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One-pot multienzyme (OPME) synthesis of human blood group H antigens and a human milk oligosaccharide (HMOS) with highly active *Thermosynechococcus elongatus* α 1–2-fucosyltransferase

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Abstract

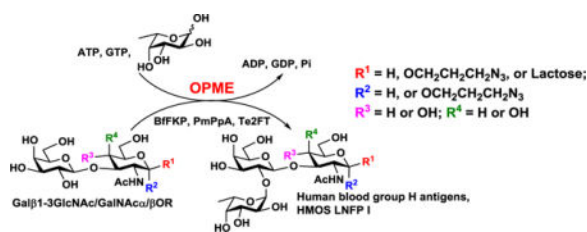
A novel α 1–2-fucosyltransferase from *Thermosynechococcus elongatus* BP-1 (Te2FT) with high fucosyltransferase activity and low donor hydrolysis activity was discovered and characterized. It was used in an efficient one-pot multienzyme (OPME) fucosylation system for high-yield synthesis of human blood group H antigens containing β 1–3-linked galactosides and an important human milk oligosaccharide (HMOS) lacto-*N*-fucopentaose I (LNFP I) in preparative and gram scales. LNFP I was shown to be selectively consumed by *Bifidobacterium longum* subsp. *infantis* but not *Bifidobacterium animalis* subsp. *lactis* and is a potential prebiotic.

TOC image

Lacto-*N*-fucopentaose I (LNFP I) and human blood H group antigens were synthesized efficiently by one-pot multienzyme (OPME) fucosylation with a bacterial α 1-2-fucosyltransferase.

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Electronic Supplementary Information (ESI) available: Experimental details for cloning and characterization of Te2FT, chemical and enzymatic synthesis, NMR and HRMS data, NMR spectra, and bacterial growth study procedures. See DOI: 10.1039/x0xx00000x



α 1–2-Linked fucose is a major structural component of all human histo-blood group ABH antigens, some Lewis antigens such as Lewis b and Lewis y,¹ and many neutral human milk oligosaccharides (HMOS) where they have been found to possess prebiotic, antiadhesive antimicrobial, and immunomodulating activities which contribute significantly to the benefits of breast feeding.^{2–5}

To facilitate enzymatic and chemoenzymatic synthesis of α 1–2-fucosides, several bacterial α 1–2-fucosyltransferases (2FTs) have been cloned and characterized. These include *Helicobacter pylori* (*H. pylori*) FutC (HpFutC or Hp2FT),^{6, 7} *Escherichia coli* (*E. coli*) O86:B7 WbwK,⁸ *E. coli* O86:K62:H2 WbnK,⁹ *E. coli* O128 WbsJ,^{10–12} and *E. coli* O127:K63(B8) WbiQ.¹³ However, their expression levels are usually low which limit their application in synthesis. A more recently characterized *E. coli* O126 WbgL^{14, 15} has a reasonable expression level but has a preference towards β 1–4-linked galactosides as acceptor substrates. The access to α 1–2-fucosylated β 1–3-linked galactosides in large scales has been greatly hampered by the lack of a 2FT that has a high activity and can be obtained in large amounts. Here we report a novel α 1–2-fucosyltransferase (encoded by gene *tl0994*) from thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1¹⁶ (Te2FT). It has a high expression level in *E. coli* and a high specific activity. It is a powerful catalyst for highly efficient enzymatic synthesis of α 1–2-fucosylated β 1–3-linked galactosides in preparative and large scales.

Te2FT shows 27–33% identities and 44–48% similarities to previously reported bacterial α 1–2-fucosyltransferases and belongs to Carbohydrate Active enZyme (CAZy)^{17, 18} glycosyltransferase family 11 (GT11) (Fig. S1). Te2FT was cloned as an N-His₆-tagged recombinant protein (His₆-Te2FT) in pET15b vector, as well as an N-maltose binding protein (MBP)-fused and C-His₆-tagged recombinant protein (MBP-Te2FT-His₆) in pMAL-c4X vector. Ni²⁺-column purified proteins have molecular weights close to the calculated values of 36.2 KDa (His₆-Te2FT) and 75.8 KDa (MBP-Te2FT-His₆), respectively (Fig. S2). When expressed under optimal conditions at 16 °C for 20 hours with shaking at 120 rpm, the expression levels of His₆-Te2FT and MBP-Te2FT-His₆ were 15 and 16 mg per liter *E. coli* culture (the amounts of purified proteins determined by bicinchoninic acid assays), respectively. The expression level of His₆-Te2FT (15 mg/L culture) was significantly higher than other reported α 1–2FTs¹⁴ and was comparable to recombinant C-truncated *Helicobacter pylori* α 3FT¹⁹ which, partially due to its easy accessibility by recombinant expression, has been crystallized²⁰ and broadly used in enzymatic and chemoenzymatic synthesis of α 1–3-linked fucosides.^{21–24} Due to its good expression level and lower molecular weight compared to MBP-Te2FT-His₆, His₆-Te2FT was used further for detailed characterization and synthesis.

Initial substrate specificity studies using various disaccharides as acceptor substrates indicated that His₆-Te2FT worked well with type I (Galβ1–3GlcNAcβOR) and its derivative (Galβ1–3GlcNAcαOR), Type III (Galβ1–3GalNAcαOR), and type IV (Galβ1–3GalNAcβOR) acceptors for adding a fucose α1–2-linked to the terminal galactose (Gal) residue. However, type II (Galβ1–4GlcNAcβOR) and type V (Galβ1–4GlcβOR)^{25, 26} glycans were not good acceptors.

High performance liquid chromatography (HPLC)-based pH profile study using Galβ1–3GlcNAcβ2AA (a type I glycan with a fluorescent 2-anthranilic acid aglycone) as an acceptor showed that His₆-Te2FT was active in a broad pH range of 4.0–10.0 with optimal activities at pH 4.5–6.0 (Fig. S3). About 85% of the optimal activity was found at pH 6.5 under the experimental conditions used. As significant guanosine 5'-diphosphate-fucose (GDP-fucose, the donor substrate for fucosyltransferases) hydrolysis activity of fucosyltransferases could lower synthetic yields,²⁷ the donor hydrolysis activity assays of His₆-Te2FT were carried out. To our delight, using 15-fold more enzyme and 3-fold longer reaction time than those in the α1–2-fucosyltransferase activity assays indicated that the donor hydrolysis activity of His₆-Te2FT was minimum in the pH range (5.0–8.0) that would normally be used for the α1–2-fucosyltransfer reactions. An optimal pH at 9.5 was observed for the GDP-fucose hydrolysis activity.

A divalent metal ion was not required for the α1–2-fucosyltransferase activity of His₆-Te2FT. Adding 10 mM of ethylenediaminetetraacetic acid (EDTA) decreased its activity only slightly (Fig. S4). However, its activity was enhanced by about two-fold when 5–20 mM of Mg²⁺ or Mn²⁺ was added. The addition of 10 mM of dithiothreitol (DTT) decreased His₆-Te2FT activity moderately, indicating disulfide bond formation was not essential for the enzyme activity despite of the presence of 5 cysteine residues in the Te2FT protein sequence.

Since His₆-Te2FT was originated from a thermophilic cyanobacterium, its temperature profile was investigated. It was active in a broad temperature range of 25–60 °C with optimal activities observed in the range of 30–45 °C (Fig. S5). Low activity was observed at 15 °C and 20 °C and minimal activity was retained at 65 °C or 70 °C. His₆-Te2FT could survive freeze-dry treatment (Fig. S6), indicating its potential for long term storage.

Kinetics studies of His₆-Te2FT using Galβ1–3GlcNAcβ2AA as an acceptor (Table 1) indicated its superior α1–2-fucosyltransferase activity. Compared to GST-WbsJ,^{11, 12} His₆Prop-WgbL,¹⁴ and Hp2FT,⁷ His₆-Te2FT showed a significantly higher k_{cat} value for GDP-fucose (201-, 6.7-, and 14.6-fold, respectively) and a higher affinity for the acceptor. The catalytic efficiency of His₆-Te2FT was similar to that of Hp2FT but is superior to WbsJ and WbgL as reflected by a higher k_{cat}/K_M value for GDP-Fuc (29.2-fold and 2.5-fold higher). The GDP-fucose (donor) hydrolysis activity ($k_{cat}/K_M = 0.54 \text{ min}^{-1} \text{ mM}^{-1}$) of His₆-Te2FT was 47-fold weaker than its α1–2-fucosyltransferase activity and much lower than the donor hydrolysis activity of Hp2FT ($6.07 \text{ min}^{-1} \text{ mM}^{-1}$).⁷ These data indicate that His₆-Te2FT is a superior catalyst for enzymatic synthesis of α1–2-linked fucosides.

The synthetic application of Te2FT was explored in an efficient one-pot three-enzyme (OP3E) fucosylation system (Scheme 1) for synthesizing various α 1–2-linked fucosides. In this system, recombinant bifunctional L-fucokinase/GDP-fucose pyrophosphorylase from *Bacteroides fragilis* strain NCTC9343 (BfFKP)²⁸ was used for catalyzing the formation of GDP-fucose from fucose, adenosine 5'-triphosphate (ATP), and guanidine 5'-triphosphate (GTP) to provide the donor substrate for Te2FT. *Pasteurella multocida* inorganic pyrophosphatase (PmPpA)²⁹ was used to break down the pyrophosphate by-product to shift the reaction towards the formation of GDP-fucose. As shown in Table 2, the OP3E fucosylation of β 1–3-linked galactosides was successfully accomplished with 96%, 95%, 95%, and 98% yields, respectively, to produce desired Fuc α 1–2Gal β 1–3GlcNAc β ProN₃ (**1**, a type I H antigen), Fuc α 1–2Gal β 1–3GlcNAc α ProN₃ (**2**, a type I H antigen derivative), Fuc α 1–2Gal β 1–3GalNAc α ProN₃ (**3**, a type III H antigen), and Fuc α 1–2Gal β 1–3GalNAc β ProN₃ (**4**, a type IV H antigen). Moreover, a human milk tetrasaccharide lacto-*N*-tetraose (LNT) Gal β 1–3GlcNAc β 1–3Gal β 1–4Glc was also an excellent acceptor for His₆-Te2FT and the OP3E synthesis of Fuc α 1–2LNT (**5**), a human milk pentasaccharide also known as lacto-*N*-fucopentaose I (LNFP I), was achieved in a preparative scale (68.1 mg) with an excellent 94% yield.

To demonstrate the efficiency of the OP3E fucosylation system and the activity of Te2FT, gram-scale synthesis of Fuc α 1–2LNT (LNFP I) pentasaccharide was carried out. Pure LNFP I in an amount of 1.146 gram was successfully obtained with an excellent 95% yield. It should be noted that the purification of the product in the gram-scale synthesis of LNFP I was greatly simplified by using activated charcoal which was very efficient in removing nucleotides in the reaction mixtures. Due to the complete consumption of the acceptor LNT in the reaction mixture, separating the pentasaccharide product from other components with smaller molecular weights was conveniently done by a gel filtration column which served similarly as desalting.

The facile synthesis of lacto-*N*-fucopentaose I (LNFP I) is significant. LNFP I was identified in 1956 as one of the HMOS structures.³⁰ It is missing in the milk of Le^{a+b-} non-secretors,³¹ otherwise it is an abundant HMOS species (1.2–1.7 g/liter) in pooled human milk.⁴ It is not presented in the milk or the colostrum of cows,^{32, 33} pigs,³⁴ or other domestic animals.³³ Therefore it is not readily accessible from natural sources by purification. Chemical synthesis of LNFP I with a β -linked pentanilamino aglycon from a protected tetrasaccharide precursor obtained by a one-pot chemical synthetic procedure was achieved in four steps with 49% yield.³⁵ LNFP I was also synthesized from LNT and GDP-fucose using a recombinant human FUT1 expressed in a baculovirus system (3.0 mg, 71% yield)³⁶ and in whole-cell recombinant *E. coli* expressing HpFutC (59.4 mg).³⁷ The OP3E system presented here is a more efficient and an economically feasible approach for large-scale production of LNFP I.

Obtaining large amounts of LNFP I by highly efficient OP3E enzymatic process presented here allows downstream investigation of its potential prebiotic application.

The ability of LNFP I in serving as the sole carbon source for the growth of bifidobacteria was examined. Media containing HMOS (a mixture of oligosaccharides isolated from

human milk)³⁸ or glucose were used as controls. *Bifidobacterium longum* subsp. *infantis* (*B. infantis*) ATCC 15697 grew well on LNFP I and HMOS with a similar pattern which was remarkably faster than its growth on glucose (Fig. 1A). To establish specificity, LNFP I was tested for its ability to support the growth of *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*) ATCC 27536, a subspecies that did not grow on HMOS.³⁹ *B. lactis* failed to grow on either LNFP I or HMOS, while it showed a similar growth pattern to *B. infantis* in the presence of glucose (Fig. 1B). Selective growth of *B. infantis* on a specific fucosylated HMOS species like LNFP I provides a molecular rationale for the unique enrichment of *B. infantis* and other infant-borne bifidobacteria in the nursing infant gastrointestinal tract by breastfeeding.^{40, 41}

In conclusion, Te2FT with a good recombinant expression level and high activity is an important tool for large-scale enzymatic synthesis of various biologically important α 1–2-fucosides. We demonstrated again that the one-pot multienzyme (OPME) fucosylation is a highly effective system for chemoenzymatic and enzymatic synthesis of fucosides. The gram-scale synthesis of fucosylated human milk oligosaccharide LNFP I allowed the performance of bacteria growth study which showed that LNFP I was selectively consumed as a carbon source by *B. infantis* but not *B. lactis*. Therefore, LNFP I is a potential prebiotic candidate for further development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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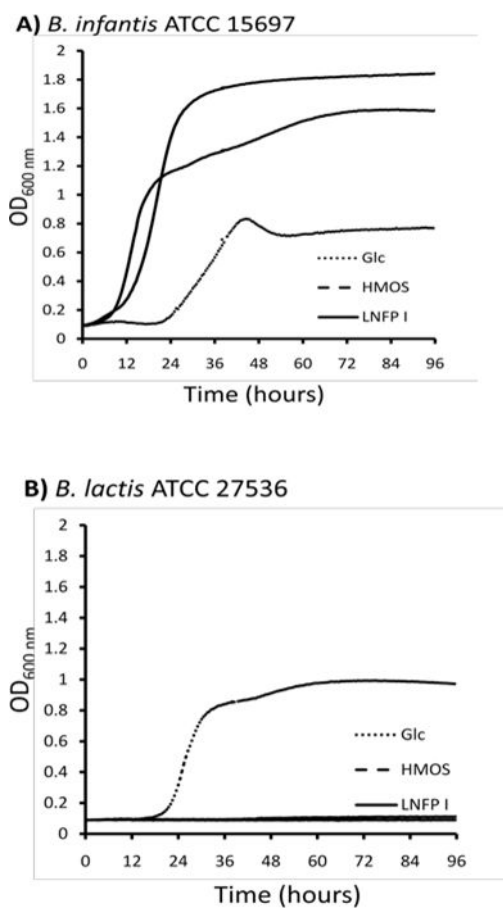
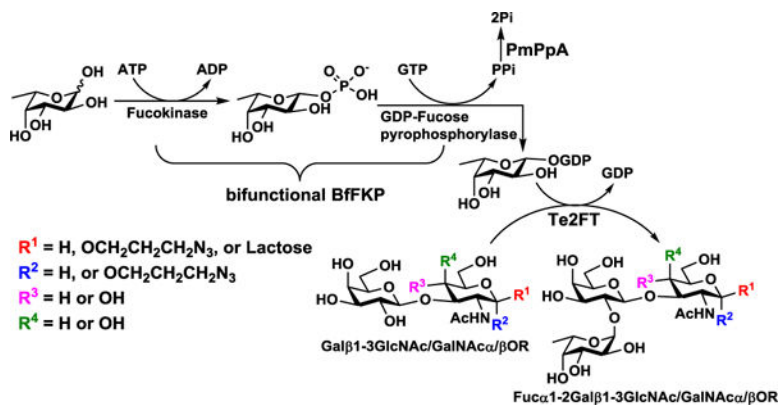


Fig. 1. Growth of bifidobacterial strains *B. infantis* (A) and *B. lactis* (B) on mMRS medium supplemented with 2% (wt/vol) glucose (Glc, dotted line), human milk oligosaccharides (HMOS, dashed line) or LNFP I (solid line).

**Scheme 1.**

One-pot three-enzyme (OP3E) synthesis of α 1–2-fucosides. Enzymes and abbreviations: BfFKP, *Bacteroides fragilis* strain NCTC9343 bifunctional L-fucokinase/GDP-fucose pyrophosphorylase;²⁸ PmPpA, *Pasteurella multocida* inorganic pyrophosphorylase;²⁹ and Te2FT, *Thermosynechococcus elongatus* α 1–2-fucosyltransferase.

Table 1Apparent kinetic parameters of His₆-Te2FT.

Activity	Substrate	k_{cat} (min ⁻¹)	K_{M} (mM)	$k_{\text{cat}}/K_{\text{M}}$ (min ⁻¹ mM ⁻¹)
2FT	Galβ1-3GlcNAcβ2AA	20.0±0.7	0.79±0.11	25.3
	GDP-fucose	26.3±0.8	0.73±0.08	36.1
GDP-Fuc hydrolysis	GDP-fucose	0.30±0.06	0.56±0.21	0.54

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Table 2One-pot three-enzyme (OP3E) synthesis of α 1-2-linked fucosides.

Acceptor	Product	Yield (%)	Amount (mg)
Gal β 1-3GlcNAc β ProN ₃	Fuca1-2Gal β 1-3GlcNAc β ProN ₃ (1)	96	50.5
Gal β 1-3GlcNAc α ProN ₃	Fuca1-2Gal β 1-3GlcNAc α ProN ₃ (2)	95	51.3
Gal β 1-3GalNAc α ProN ₃	Fuca1-2Gal β 1-3GalNAc α ProN ₃ (3)	95	35.7
Gal β 1-3GalNAc β ProN ₃	Fuca1-2Gal β 1-3GalNAc β ProN ₃ (4)	98	43.8
LNT	Fuca1-2LNT or LNFP I (5)	94	68.1
		95	1,146

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