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Synthesis of Fucosyl-*N*-Acetylglucosamine Disaccharides by Transfucosylation Using α-L-Fucosidases from *Lactobacillus casei*

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AlfB and AlfC α-L-fucosidases from *Lactobacillus casei* were used in transglycosylation reactions, and they showed high efficiency in synthesizing fucosyldisaccharides. AlfB and AlfC activities exclusively produced fucosyl-α-1,3-*N*-acetylglucosamine and fucosyl-α-1,6-*N*-acetylglucosamine, respectively. The reaction kinetics showed that AlfB can convert 23% *p*-nitrophenyl-α-L-fucopyranoside into fucosyl-α-1,3-*N*-acetylglucosamine and AlfC at up to 56% into fucosyl-α-1,6-*N*-acetylglucosamine.

Fucosyl-oligosaccharides (FUS) are coming to be of great interest due to their presence in human milk and their antiadhesive activity against pathogens. This last property is characterized by blocking the attachment of intestinal pathogens to host cells due to FUS structural similarity with cell-surface glycoconjugate receptors (1–4). In addition, genes encoding α-L-fucosidases (EC 3.2.1.51) have recently been found in genomes of bifidobacteria (5, 6) and lactobacilli (7), which raises issues regarding the role of these enzymes in the metabolism of fucose-containing structures and opens up the possibility of using FUS as novel prebiotics (8). To fully establish the biological function of FUS as antiadhesins and prebiotics, extensive studies are required, for which synthesis of sufficient amounts would be necessary. Chemical synthesis of FUS such as ABH and Lewis blood group antigens has long been performed, but it is a tedious and expensive process since it requires multiple protection and deprotection steps to achieve the desired selectivity (9, 10). The enzymatic synthesis of FUS is an alternative to chemical methods, and it offers the advantage of forming specific glycosidic linkages in the presence of other reactive functional groups. There are two types of enzymes that can carry out fucosylation: fucosyltransferases and fucosidases. Fucosyltransferases present high specificity toward the acceptor and do not hydrolyze the product (11, 12). However, those enzymes are generally difficult to express and they require an expensive sugar nucleotide or a complex multienzymatic system for the regeneration (11, 13). In contrast, oligosaccharides have been largely produced using the transglycosylation activity of glycosidases and engineered glycosynthases (14, 15). Only a few works describe the synthesis of FUS by transglycosylation using α-L-fucosidases; however, they cover a wide range of sources such as mammalian tissues (16), fungi (17), and bacteria (18, 19). The transglycosylation activity of α-L-fucosidases is generally moderate compared with the hydrolysis activity, although it is variable and depends on the origin of the enzymes (20).

We have previously isolated three α-L-fucosidases (AlfA, AlfB, and AlfC) from *Lactobacillus casei* that cleaved α-linked L-fucose from natural FUS (7). AlfB and AlfC showed high specific activity on fucosyl-α-1,3-*N*-acetylglucosamine (Fuc-α-1,3-GlcNAc) and fucosyl-α-1,6-*N*-acetylglucosamine (Fuc-α-1,6-GlcNAc), respectively. These substrate specificities led us in the present work to explore the likely use of *L. casei* α-L-fucosidases to synthesize those FUS. AlfB and AlfC were expressed in *Escherichia coli* as 6×(His)-tagged proteins and purified to near homogeneity as previously described (7). Transfucosylation activity of 6×(His)AlfB and 6×(His)AlfC was analyzed in 100 mM sodium phosphate buffer at pH 7.0. The reactions mixtures contained 50 mM *p*-nitrophenyl-α-L-fucopyranoside (*p*NP-fuc) as the donor and 200 mM *N*-acetylglucosamine (GlcNAc) as the acceptor. The mixtures were heated at 100°C to overcome the poor solubility of *p*NP-fuc and then cooled to the reaction temperature of 42°C. The reactions were started by the addition of 50 U/ml AlfB or 100 U/ml AlfC (1 U was defined as the amount of enzyme able to release 1 μmol of *p*NP in 1 h). Double the amount of AlfC compared to AlfB was needed to achieve the maximum disaccharide synthesis, due to the

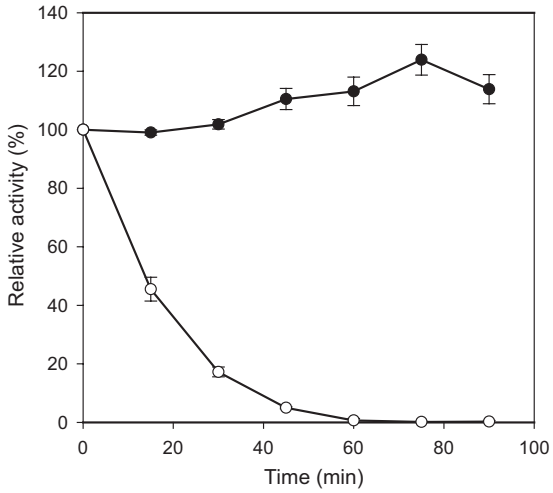

FIG 1 Stability of the α-L-fucosidases AlfB (●) and AlfC (○). The enzymes (10 μg/ml) were incubated in 100 mM sodium phosphate buffer at pH 7.0 and 42°C, and the residual activities were determined at different time intervals with 2 mM *p*-nitrophenyl α-L-fucopyranoside as the substrate. The initial activity was defined as 100%. The data presented are mean values from three replicate experiments. The errors bars represent the standard deviations.

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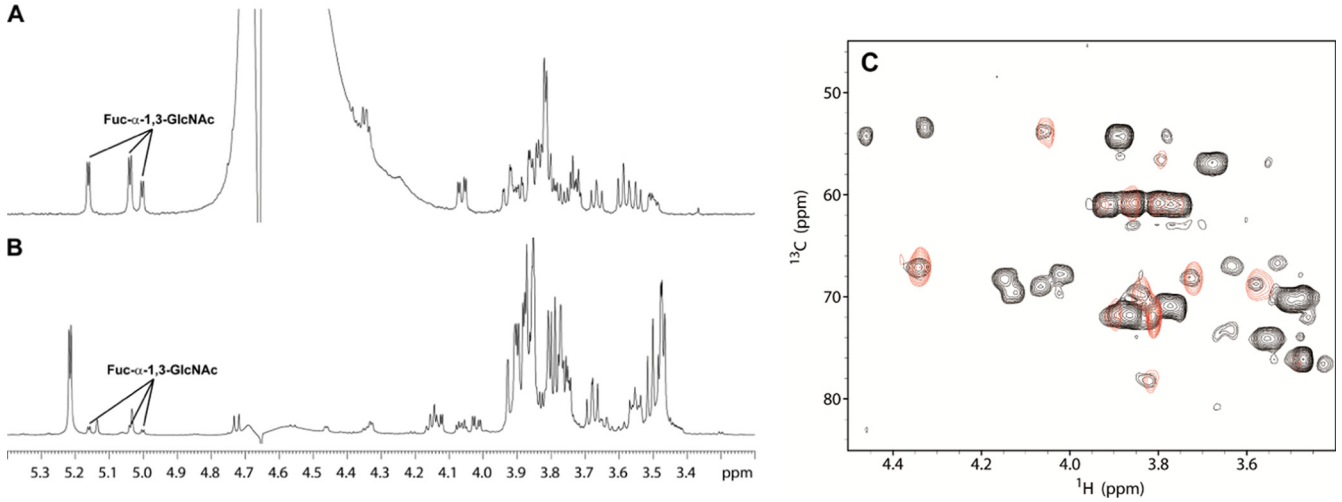

FIG 2 (A and B) Partial 1D ^{1}H NMR spectra (600 MHz, 313 K) covering the anomeric region of commercial fucosyl-α-1,3-*N*-acetylglucosamine (Fuc-α-1,3-GlcNAc) (A) and the transfucosylation reaction with AlfB (B). (C) ^{1}H, ^{13}C-HSQC of Fuc-α-1,3-GlcNAc (red) and the reaction mixture (black).

lower stability for AlfC, which is completely inactivated after 60 min at 42°C (Fig. 1). Samples (10 μl) were heated at 100°C for 3 min to stop the reaction, dried, and dissolved in 500 μl of D_2O. The reaction products were analyzed by nuclear magnetic resonance (NMR) spectroscopy. NMR spectra were recorded at 313 K using a Bruker Avance Ultrashield Plus 600 spectrometer equipped with a 5-mm TCI cryoprobe. Chemical shifts were referenced using trimethylsilyl propionate (TSP) as an internal reference. One-dimensional (1D) ^{1}H and ^{1}H, ^{13}C heteronuclear single quantum coherence–heteronuclear multiple-bond correlation (HSQC/HMBC) experiments were acquired for the reaction mixtures and for each of the substrates and products. Typical parameters for the 2D experiments were as follows: for HSQC, 256 and 2,048 points in F1 and F2, respectively (48 transients each); and for HMBC, 512 and 2,048 points in F1 and F2, respectively (64 transients each). NMR spectra were processed using the program Topspin 1.3 (Bruker GmbH, Karlsruhe, Germany). The structural analysis of the reaction products showed that the enzymes AlfB (Fig. 2) and AlfC (Fig. 3) generate by their transglycosylation activity the disaccharides Fuc-α-1,3-GlcNAc and Fuc-α-1,6-GlcNAc, respectively. No byproducts other than the hydrolyzed fucose and *p*NP were detected in the reactions. This is the first reported example of synthesis of Fuc-α-1,6-GlcNAc by transglycosylation. The synthesis of Fuc-α-1,3-GlcNAc has been previously described by using an α-L-fucosidase partially purified from a culture broth of *Penicillium multicolor* (17). However, the substrate specificity demonstrated for this enzyme suggests that a mixing of transient disaccharides occurred in the transfucosylation reaction. Both disaccharides synthesized here are important molecules that form part of glycoconjugates present at mucosal surfaces. Fuc-α-1,3-GlcNAc forms part of the Lewis X antigen core, which is found in many human glycoproteins at mucosal surfaces (21) and also forms part of human milk oli-

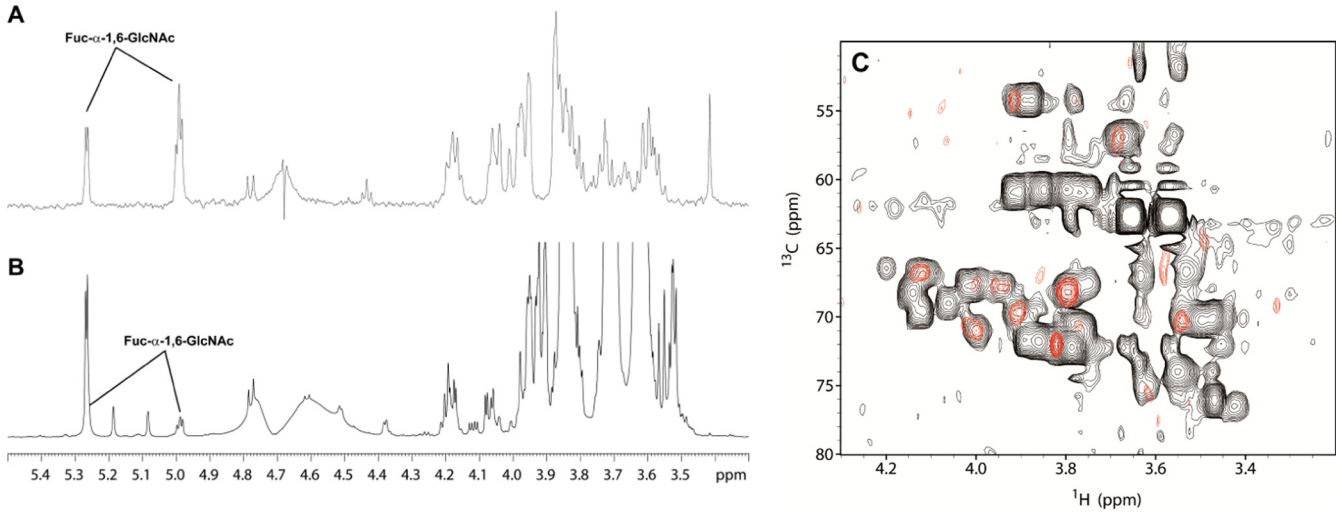

FIG 3 (A and B) Partial 1D ^{1}H NMR spectra (600 MHz, 313 K) covering the anomeric region of commercial fucosyl-α-1,6-*N*-acetylglucosamine (Fuc-α-1,6-GlcNAc) (A) and the transfucosylation reaction with AlfC (B). (C) ^{1}H, ^{13}C-HSQC of Fuc-α-1,6-GlcNAc (red) and the reaction mixture (black).

TABLE 1 Transglycosylation and hydrolysis activities of AlfB and AlfC

Enzyme	Transglycosylation (μmol/min/mg protein)	Hydrolysis (μmol/min/mg protein)	V_{trans}/V_{hyd} [a]
AlfB	0.95	1.50	0.63
AlfC	12.79	2.63	4.86

[a] Tranglycolylation/hydrolysis (V_{trans}/V_{hyd}) ratios were calculated from the initials rates of formation of transglycosylation products over the initial rates of hydrolysis.

gosaccharides (22). Fuc-α-1,6-GlcNAc is part of the core sugar in protein N-glycosylation also at mucosal surfaces (23).

In order to analyze the transfucosylation kinetics of the AlfB and AlfC enzymes, a time course analysis was performed. 1D ^{1}H spectra were collected for several reaction time points, with 64 transients, 32 K points, and a recovery time of 5 s, using water presaturation to suppress the residual HOD signal. Compound yields at each time point were determined from the integration of characteristic nonoverlapped proton signals for each component present in the reaction mixture. The total integral was normalized and the concentration of each molecule calculated using as a reference the added TSP (100 μM). The transglycosylation/hydrolysis ratio was 7.7-fold higher for AlfC than for AlfB (Table 1). This resulted in maximum transient yields of approximately 23% for AlfB and 56% for AlfC with respect to the added *p*NP-fuc (Fig. 4). The efficiency of transfucosylation is largely dependent on the type of enzyme. Thus, α-L-fucosidases from some bifidobacteria are completely devoid of transfucosylation activity unless they are mutated at specific amino acid sites (19). The reaction efficiency of AlfB was similar to the efficiencies of other α-L-fucosidases found in the literature, which ranged from 3% to 30% (18, 20, 24). Interestingly, the 56% AlfC yield is the maximum percentage described for a wild-type α-L-fucosidase, showing that this enzyme has very high transfucosylation activity which resembles that of engineered α-L-fucosidases; i.e., yields of *p*NP-fucosyl-α-1,2-galactose that ranged from 28% to 65% compared to 7% with wild-type enzyme have been obtained with mutants of an α-L-fucosidase from *Thermotoga maritima* (18). We have recently reported that probiotic bacterium *L. casei* strain BL23 can use Fuc-α-1,3-GlcNAc as a carbon source and that AlfB is necessary for this. However, Fuc-α-1,6-GlcNAc cannot be metabolized by *L. casei* (25). This suggests that, in spite of the high specific activity of AlfC on Fuc-α-1,6-GlcNAc (13.6 μmol fucose liberated/min · mg protein, compared to 27.5 μmol fucose liberated from Fuc-α-1,3-GlcNAc/min · mg protein for AlfB [7]), this disaccharide is not the natural substrate of AlfC. This could explain the elevated capacity of AlfC to synthesize Fuc-α-1,6-GlcNAc.

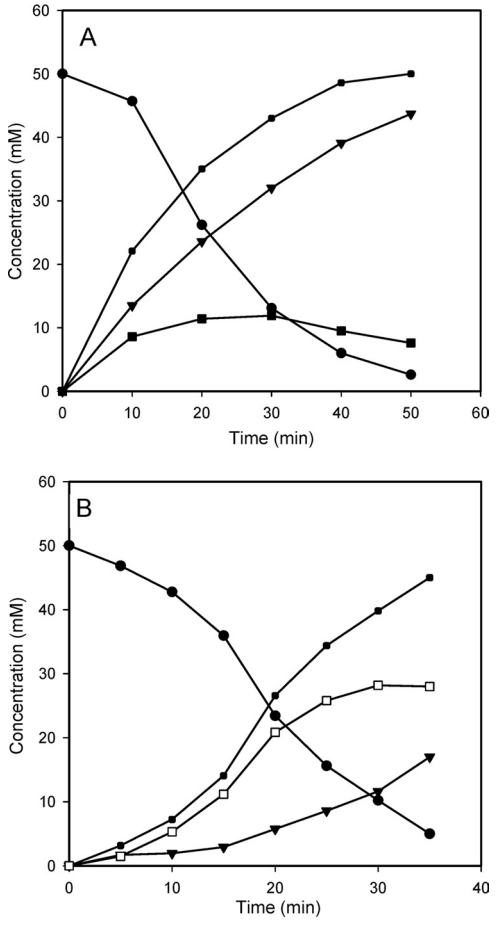

FIG 4 Time course of the substrate consumption and the product formation by transfucosylation catalyzed by α-L-fucosidases AlfB (A) and AlfC (B). ●, *p*-nitrophenyl-α-L-fucopyranoside; ◆, *p*-nitrophenol; ▼, L-fucose; ■, fucosyl-α-1,3-*N*-acetylglucosamine; □, fucosyl-α-1,6-*N*-acetylglucosamine. Data from a representative experiment are shown.

In this report, we have shown that the high transglycosylation activity of AlfB and AlfC enables the enzymatic synthesis of two important fucosyldisaccharides. The use of glycosidases from probiotic species is within the rational strategies for efficient prebiotic oligosaccharide production, because all the machinery necessary for uptake and degradation is already present in these bacteria and also constitutes a tool for the synthesis of FUS to be used as anti-adhesin ingredients.

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