40 \times 40 \times 40$ points covering the substrate-binding pocket with a point spacing of 0.375 Å. For the Lamarckian genetic algorithm search, the size of the initial random population was 150 individuals, the maximal number of energy evaluations was 2.5×10^6, the maximal number of generations was 27,000, the number of top individuals that survived into the next generation was 1, the rate of mutation was 0.02, the rate of crossover was 0.80, and the average of the worst energy was calculated over a window of 10 generations. After 256 docking runs, all structures generated for a single compound were assigned to clusters based on a tolerance of 2.0 Å for all atom root mean square deviations from the lowest energy structure.

RESULTS

*Conversion of 1,3-1,4-α-L-Fucosidase to 1,3-1,4-α-L-Fucosynthase—Bb*AfcB belongs to GH29 (26). The members of this family hydrolyze α-L-fucosidic bonds via a retaining mechanism (43, 44). We replaced Asp-703, which is assumed to be a nucleophile by sequence alignment with well studied GH29 members (discussed later) (45), with alanine, cysteine, glycine, and serine and examined the hydrolytic activity of these mutants. Note that only the specific activities were determined because the kinetic parameters could not be calculated (supplemental Table S1). The specific activities of D703A, D703C, D703G, and D703S were found to decrease by 160,000-, 46,000-, 17,000-, and 76,000-fold, respectively, as compared with that of the WT enzyme.

The glycosynthase activities of these mutants were then examined using 20 mM FucF and 100 mM LNB or LacNAc as the donor and the acceptor, respectively. Peaks corresponding to Le^a and Le^x trisaccharides appeared for the D703G/S mutants in the HPLC analysis of the reaction products, whereas no peak was observed for WT and the D703A/C mutants (supplemental Fig. S1). In either case, D703S gave a slightly larger peak area than D703G, and hence, D703S was further analyzed.

The optimal pH for the synthase reaction was found to be 5.0 (data not shown). The synthase activity was observed only when the reaction was carried out in the presence of FucF, LNB/LacNAc, and the enzyme. No peaks other than the added substrates were detected when the donor (FucF), the acceptor (LNB/LacNAc), or the enzyme was omitted (Fig. 1, *a* and *b*). Strikingly, no by-products other than the decomposed Fuc were detected in the reaction as revealed by HPLC-CAD. Identification of the reaction products is described later.

The kinetic parameters of the reaction could not be calculated because of the rapid spontaneous decomposition of FucF. The half-life of FucF under the reaction conditions was determined to be 20 min (data not shown). In the time course of the optimized reaction, the concentrations of the products Le^a and Le^x reached a maximum at ~40 and 60 min, respectively (supplemental Fig. S2). The reaction efficiency was estimated to be 56% against the added FucF when the reaction was carried out under the optimized conditions, *i.e.* in the presence of 40 mM FucF (donor), 200 mM LNB or 100 mM LacNAc (acceptor), and a 17 μM concentration of the enzyme. None of the *Bb*AfcB Asp-703 variants used β-L-fucopyranosyl azide as a donor (18) or were rescued by sodium azide (data not shown).

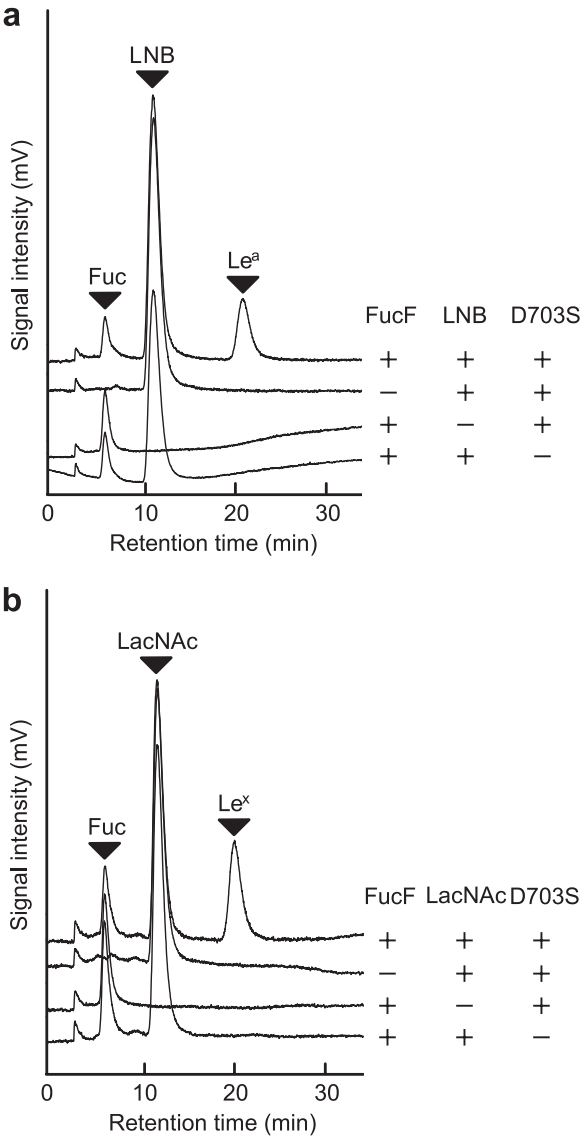

FIGURE 1. **Specific syntheses of Le^a (*a*) and Le^x (*b*) trisaccharides by the *Bb*AfcB D703S mutant.** *a*, the reaction was carried out for 40 min at 30 °C in 100 mM MES buffer (pH 5.0) containing 40 mM FucF (donor), 200 mM LNB (acceptor), and a 17 μM concentration of the *Bb*AfcB D703S mutant. For the control experiments, the substrate or enzyme was omitted from the reaction. *b*, for the synthesis of Le^x, 100 mM LacNAc was used as the acceptor. The reaction products were analyzed by HPLC-CAD. The peaks of L-fucose (*Fuc*), LNB, LacNAc, Le^a, and Le^x are shown. Note that FucF is decomposed when terminating the reaction by trichloroacetic acid.

Acceptor Specificity—The acceptor specificity of *Bb*AfcB D703S was examined using various mono- and oligosaccharides at a fixed concentration (100 mM) (Table 1, Fig. 2, and supplemental Fig. S3). In addition to LNB and LacNAc, the enzyme recognized Lac, 2′-FL, and LNT as acceptors and specifically produced 3-FL, lactodifucotetraose, and LNFP II in yields of 13, 5.5, and 41% against the added FucF, respectively. In contrast, the enzyme did not accept monosaccharides (Glc, Gal, GlcNAc, and GalNAc), cellobiose, GlcGlcNAc, *N*,*N*′-diacetylchitobiose, or galacto-*N*-biose as a substrate.

Regio- and Stereospecificity—We then analyzed the reaction products eluted from HPLC using electrospray ionization-MS and ^1H and ^{13}C NMR spectroscopy. The MS spectra indicated that one fucosyl residue was attached to LNB and LacNAc (cal-

TABLE 1

Acceptor specificity of 1,3-1,4-α-L-fucosynthase (*Bb*AfcB D703S)

The reaction was carried out in 100 mM MES buffer (pH 5.0) containing 40 mM FucF (donor) and 100 mM of each acceptor for 40 min at 30 °C in the presence and absence of 17 μM D703S. The reaction products were analyzed by HPLC-CAD (Fig. 2 and supplemental Fig. S3).

Substrates		Synthesized compounds		
Name	Structure	Name	Structure	Yield[a]
				%
Monosaccharides				
Glucose				0
Galactose				0
N-Acetylglucosamine				0
N-Acetylgalactosamine				0
Disaccharides				
Cellobiose	Glcβ1–4Glc			0
GlcGlcNAc[b]	Glcβ1–4GlcNAc			0
N,N′-Diacetylchitobiose	GlcNAcβ1–4GlcNAc			0
Lac	Galβ1–4Glc	3-FL	Galβ1–4 (Fucα1–3)Glc	13
LNB[b]	Galβ1–3GlcNAc	Le[a] trisaccharide	Galβ1–3 (Fucα1–4)GlcNAc	47
LacNAc	Galβ1–4GlcNAc	Le[x] trisaccharide	Galβ1–4 (Fucα1–3)GlcNAc	55
Galacto-*N*-biose[b]	Galβ1–3GalNAc			0
Tri- and tetrasaccharides				
2′-FL	Fucα1–2Galβ1–4Glc	Lactodifucotetraose	Fucα1–2Galβ1–4 (Fucα1–3)Glc	5.5
LNT	Galβ1–3GlcNAcβ1–3Galβ1–4Glc	LNFP II	Galβ1–3 (Fucα1–4)GlcNAcβ1–3Galβ1–4Glc	41

[a] The product yield against the added FucF.
[b] These substrates were prepared as described previously (31–33).

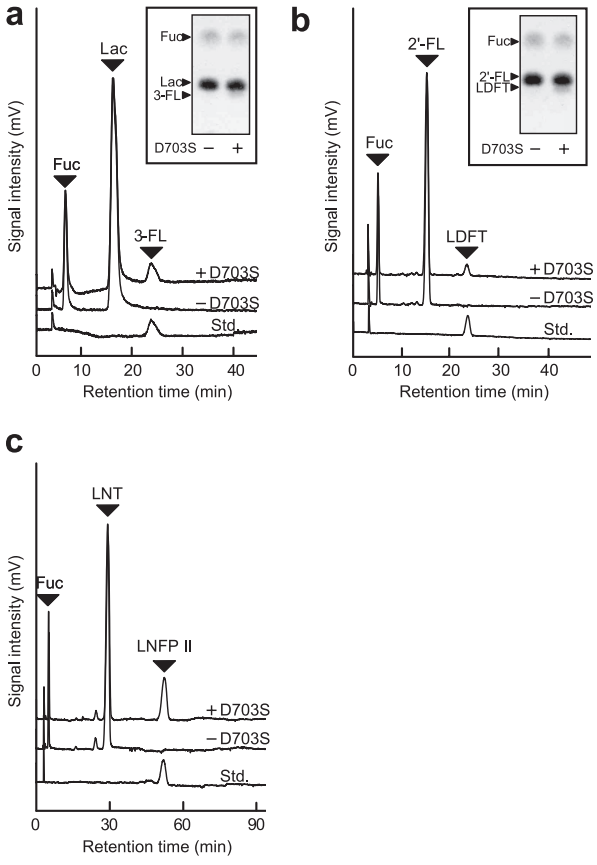

FIGURE 2. **Acceptor specificity of *Bb*AfcB D703S glycosynthase.** The reaction was carried out in 100 mM MES buffer (pH 5.0) containing 40 mM FucF and a 100 mM concentration of each acceptor for 40 min at 30 °C in the presence and absence of the D703S mutant (17 μM). The acceptors used were as follows: Lac (*a*), 2′-FL (*b*), and LNT (*c*). The reaction products were analyzed by HPLC-CAD. The peaks of L-fucose (*Fuc*), acceptor, and standard sugars (*Std.*) are indicated. *Inset* in *a* and *b* are the results of the thin-layer chromatography (TLC) analysis of the reaction products. See also Table 1 and supplemental Figs. S3–S7.

culated for the sodium adduct $[M + Na]^+$, 552.19; observed, 552.18; supplemental Fig. S4). Both of the ^1H and ^{13}C NMR spectra were identical to the spectra of the authentic Le^a and Le^x trisaccharides (supplemental Figs. S5 and S6). In the case where LNT was used as an acceptor, only LNFP II, but not LNFP V (Galβ1–3GlcNAcβ1–3Galβ1–4(Fucα1–3)Glc) or lacto-*N*-difucohexaose II (Galβ1–3(Fucα1–4)GlcNAcβ1-3Galβ1–4(Fucα1–3)Glc), was synthesized as revealed by ^1H NMR analysis (supplemental Fig. S7).

Crystallography of 1,3-1,4-α-L-Fucosidase—To elucidate the structural basis underlying the unique substrate specificity of this synthase, we tried to crystallize *Bb*AfcB and its variants; however, no crystal was obtained. *B. longum* subsp. *infantis* possesses the paralogue (locus_tag 2336) of *Bb*AfcB (46), and we found that this paralogue (referred to as *Bi*AfcB) has a substrate specificity identical to that of *Bb*AfcB (supplemental Fig. S8). The Pfam database suggested that *Bb*AfcB is a multimodular enzyme, whereas *Bi*AfcB possesses a simple domain organization. The structure of a ligand-free form of *Bi*AfcB has been recently determined (Protein Data Bank code 3MO4) (37). We obtained the crystals of *Bi*AfcB under different conditions from those used by Sela *et al.* (37) and determined two complex structures of *Bi*AfcB: WT complexed with DFJ and EG (WT-DFJ-EG; 1.6-Å resolution) and an inactive mutant complexed with LNFP II (D172A/E217A-LNFP II; 2.1-Å resolution). Asp-172 and Glu-217 are a nucleophile and a general acid/base, respectively (described later). Data collection and refinement statistics are shown in Table 2. *Bi*AfcB forms a dimer in solution (data not shown), and all three *Bi*AfcB crystals contain homodimers in the asymmetric unit. Because the two chains (A and B) were almost identical in every case, we describe the chain A of each structure. The DFJ and ethylene glycol (cryoprotectant) molecules in the WT-DFJ-EG structure and the Fuc and Gal moieties in the D172A/E217A-LNFP II structure were clearly observed (supplemental Fig. S9). However, the GlcNAc moiety in LNFP II was partially ambiguous, and the β1,3-linked Lac moiety at the reducing end was not visible. Therefore, we included the Le^a trisaccharide structure (Galβ1–3(Fucα1-4)GlcNAc) in the model. We also determined a complex structure of D172A/E217A with the Le^a trisaccharide, and the result-

TABLE 2

Crystallographic data collection and refinement statistics

r.m.s.d., root mean square deviation. Values in parentheses are for the highest-resolution shell.

Data set	WT-DFJ-EG	D172A/E217A-LNFP II
Data collection		
Protein Data Bank code	3UES	3UET
Beamline	NW12A	NW12A
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit cell (Å)	$a = 64.0$, $b = 120.6$, $c = 142.5$	$a = 82.6$, $b = 106.0$, $c = 120.7$
Matthews coefficient (Å^3/Da)	2.59	2.49
Solvent content (%)	52.5	50.6
Resolution (Å)	50.00-1.60 (1.66-1.60)	50.00-2.10 (2.14-2.10)
Total reflections	1,029,177	358,658
Unique reflections	146,170	60,403
Completeness (%)	99.9 (100.0)	96.4 (95.4)
Redundancy	7.0 (6.7)	5.9 (5.2)
Mean $I/\sigma(I)$	36.2 (4.1)	16.6 (2.6)
R_{sym} (%)	5.9 (33.5)	10.4 (47.4)
Refinement		
Resolution (Å)	25.91-1.60	32.05-2.10
No. of reflections	138,464	57,112
R-factor/R_{free} (%)	16.0/19.1	17.3/22.3
No. of atoms	8,320	7,683
r.m.s.d. from ideal values		
Bond lengths (Å)	0.029	0.020
Bond angles (°)	2.561	1.987
Ramachandran plot (%)		
Favored	98.0	97.1
Allowed	1.8	2.7
Outlier	0.2	0.2

ing electron density map was virtually identical to that of the D172A/E217A-LNFP II structure (data not shown).

Fig. 3 shows the comparison between the three *Bi*AfcB structures. Interestingly, upon ligand binding, *Bi*AfcB undergoes a large conformational change at two loop regions (173–182 and 215–220) (Fig. 3*a*), and to our surprise, the acid/base catalyst Glu-217 is included in this induced fit motion. In the substrate-free structure, the catalytic residues (Asp-172 and Glu-217) are not appropriately poised (separated by 20.8 Å), and a tyrosine molecule occupies subsite −1 (Fuc-binding site) (Fig. 3*b*). In the WT-DFJ-EG structure, the loop (215–220) covers the catalytic pocket so that Glu-217 can be suitably poised to act as an acid/base. Consequently, the distance between the Oδ atom of Asp-172 and the Oϵ atom of Glu-217 becomes 5.8 Å, which is the typical length observed in retaining glycosidases (43). The Oδ atom of Asp-172 is located at a distance of 3.1 Å from C1 of the DFJ. Alanine replacement of each of these residues resulted in a ~20,000-fold decrease of the hydrolytic activity (data not shown). The DFJ and ethylene glycol molecules are tightly bound by many hydrogen bonds with the protein. In the D172A/E217A-LNFP II structure, Fuc and Gal moieties of LNFP II are extensively recognized through the formation of hydrogen bonds, whereas the GlcNAc moiety does not make any notable interactions with the protein. The Fuc makes direct hydrogen bonds with His-36, Trp-47, His-85, His-86, and Tyr-131, and its C6 methyl group makes hydrophobic interactions with Phe-34, Trp-170, and Trp-290. The Gal moiety forms hydrogen bonds with the nitrogen atom of the main chain of Gly-173 and the side chains of Glu-237 and Asp-283, and its hydrophobic β-face is stacked by Trp-213 (Fig. 3*c*). The ethylene glycol in the WT-DFJ-EG complex occupies the Gal-binding site and interacts with the catalytic residues (Asp-172 and Glu-217) and the main chain of Gly-173. A water-mediated hydrogen bond is also formed with the side chain of Glu-237. However, its two hydroxyl groups do not overlap any of the Gal hydroxyl groups (Fig. 3, *b* and *c*).

Sequence identity between *Bb*AfcB and *Bi*AfcB is modest (34%) in the alignment (495–998 for *Bb*AfcB and 4–472 for *Bi*AfcB); however, the residues involved in substrate binding are essentially conserved to reflect the same substrate specificity (supplemental Fig. S10). Asp-172, Gly-173, Trp-213, Glu-217, and Asp-283 of *Bi*AfcB correspond to Asp-703, Gly-704, Trp-742, Glu-746, and Asp-807 in *Bb*AfcB, respectively. *Bb*AfcB does not have a residue corresponding to Glu-237 of *Bi*AfcB located in the disordered loop (236–254), but instead, three acid residues (Asp-763, Asp-766, and Asp-778) are present in the corresponding region. The specific activities of W742A, E746A, and D807A of *Bb*AfcB for the hydrolysis of 3-FL decreased by 1,800-, 6,900-, and 80-fold, respectively, as compared with that of the WT enzyme (supplemental Table S1). Alanine substitution for Asp-763, Asp-766, and Asp-778 slightly lowered the enzyme activity.

DISCUSSION

A powerful tool to regiospecifically and stereospecifically install Fuc residues into glycoconjugates was developed. The *Bb*AfcB D703S mutant can introduce Le^a and Le^x epitopes into type-1 and type-2 chains at the non-reducing ends, respectively, without producing by-products. The yields were ~60% with respect to the added FucF. The reaction efficiency of this glycosynthase may be slightly lower than those of typical glycosynthases (80% or more), and this could be due to the spontaneous decomposition of FucF during the reaction as described above. Recently, Cobucci-Ponzano *et al.* (18, 19) demonstrated that some glycosynthases can accept glycosyl azide as a donor molecule. This finding is particularly valuable for oligosaccharide synthesis using α-glycosynthases because β-glycosyl azides are considerably more stable than β-glycosyl fluorides in aqueous solution. We examined the usage of β-L-fucopyranosyl azide in the synthase reaction of *Bb*AfcB mutants, but no product was obtained in that reaction.

The glycosynthase exclusively recognized LNB, LacNAc, and Lac disaccharide structures (Galβ1–3/4GlcNAc(Glc)) at the non-reducing ends and did not recognize monosaccharides. Indeed, WT *Bb*AfcB did not hydrolyze Fucα1–3/4GlcNAc disaccharides (supplemental Fig. S8), which had not been examined in our previous study (29). These results indicate that the presence of the branched Gal residue is critical for the catalytic activities of *Bb*AfcB and the glycosynthase mutant.

Structural Basis for Substrate Specificity of 1,3-1,4-α-L-Fucosynthase—*Bi*AfcB was identified as a paralogue of *Bb*AfcB (46), and it showed the same substrate specificity as *Bb*AfcB (supplemental Fig. S8). Structural analysis of *Bi*AfcB enabled us to consider the mechanism of the unique enzymatic activity of 1,3-1,4-α-L-fucosidase(synthase). Binding of Fuc(F) and the Gal moiety of LNB/LacNAc would cause the induced fit movement at the catalytic site. The ethylene glycol molecule in the WT-DFJ-EG complex may stabilize the induced fit structure in the

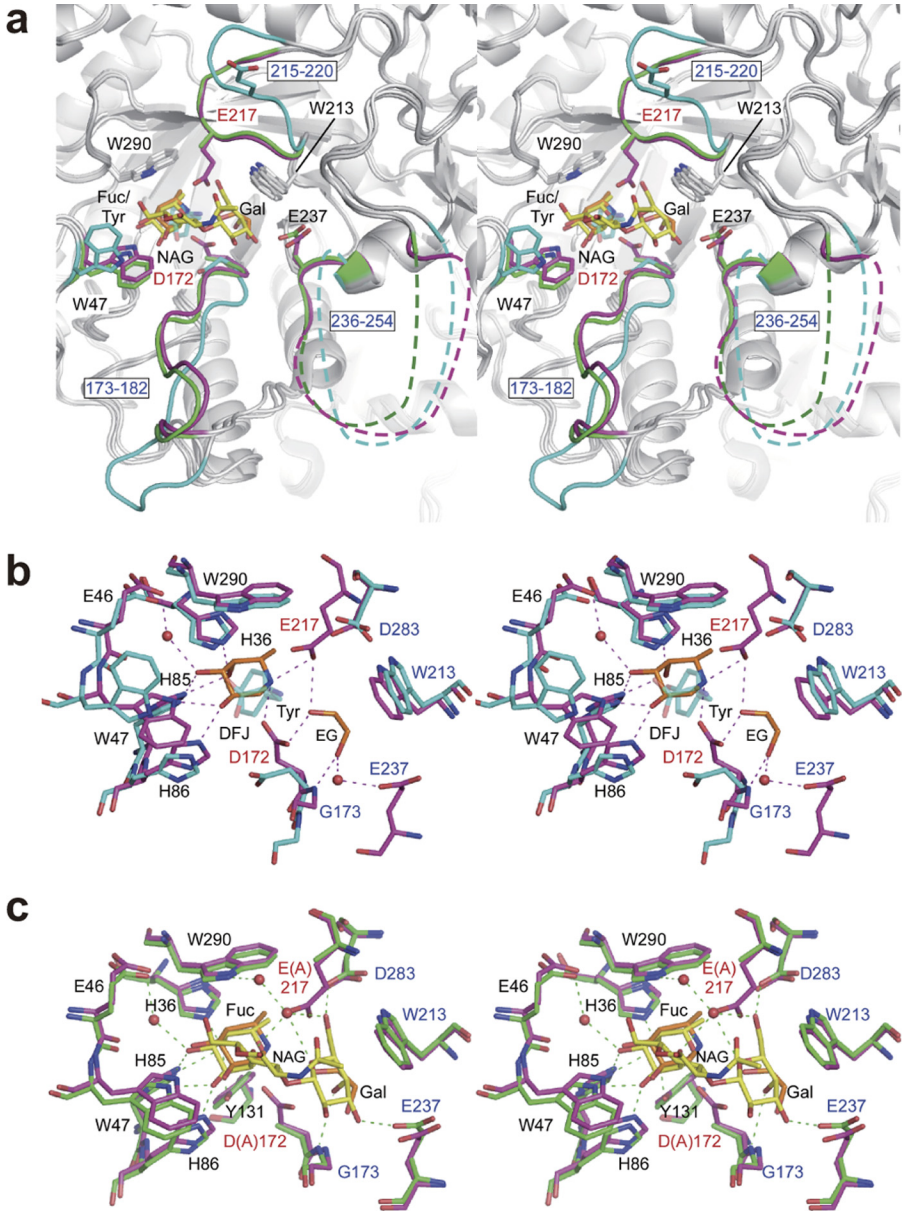

FIGURE 3. **Induced fit movement of *Bi*AfcB upon substrate binding (stereoviews).** *a*, superimposed structures of the ligand-free form (*cyan*; Protein Data Bank code 3MO4) (37), WT-DFJ-EG complex (protein in *magenta*; ligand in *orange*), and D172A/E217A-LNFP II complex (protein in *green*; ligand in *yellow*). The ligand-free structure contains a tyrosine molecule at the Fuc-binding site (subsite −1). The side chain of Trp-47 and two loop regions (173–182 and 215–220) are significantly displaced. Disordered regions of the 236–254 loop are shown as *dotted lines*. *b* and *c*, the structure of the substrate-binding site. *b*, ligand-free (*cyan*) and WT-DFJ-EG complex (protein in *magenta*; ligand in *orange*). The tyrosine molecule bound to the ligand-free structure is shown transparently. Water molecules and hydrogen bonds in the WT-DFJ-EG complex are shown. *c*, WT-DFJ-EG complex (protein in *magenta*; ligand in *orange*) and D172A/E217A-LNFP II complex (protein in *green*; ligand in *yellow*). Water molecules and hydrogen bonds in the LNFP II complex are shown. Ethylene glycol and GlcNAc are labeled as *EG* and *NAG*, respectively.

crystal. The Gal-binding site could be highly specific to Gal as the axial O4 hydroxyl group is recognized by the main chain (Gly-173 in *Bi*AfcB and Gly-704 in *Bb*AfcB), which is next to the nucleophile residue and is included in the mobile loop (Fig. 3*c* and supplemental Fig. S10). The glycosynthase neither accepted cellobiose nor GlcGlcNAc (Table 1). The Gal O2 hydroxyl group is not recognized by any protein residues and is exposed to the solvent (Fig. 3*c*), which rationalizes that the enzyme used 2′-FL as the acceptor. The GlcNAc-binding site appears to be rather promiscuous. It is interesting to note that the faces of the GlcNAc sugar rings are inverted between the LNB and LacNAc disaccharides, and consequently, the geometric positions of the respective O4 and O3 hydroxyl groups of the GlcNAc residues become identical (Fig. 4). When automated docking analysis was performed using Le^x trisaccharide, the best docking result was found in the first ranked cluster containing the majority of the 256 docking run conformations. The number of conformations in this cluster, lowest binding energy, and mean binding energy were 81, −6.30, and −3.81 kcal/mol, respectively. In the predicted mode of Le^x binding, the Fuc, Gal, and GlcNAc moieties of the molecule overlap well with those of the Le^a trisaccharide observed in the D172A/E217A-LNFP II crystal except for the hydroxymethyl and *N*-acetyl groups of the GlcNAc (Fig. 4*b*). The O1 atoms of the

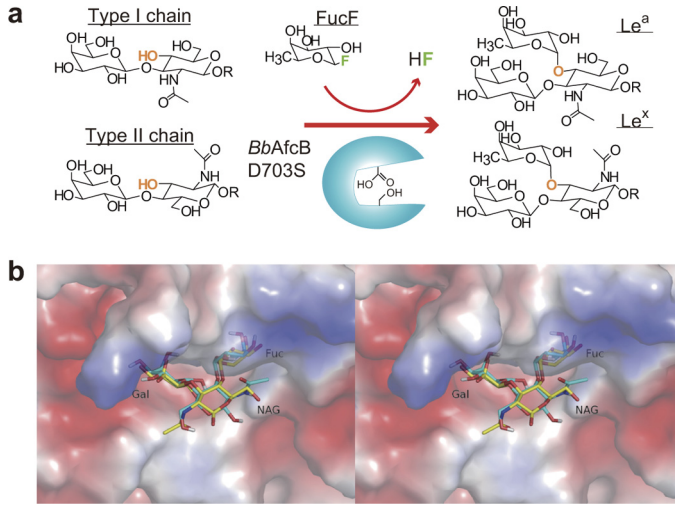

FIGURE 4. *a*, regio- and stereospecific installation of a Fuc residue into type-1/2 chains catalyzed by the *Bb*AfcB D703S glycosynthase. *b*, the predicted mode of binding of Le^x trisaccharide (*cyan*) and its comparison with the Le^a trisaccharide (*yellow*) observed in the D172A/E217A-LNFP II structure (electrostatic surface potential map) (stereoview). *NAG*, GlcNAc.

inverted GlcNAc residues are exposed to the solvent in both cases and rather protrude from the catalytic pocket. These results explain why this synthase accepts LNB, LacNAc, and LNT almost equally (Table 1). The enzyme probably has no structural constraint at the reducing ends of the LNB/LacNAc disaccharides. Indeed, the Lac moiety is invisible in the D172A/E217A-LNFP II structure, and the rate of hydrolysis by *Bb*AfcB is the same between LNFP II (type-1) and LNFP III (Galβ1–4(Fucα1–3)GlcNAcβ1–3Galβ1–4Glc) (type-2) (29). Galacto-*N*-biose cannot be used in the synthase reaction because the axial O4 hydroxyl group of the GalNAc moiety is not appropriately placed to make a glycosidic bond with Fuc. The GlcNAc-binding site can accept both GlcNAc and Glc, but the product yields in the synthetic reactions significantly differed between LNB and Lac (Table 1). The *N*-acetyl group of GlcNAc in the D172A/E217A-LNFP II structure does not form strong interactions with the protein, but the presence of the large *N*-acetyl group may stabilize the acceptor binding through a weak hydrophobic interaction with Trp-47 or Trp-213 (Fig. 3*c*). The enzyme synthesized LNFP II when LNT (Galβ1–3GlcNAcβ1–3Galβ1–4Glc) was included in the reaction mixture. LNT has two potential sites to be fucosylated by the enzyme, but in the analysis of the isolated product, we only detected LNFP II. This is because the substitution of the O3 hydroxyl group of the penultimate Gal impaired the accommodation and/or constrained the induced fit movement. Accordingly, when lacto-*N*-difucohexaose II (Galβ1–3(Fucα1–4)GlcNAcβ1–3Galβ1–4(Fucα1–3)Glc) was incubated with the WT AfcBs, the Fucα1–4GlcNAc linkage was hydrolyzed about 100-fold faster than the Fucα1–3Glc linkage (data not shown).

The mutational study of *Bb*AfcB confirmed that Asp-703 and Glu-746 are the catalytic nucleophile and acid/base catalyst of the enzyme, respectively (supplemental Table S1). The amino acid replacement at the Gal-binding site (Trp-742 and Asp-807) resulted in a significant reduction (by 1800- and 80-fold, respectively) in the hydrolytic activity for 3-FL. It is interesting to note that the specific activities of the mutants decreased only by 2–4-fold for the *p*NP-Fuc hydrolysis (supplemental Table S1), which agrees with the finding that these residues are involved in the interaction with the Gal moiety and not in the Fuc binding. The presence of the branched Gal residue is important not only for the induced fit movement but also for fixing the substrate at the catalytic site. In the D172A/E217A-LNFP II structure, Glu-237 of *Bi*AfcB interacts with the Gal O3 hydroxyl group. *Bb*AfcB appears not to possess the corresponding residue as the alanine substitutions at Asp-763, Asp-766, and Asp-778 did not significantly affect hydrolytic activity. The sequence of the loop (236–254 in *Bi*AfcB and 758–773 in *Bb*AfcB) is not conserved between the two enzymes, and the loop is disordered in the *Bi*AfcB structures (Fig. 3*a* and supplemental Fig. S10). BT_2192, an another close homologue of *Bi*AfcB (see Fig. 5; described later), also does not possess the corresponding residue in its sequence, but the side chain of Glu-254 occupies the site in the structure (supplemental Figs. S11 and S12). These results suggest an auxiliary role for this loop region in the substrate binding.

Comparison with Other α-L-Fucosidases—GH29 can be divided into two subfamilies by phylogenetic analysis (Fig. 5*a*), and this classification apparently correlates with the difference of the substrate specificities between the two groups (29). One subfamily (hereafter referred to as GH29-A) comprises enzymes that show relatively relaxed substrate specificity and act efficiently on a chromogenic substrate (*e.g. p*NP-Fuc) as do human and *Thermotoga maritima* (*Tm*Fuc) enzymes (α-L-fucosidases; EC 3.2.1.51) (44, 47, 48). In contrast, the members of the other subfamily specifically hydrolyze the terminal α-(1,3/4)-fucosidic linkages and hardly act on *p*NP-Fuc (referred to as GH29-B) (1,3-1,4-α-L-fucosidases; EC 3.2.1.111) (49, 50).[3] *Bb*AfcB and *Bi*AfcB are thus categorized into the GH29-B subfamily (29). The overall structure of *Bi*AfcB shows a resemblance to that of *Tm*Fuc (GH29-A) (Protein Data Bank code 1ODU) (root mean square deviation, 3.5 Å for 289 Cα atoms; Z score, 28.5; sequence identity, 24%). In particular, the structure of the Fuc-binding site (subsite −1) is similar between the two enzymes (Fig. 5*b*). The catalytic nucleophile Asp-224 and the acid/base catalyst Glu-266 of *Tm*Fuc overlap with Asp-172 and Glu-217 of *Bi*AfcB (by induced fit movement), respectively (44, 45). *Tm*Fuc, however, does not have a Gal-binding site, and the side chain of Arg-254 occupies the corresponding site and forms a hydrogen bond with the endocyclic oxygen atom of Fuc. This Arg residue is highly conserved in GH29-A enzymes (13 of 14 members listed in Fig. 5*a*), whereas the Gal-binding residues (corresponding to Gly-173, Trp-213, and Asp-283 of *Bi*AfcB) are invariable in the five characterized members of GH29-B. In addition, the constitution of the catalytic pocket is structurally conserved in the respective subfamilies (supplemental Figs. 11 and 12) (51). The structural difference observed at that site may therefore be the basis that differentiates the substrate specificity between GH29-A and GH29-B enzymes. But it is interesting to note that, in both *Tm*Fuc (GH29-A) and *Bi*AfcB (GH29-B), two mobile loops (including or close to an acid/base residue) play critical roles in the catalytic processes (52) (supplemental Fig. S11).

[3] Unpublished results for BT_2192, H. Sakurama and T. Katayama.

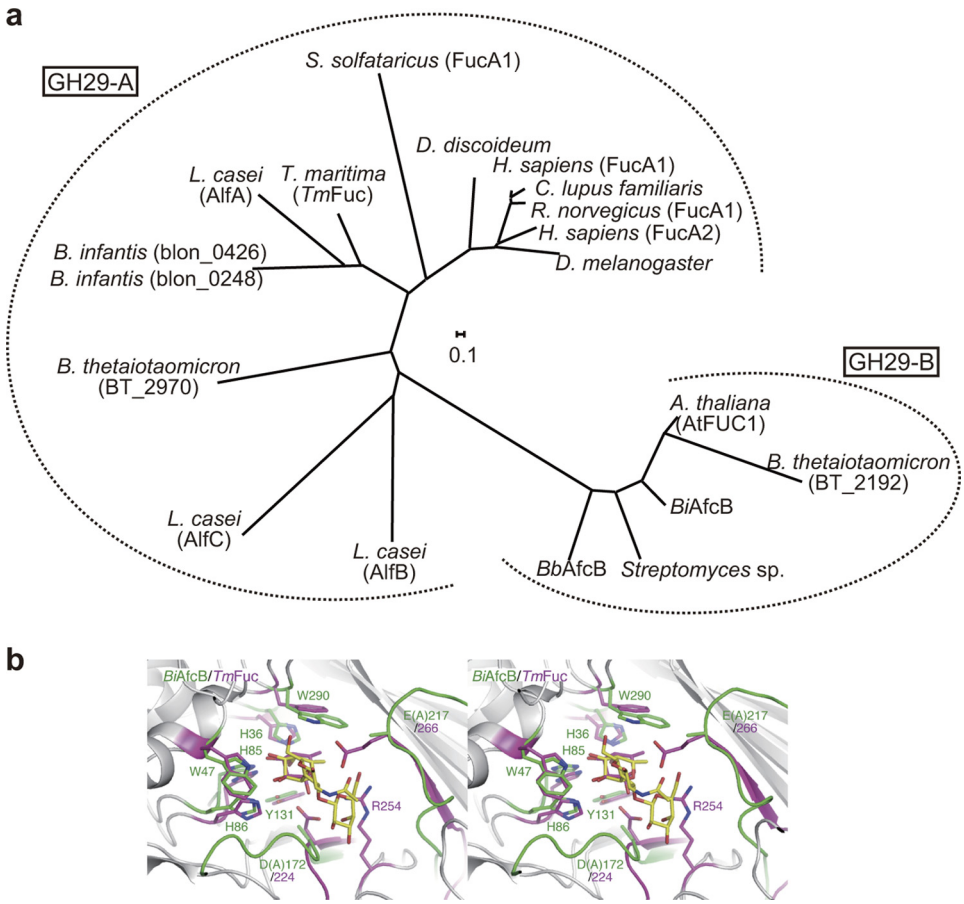

FIGURE 5. **Phylogenetic and structural difference between two subfamilies of GH29.** *a*, the phylogenetic analysis of enzymatically characterized GH29 α-L-fucosidases. The tree was constructed using the ClustalW program with a neighbor-joining method (55). *b*, comparison of the catalytic sites of *Bi*AfcB (D172A/E217A-LNFP II complex; protein in *green*; ligand in *yellow*) and *Tm*Fuc (Fuc complex; *magenta*; Protein Data Bank code 1ODU) (stereoview) (44). The numbers of the residues of *Bi*AfcB and *Tm*Fuc are labeled in *green* and *magenta*, respectively. See also supplemental Figs. S11 and S12. *A.*, *Arabidopsis*; *H.*, *Homo*; *R.*, *Rattus*; *D. discoideum*, *Dictyostelium discoideum*; *C.*, *Canis*; *L.*, *Lactobacillus*; *B. infantis*, *Bifidobacterium longum* subsp. *infantis*; *B. thetaiotaomicron*, *Bacteroides thetaiotaomicron*; *D. melanogaster*, *Drosophila melanogaster*.

Transglycosylation activity of α-L-fucosidases from *Alcaligenes* sp., *Aspergillus niger*, *Penicillium multicolor*, and *T. maritima* has been used to produce fucosyl oligosaccharides using *p*NP-Fuc as a donor (22–25). Recently, the glycosynthase methodology was introduced in α-L-fucosidase from *Sulfolobus solfataricus* (*Ss*Fuc D242S) and *T. maritima* (*Tm*Fuc D224G) (18). These enzymes/mutants are efficient catalysts that attach an α-L-fucosyl residue(s) to various acceptor molecules; however, because they belong to the GH29-A subfamily, they intrinsically fail to control the regioselectivity and acceptor specificity even though they have some preference. For example, *Tm*Fuc synthesized a mixture of Fucα1–3Fucα-*p*NP and Fucα1–2Galβ-*p*NP as a result of transglycosylation when incubated with *p*NP-Fuc (donor) and *p*NP-β-Gal (acceptor) (25). The *Ss*Fuc glycosynthase mutant produced Fucα1–6Galβ-*p*NP, Fucα1–3Galβ-*p*NP, Fucα1–4Galβ-*p*NP, and Fucα1–2(Fucα1–3)Galβ-*p*NP when incubated with β-L-fucopyranosyl azide and Galβ-*p*NP as the donor and the acceptor, respectively (18).

The strict specificity exhibited by the *Bb*AfcB D703S mutant is thus a quite unusual feature. The synthase should therefore serve as a powerful tool for introducing the Le^a and Le^x antigens into the type-1 and type-2 chains at the non-reducing ends of various glycoconjugates including glycoprotein and glycolipids without attaching undesirable and unexpected α-L-fucosyl residues. Considering that 1,3-1,4-α-L-fucosynthase can recognize 2′-FL as the acceptor, the combination of this synthase with 1,2-α-L-fucosynthase, which we have already developed (30), may afford the one-pot tailor-made synthesis of all types of Le antigens including Le^b and Le^y. Mining glycosidases with strict specificity and exploiting those enzymes could be the most efficient route to the specific synthesis of glycosides. In this sense, intestinal microorganisms including bifidobacteria represent good enzyme resources as they are found to possess various glycosidases specific to host glycans (46, 53, 54).

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