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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 α1–2-fucosyltransferase

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A novel α1–2-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 β1–3-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.

α1–2-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 α1–2-fucosides, several bacterial α1–2-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 WbKL,8 E. coli O86:K62:H2 WbKK,9 E. coli O128 WbsJ,10–12 and E. coli O127:K63(B8) WbiQ,13 However, their expression levels are usually low which limits their application in synthesis. A more recently characterized E. coli O126 WbgL14, 15 has a reasonable expression level but has a preference towards β1–4-linked galactosides as acceptor substrates. The access to α1–2-fucosylated β1–3-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 α1–2-fucosyltransferase (encoded by gene tll0994) 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 α1–2-fucosylated β1–3-linked galactosides in preparative and large scales.

Te2FT shows 27–33% identities and 44–48% similarities to previously reported bacterial α1–2-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 value 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 α1–2FTs14 and was comparable to recombinant C-truncated Helicobacter pylori α3FT19 which, partially due to its easy accessibility by recombinant expression, has been used in enzymatic and chemoenzymatic synthesis of α1–3-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 (Galβ1–3GlicNAcβ1) and its derivative...
survive freeze-dry treatment (Fig. S4). The addition of 10 mM of dithiothreitol (DTT) decreased His66–Te2FT activity. Compared to GST-WbsJ, an acceptor (Table 1) indicated its superior α1–2-fucosyltransferase activity. Since His66–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. 55). Low activity was observed at 15°C and 20°C and minimal activity was retained at 65°C or 70°C. His66–Te2FT could survive freeze-dry treatment (Fig. 56), indicating its potential for long-term storage.

Kinetics studies of His66–Te2FT using Galβ1–3GalNAcβ1–2AA as an acceptor (Table 1) indicated its superior α1–2-fucosyltransferase activity. Compared to GST-WbsJ, His66Prop-WbgL,14 and Hp2FT,7 His66–Te2FT showed a significantly higher $k_{cat}$ value for GDP-fucose (201±, 6.7±, and 14.63-fold, respectively) and a higher affinity for the acceptor. The catalytic efficiency of His66–Te2FT was similar to that of Hps2FT 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 min$^{-1}$·mM$^{-1}$) of His66–Te2FT was more than 47-fold weaker than its α1–2-fucosyltransferase activity and much lower than that of Hps2FT.7 These data indicate that His66–Te2FT is a superior catalyst for enzymatic synthesis of α1–2-fucosides.

The synthetic application of Te2FT was explored in an efficient one-pot three-enzyme (OP3E) fucosylation system (Scheme 1) for synthesizing various α1–2-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 (PnPpa)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 β1–3-linked galactosides was successfully accomplished with 96%, 95%, 95%, and 98% yields, respectively, to produce desired Fusα1–2Galβ1–3GlcNAcβ1–2OR (1, a type I H antigen), Fusα1–2Galβ1–3GlcNAcβ1–2ProN (2, a type I H antigen derivative), Fusα1–2Galβ1–3GlcNAcβ1–2ProN (3, a type III H antigen), and Fusα1–2Galβ1–3GlcNAcβ1–2ProN (4, a type IV H antigen). Moreover, a human milk tetrasaccharide lacto-N-tetraose (LNT) Galβ1–4Glcα1–6Galβ1–3Galβ1–4Glc was also an excellent acceptor for His66–Te2FT and the OP3E synthesis of Fusα1–2LNT (5), a human milk pentasaccharide also known as lacto-N-fucopentaose I (LFP I), was achieved in a preparative scale (68.1 mg) with an excellent 94% yield.

Table 1. Apparent kinetic parameters of His66–Te2FT.

<table>
<thead>
<tr>
<th>Activity</th>
<th>Substrate</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$K_{M}$ (mM)</th>
<th>$k_{cat}/K_{M}$ (min$^{-1}$·mM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2FT</td>
<td>Galβ1–3GalNAcβ1–2AA</td>
<td>20.0±0.7</td>
<td>0.79±0.11</td>
<td>25.5</td>
</tr>
<tr>
<td></td>
<td>GDP-fucose</td>
<td>26.3±0.8</td>
<td>0.73±0.08</td>
<td>36.1</td>
</tr>
</tbody>
</table>

GDP-Fuc hydrolysis
GDP-fucose 0.30±0.08 0.58±0.21 0.54

Scheme 1. One-pot three-enzyme (OP3E) synthesis of α1–2-fucosides. Enzymes and abbreviations: BfFKP, Bacteroides fragilis strain NCTC9343 bifunctional L-fucokinase/GDP-fucose pyrophosphorylase;28 PnPpa, Pasteurella multocida inorganic pyrophosphatase;29 and Te2FT, Thermosynechococcus elongatus α1–2-fucosyltransferase.

To demonstrate the efficiency of the OP3E fucosylation system and the activity of Te2FT, gram-scale synthesis of
Fucox1–2LNT (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.

Table 2. One-pot three-enzyme (OP3E) synthesis of α1–2-linked fucosides.

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>Product</th>
<th>Yield (%)</th>
<th>Amount (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galβ1–3GlcNAcβ1-ProN2</td>
<td>Fuco1–2Galβ1–3GlcNAcβ1-ProN2</td>
<td>96</td>
<td>50.5</td>
</tr>
<tr>
<td>Galβ1–3GlcNAcβ1-ProN2</td>
<td>Fuco1–2Galβ1–3GlcNAcβ1-ProN2</td>
<td>95</td>
<td>51.3</td>
</tr>
<tr>
<td>Galβ1–3GlcNAcβ1-ProN2</td>
<td>Fuco1–2Galβ1–3GlcNAcβ1-ProN2</td>
<td>95</td>
<td>35.7</td>
</tr>
<tr>
<td>Galβ1–3GlcNAcβ1-ProN2</td>
<td>Fuco1–2Galβ1–3GlcNAcβ1-ProN2</td>
<td>95</td>
<td>43.8</td>
</tr>
<tr>
<td>LNT</td>
<td>Fuco1–2LNT or LNFP I</td>
<td>94</td>
<td>68.1</td>
</tr>
<tr>
<td></td>
<td>(5)</td>
<td>95</td>
<td>1,146</td>
</tr>
</tbody>
</table>

The facile synthesis of lacto-N-fucopentaose I (LNFP I) is significant. LNFP I was identified in 1956 as one of the HMOS structures. Otherwise, it is missing in the milk of Lea non-secretors, otherwise it is an abundant HMOS species in pooled human milk. It is not presented in the milk or the colostrum of cows, pigs, or other domestic animals. Therefore it is not readily accessible from natural sources by purification. Chemical synthesis of LNFP I with a β-linked pentanamino aglycon from a protected tetrasaccharide precursor obtained by a one-pot chemical synthetic procedure was achieved in four steps with 49% yield. 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) and in whole-cell recombinant E. coli expressing HpFucT (59.4 mg). 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) 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. 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.

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 α1–2-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.

This work was supported by NIH grant R01HD065122 (to X.C.), FAFU grants XJQ201417 and 612014043 (to C.Z. and B.L.), Scholarships of Education Department of Fujian Province (to C.Z. and Y.W.), and Scholarships of China (to J.Z.).

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).
Avance-800 NMR spectrometer was funded by NSF grant DBIO-722538. H.Y., Y.L., and X.C. are co-founders of Glycobub, Inc., a company focused on the development of carbohydrate-based reagents, diagnostics, and therapeutics. D.A.M. is a co-founder of Evolve Biosystems, a company focused on diet-based manipulation of the gut microbiota. Glycobub, Inc. and Evolve Biosystems played no role in the design, execution, interpretation, or publication of this study.

Notes and references