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Search for bacterial α1,2-fucosyltransferases for whole-cell biosynthesis of 2′-fucosyllactose in recombinant *Escherichia coli*

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ABSTRACT

2′-Fucosyllactose (2′-FL) is the most abundant human milk oligosaccharide and is important for infant nutrition and health. Because 2′-FL has potential as a functional ingredient in advanced infant formula and as a prebiotic in various foods, a cost-effective method for 2′-FL production is desirable. α1,2-Fucosyltransferase (α1,2-FT) is one of the key enzymes enabling the microbial biosynthesis of this complex sugar. However, the α1,2-FTs reported so far for the whole-cell biosynthesis of 2′-FL originate from pathogens, posing a potential hurdle for approval as a food production method depending on countries. In this study, 10 α1,2-FT genes from bacteria of biosafety level one were identified, and the main features of the deduced amino acid sequences were characterized. Four codon-optimized α1,2-FT genes were synthesized and introduced into *Escherichia coli* ΔL M15 strain containing the plasmid pBCGW encoding guanosine 5′-diphosphate-L-fucose biosynthetic enzymes. Among the four genes, 2′-FL was produced only by the α1,2-FT from *Thermosynechococcus elongatus* (Te2FT). *Bifidobacterium thermacidophilum* α1,2-FT (Bt2FT) showed high expression but was not active in *E. coli* ΔL M15. The other two α1,2-FTs were not expressed to a detectable level. During batch flask fermentation of Te2FT-expressing *E. coli* ΔL M15 cells, 0.49 g/L 2′-FL was obtained after 72 h of induction. This is comparable to the values previously reported for α1,2-FTs from *Helicobacter pylori* and *Bacteroides fragilis*.

1. Introduction

For a period of time, a mother's milk is the sole source of macro- and micronutrients for breast-fed infants. Human milk oligosaccharides (HMOs) are a group of complex carbohydrates unique to breast milk. HMOs pass the gut and small intestine undigested (Castanys-Munoz et al., 2013). However, they maintain the health of newborns in several ways. In addition to their prebiotic effects, HMOs exhibit protective activity against pathogenic bacteria and their toxins, contributing significantly to the prevention of infectious diseases in the gut, respiratory and urinary tracts of infants (Bode, 2015; Castanys-Munoz et al., 2013; Newburg et al., 2005). Nearly half of the total HMOs are fucosylated. Alpha-1,2-linked fucose is a major structural component of 2′-fucosyllactose (2′-FL), which is one of the key HMOs constituting up to 30% of their total amount in secretor women (Petschacher and Nidetzky, 2016; Sprenger et al., 2017).

Numerous studies have been performed to gain insight into the variability of HMOs in the milk of women living in different geographical regions. Although 2′-FL is the most abundant HMO, it was detected in only 46% of milk samples collected from some geographic populations (Castanys-Munoz et al., 2013). Furthermore, clinically, quantitative variation in 2′-FL may affect the capacity of human milk to protect a nursing infant (Chaturvedi et al., 2001). A low content of this fucosyl-oligosaccharide in the milk of sore mothers is associated with an increased risk of bacterial diarrhea in breast-fed infants, which is among the most common causes of infant mortality (Morrow et al., 2004). Because of its various beneficial effects on infant health, 2′-FL has several promising applications, for instance, supplementation of neonate formulas as a prebiotic, and anti-adhesive antimicrobial agent (Bode, 2015; Castanys-Munoz et al., 2013; Newburg et al., 2005; Petschacher and Nidetzky, 2016; Sprenger et al., 2017). The nutraceutical and pharmaceutical potential of this functional

Abbreviations: Amp, ampicillin; α1, 2-FT, α1,2-fucosyltransferase; 2′-FL, 2′-fucosyllactose; GDP-L-fucose, guanosine 5′-diphosphate-L-fucose; GRAS, generally recognized as safe; HMO, human milk oligosaccharide; IPTG, isopropyl β-D-1-thiogalactopyranoside; Kan, kanamycin; TMD, transmembrane domain

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oligosaccharide necessitates the development of a method for its large-scale production.

The usage of this functional oligosaccharide is limited mainly because of its high production cost. Therefore, efficient and economic production methods should be developed to produce the necessary (*i.e.*, tons/year) quantities of 2′-FL. Human milk can almost be excluded as source material because collection of breast milk and extraction of 2′-FL from it is extremely tedious and raises ethical issues. Extracting this compound from animal milk is not an economically feasible option as only trace amounts of fucosylated oligosaccharides are usually present in it (Bode et al., 2016; Petschacher and Nidetzky, 2016; Sprenger et al., 2017). Chemical synthesis is possible but challenging at this moment because of the complex multistep routes and toxic reagents required (Han et al., 2012; Petschacher and Nidetzky, 2016; Sprenger et al., 2017; Zhao et al., 2017). The disadvantages of chemical synthesis might be overcome by the enzymatic synthesis. Therefore, the use of enzymes in the synthesis of HMOs has attracted growing interest as an alternative to chemical synthesis. As a pioneering work, Albermann and co-workers reported the enzymatic formation of 2′-FL by using the *Helicobacter pylori* α1,2-fucosyltransferase, enzymatically synthesized GDP-L-fucose and lactose (Albermann et al., 2001). Currently, development of one-pot multienzyme-catalyzed syntheses methods is still needed to efficiently reduce substrate costs for *in vitro* production of fucosylated HMOs. Chemo-enzymatic approaches also offer several different opportunities to produce HMOs (Bode et al., 2016; Sprenger et al., 2017; Zhao et al., 2017). Despite the clear advantages offered by enzyme-catalyzed and chemo-enzymatic syntheses, the production or at least partial purification of the enzymes needed are labor-intensive and scale-up is challenging (Bode et al., 2016; Han et al., 2012; Petschacher and Nidetzky, 2016; Sprenger et al., 2017; Zhao et al., 2016, 2017). In recent years, fermentative production of 2′-FL using genetically engineered strains of microorganisms has gained much interest. Various aspects of the microbial synthesis of 2′-FL are covered in several excellent reviews (Bode et al., 2016; Han et al., 2012; Petschacher and Nidetzky, 2016; Sprenger et al., 2017). There are two main advantages of this *in vivo* approach: (1) enzymes are synthesized in the cells and (2) the fucosyl donor (GDP-L-fucose) is provided by cellular metabolism from inexpensive carbon and energy sources.

GDP-L-fucose can be derived from two metabolic pathways: the *de novo* or the *salvage* pathway (Andrianopoulos et al., 1998; Coyne et al., 2005; Stevenson et al., 1996). The *de novo* pathway is naturally present in *E. coli* and branches off from fructose-6-phosphate. In the *salvage* pathway, exogenous L-fucose is converted inside the cells to GDP-L-fucose by a bifunctional enzyme L-fucose kinase/L-fucose-1-phosphate guanylyltransferase (Fkp). In the past, GDP-L-fucose has only been produced by expensive chemical synthesis. Albermann and co-workers have established a basis for both *in vitro* enzymatic production and for *in vivo* fucosylation system (Albermann et al., 2000). Later, several research teams demonstrated different successful strategies for enhanced production of intracellular GDP-L-fucose in engineered microbial hosts (Baumgärtner et al., 2013; Byun et al., 2007; Drouillard et al., 2006; Dumon et al., 2006; Huang et al., 2017; Lee et al., 2009, 2011,). The *salvage* pathway has been employed as an alternative or in addition to the *de novo* pathway (Baumgärtner et al., 2013; Chin et al., 2016; Guan et al., 2018; Hüfner et al., 2011). In particular, expression of the *fkp* gene from *Bacteroides fragilis* in *E. coli*, uptake of exogenous L-fucose and knock out of *fucI* and *fucK* genes to prevent its degradation resulted in an accumulation of sufficient amount of endogenous GDP-L-fucose and synthesis of 23.1 g/L of 2′-FL by engineered *E. coli* during fed-batch fermentation in a bioreactor (Chin et al., 2016). This concentration of 2′-FL was almost in the same range with 2′-FL produced in large-scale fed-batch cultivation *via* GDP-L-fucose *de novo* pathway reported earlier by Baumgärtner et al. (2013). It is noteworthy that in the case of 2′-FL synthesis, the formation of GDP-L-fucose is an energy-requiring process. Guan et al. (2018) reported that TCA cycle intermediate can be used as an alternative energy source to power 2′-FL synthesis in whole-cell biocatalysis. Yu et al. (2018) have demonstrated for the first time that 2′-FL - so far produced by engineered *E. coli* - can also be produced by metabolically engineered *Saccharomyces cerevisiae via* the *salvage* pathway, resulting in 0.092 g/L and 0.503 g/L of 2′-FL during batch and fed-batch fermentations, respectively.

In addition to intracellular GDP-L-fucose, availability of the lactose in sufficient amounts has to be secured for effective microbial production of 2′-FL. To block further lactose metabolism in whole cells of *E. coli*, β–galactosidase gene (*lacZ*) was partially disrupted or deleted while the lactose permease gene (*lacY*) was overexpressed in order to enhance lactose uptake and subsequently improve 2′-FL production (Baumgärtner et al., 2013; Chin et al., 2015; Huang et al., 2017).

Finally, α1,2-FT, the key enzyme, which transfers the fucosyl residue from the donor substrate (GDP-L-fucose) onto the acceptor molecule (lactose) during the final step of 2′-FL biosynthesis, needs to be introduced into a microbial host (Ma et al., 2006). Previously, several bacterial α1,2-FTs were successfully cloned and characterized by different research groups. These include *H. pylori* FutC (Huang et al., 2017; Wang et al., 1999), *E. coli* O128 WbsJ (Shao et al., 2003), *E. coli* O86:B7 WbwK (Li et al., 2008), and *E. coli* O127:K63(B8) WbiQ (Pettit et al., 2010). However, the expression levels of the genes were typically low, limiting their application in 2′-FL synthesis. The more recently characterized *E. coli* O126 WbgL showed a reasonable expression level but had a preference towards β1-4-linked galactosides as acceptor substrates (Engels and Elling, 2014). *Bacteroides fragilis* WcfB, reported by Chin et al. (2017), showed a promising level of 2′-FL production; however, the bacterial source of the gene is not regarded as safe for humans. Huang et al. (2017) reported several α1,2-FTs from other biosafety level two bacteria, such as FutL from *Helicobacter mustelae*, FutF from *Helicobacter bilis*, FutG from *Campylobacter jejuni*, and FutN from *Bacteroides vulgatus* ATCC 8482. It is noteworthy that several companies, for instance, Glycom A/S (Denmark), Jennewein Biotechnologie GmbH (Germany), Glycosyn, LLC (USA) and Friesland Campina (Netherlands) have registered 2′-FL produced by recombinant *E. coli* strains, expressing known α1,2-FT gene, for GRAS status with the U.S. FDA and for novel food application in the European Union, with the aim to use this compound as novel ingredient for infant and follow-on formula (Bode et al., 2016; EFSA NDA Panel, 2015; Sprenger et al., 2017; U.S. FDA, 2016a, 2016b, 2018). As previously characterized α1,2-FT genes are mostly from pathogens the use of these genes in the production host may pose a potential hurdle for approval from authorities depending on countries (Guo, 2014). Therefore, search for active α1,2-FT genes, preferably originating from safe microorganisms that naturally exist in the human gastrointestinal tract, became of interest.

To facilitate safer biotechnological production of 2′-FL, the aims of the present study were to (1) identify α1,2-FT genes from various bacteria of biosafety level one or generally recognized as safe (GRAS) and (2) evaluate their functional capacities in terms of 2′-FL production in an *E. coli* whole-cell system.

2. Materials and methods

2.1. Strains and plasmids

The strains and plasmids used in this study are listed in Table 1. *E. coli* TOP10 and *E. coli* BL21Star (DE3) cells from Invitrogen (Carlsbad, CA, USA) and *E. coli* ΔL M15 cells (Chin et al., 2015) were used for genetic manipulation, gene expression testing, and 2′-FL production, respectively (Table 1). *E. coli* TOP10 and *E. coli* BL21Star (DE3) were grown at 37 °C in Luria–Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 1% sodium chloride) or agar supplemented with appropriate antibiotics (50 μg/mL) when needed. The pUCIDT standard vector (Integrated DNA Technologies, Coralville, IA, USA) and pCOLADuet-1 expression vector (Novagen, Madison, WI, USA) were used for cloning and sub-cloning, respectively. The plasmid pBCGW was previously constructed for overexpression of the genes for GDP-L-fucose

Table 1
Strains and plasmids used in this study.

Name	Description	Source
E. coli strains		
TOP10	F^-, mcrA Δ(*mrr-hsd*RMS-*mcr*BC) *φ*80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen (USA)
BL21Star (DE3)	F^-, *omp*T *hsd*SB($r_B{}^- m_B{}^-$), *gal*, *dcm rne*131 (DE3)	Invitrogen (USA)
ΔL M15	BL21Star (DE3) Δ*lac*ZYA Tn7::*lacZ*ΔM15YA	Chin et al. (2015)
Plasmids		
pUCIDT	pMB1 replicon, Amp^R	Integrated DNA Technologies (USA)
pETDuet-1	Two T7 promoters, pBR322 replicon, Amp^R	Novagen (USA)
pCOLADuet-1	Two T7 promoters, ColA replicon, Kan^R	Novagen (USA)
pBCGW	pETDuet-1 + *man*C-*man*B (*Nco*I/*Sac*I) + *gmd-wca*G (*Nde*I/*Kpn*I)	Lee at al. (2009)
pAs2FT	pCOLADuet-1 + As2FT gene from *Acetobacter* sp. CAG:267	This study
pBt2FT	pCOLADuet-1 + Bt2FT gene from *Bifidobacterium thermacidophilum*	This study
pLa2FT	pCOLADuet-1 + La2FT gene from *Lactococcus raffinolactis*	This study
pTe2FT	pCOLADuet-1 + Te2FT gene from *Thermosynechococcus elongatus*	This study

biosynthetic enzymes (Lee et al., 2009). Recombinant DNA techniques were performed according to standard procedures (Sambrook and Russell, 2001).

2.2. Sequence analysis

Homology searching was conducted using a standard protein-BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The amino acid sequences of known α1,2-FTs, such as FutC from *Helicobacter pylori* 26,695 (Huang et al., 2017), WcfB from *Bacteroides fragilis* NCTC 9343 and Fut2 from *Homo sapiens* (Chin et al., 2017), were used as queries to identify potential candidates from safe bacterial sources. Based on the query sequences, a list of 10 α1,2-FT protein sequences from safe bacteria was generated. Next, amino acid sequences of known and candidate α1,2-FTs were aligned using the ClustalW program. The neighbor-joining tree was constructed based on multiple sequence alignment conducted with the MEGA 4.0 computer program (Tamura et al., 2007). The absence of an N-terminal signal sequence and transmembrane domain (TMD) in identified α1,2-FTs was predicted by the Protter version 1.0 program (http://wlab.ethz.ch/protter/start/).

2.3. DNA techniques

Full-length genes for selected α1,2-FTs with codons optimized for *E. coli* expression were custom-synthesized by Cosmogenetech Co., Ltd. (Seoul, Korea) based on sequence data. After gene synthesis, PCR was performed using relevant pairs of primers (Table A.1). PCR for amplifying the target genes was performed in a 50 μL reaction mixture containing 1 μL plasmid DNA (10 ng), 5 μL forward and reverse primers (10 pmole each), 4 μL dNTPs (2.5 mM each), 10 μL 5× buffer, 1 μL IP-Pfu DNA polymerase (LaboPass™, Cosmogenetech), and 24 μL molecular-grade water. The PCR procedure included an initial cycle of 5 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 55 °C, and 1 min at 72 °C, with a final extension step at 72 °C for 7 min. The resulting PCR products were purified and digested with 2 U of restriction enzymes *Nde*I and *Kpn*I (New England BioLabs, Inc., Ipswich, MA, USA) at 37 °C overnight using 10× NEBuffer. The purified and digested PCR products were then ligated with the predigested expression vector pCOLADuet-1. The recombinant plasmids were transformed into chemically competent *E. coli* TOP10 cells, and the cells were plated on LB-agar medium containing kanamycin (50 μg/mL). Positive clones were selected after screening by direct PCR amplification of colonies and verified by DNA sequencing (data not shown).

2.4. Culture conditions

To produce 2′-FL, pBCGW harboring genes for GDP-L-fucose synthesis (Lee et al., 2009) and pCOLADuet-1 harboring an appropriate α1,2-FT candidate gene were transformed into *E. coli* ΔL M15 chemically competent cells and co-expressed. After transformation of the two plasmids, transformants were selected on LB-agar medium containing ampicillin (50 μg/mL) and kanamycin (50 μg/mL). For batch fermentations, the recombinant plasmid-bearing *E. coli* cells were cultured in a 250-mL flask containing 50 mL of defined medium [13.5 g/L KH_2PO_4, 4.0 g/L $(NH_4)_2HPO_4$, 1.7 g/L citric acid, 1.4 g/L $MgSO_4{\cdot}7H_2O$, 10 mL/L trace element solution (10 g/L Fe(III) citrate, 2.25 g/L $ZnSO_4{\cdot}7H_2O$, 1.0 g/L $CuSO_4{\cdot}5H_2O$, 0.35 g/L $MnSO_4{\cdot}H_2O$, 0.23 g/L $Na_2B_4O_7{\cdot}10H_2O$, 0.11 g/L $(NH_4)_6Mo_7O_{24}$, 2.0 g/L $CaCl_2{\cdot}2H_2O$), pH 6.8] (Chin et al., 2015) supplemented with 20 g/L glycerol, ampicillin (50 μg/mL) and kanamycin (50 μg/mL) at 37 °C. The flasks were agitated at 250 rpm for the aerobic condition. When the cell optical density (OD) at 600 nm reached approximately 0.5, isopropyl β-D-1-thiogalactopyranoside (IPTG) and lactose were added at final concentrations of 0.1 mM and 5 g/L, respectively, followed by incubation at 25 °C for 84 h with agitation at 200 rpm in a shaking microbiological incubator.

2.5. Analytical methods

Cell growth was measured by recording the OD at 600 nm using a Biomate 3S UV–vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The dry cell weight was obtained by multiplication of a predetermined conversion factor of 0.36 (Chin et al., 2015) by the optical density. The concentrations of 2′-FL, lactose, and glycerol in the samples were measured with a Waters high-performance liquid chromatography (HPLC) system (Milford, MA, USA) equipped with a refractive index (RI) detector and carbohydrate analysis column (Rezex ROA-Organic Acid H^+, Phenomenex, Torrance, CA, USA). The column was eluted with 0.01 N H_2SO_4 at a flow rate of 0.6 mL/min at 50 °C (Lee et al., 2012). 2′-FL used in this study as an external standard was obtained from APTechnology (Suwon, Korea).

For extracellular metabolite (lactose, glycerol, 2′-FL) determination, fresh culture broth (1 mL) was centrifuged at 13,000 rpm for 10 min, and the supernatants were used for quantitation. To measure intracellular 2′-FL, the cell pellets collected from 5 mL of culture broth were resuspended in 2 mL of water, boiled for 10 min, and then centrifuged at 13,000 rpm for 10 min. The resulting supernatant was analyzed to determine its intracellular 2′-FL content (Huang et al., 2017). 2′-FL and other metabolites were identified using external standards. Statistical analysis of the obtained experimental results was performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA).

2.6. Analysis of expression patterns of α1,2-FT genes

Plasmid pCOLADuet-1 harboring an appropriate α1,2-FT gene was transformed into *E. coli* BL21Star (DE3) chemically competent cells, and

transformants were obtained on LB-agar plates containing kanamycin (50 μg/mL). Cultivation was performed in a 250-mL baffled flask containing 50 mL of LB medium with kanamycin (50 μg/mL) at 37 °C. The agitation speed was maintained at 250 rpm. When the cell optical density (OD_{600}) reached approximately 0.5, expression of the α1,2-FT gene was induced by adding 0.1 mM IPTG, and both the temperature and agitation speed were adjusted to 16 °C and 200 rpm, respectively. After 20 h of induction, the cells were collected by centrifugation, and their concentration was adjusted to an OD_{600} of 7 by dilution. The cells were resuspended in phosphate-buffered saline (pH 7.4) and then disrupted using an ultrasonic processor (1 s ON/3 s OFF for a total of 1 min on ice), after which the total protein fraction was collected. After centrifugation at 13,000 rpm for 20 min at 4 °C, the supernatant (soluble fraction) and debris (insoluble fraction) were collected separately. Ten microliters of the total protein fraction and the same volume of soluble fraction were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 10% polyacrylamide) to analyze the expression levels of α1,2-FT genes.

3. Results and discussion

3.1. Identification of biosafety level one bacterial α1,2-FTs

α1,2-FTs are involved in the final step of 2′-FL formation and represent one of the rate-limiting factors towards its *in vivo* synthesis (Bode et al., 2016; Han et al., 2012; Petschacher and Nidetzky, 2016; Sprenger et al., 2017). In the present study, 10 α1,2-FTs from various bacteria belonging to biosafety level one or GRAS were identified by comparing their complete amino acid sequences with protein sequences of the known α1,2-FTs, such as FutC from *H. pylori* 26,695 (Huang et al., 2017), WcfB from *B. fragilis* NCTC 9343, and Fut2 from *H. sapiens* (Chin et al., 2017) (Table 2). Protein-BLAST search was conducted, and sorting by percent identity to known α1,2-FTs was performed from the highest to the lowest value. It is noteworthy that protein sequence of WcfB from *B. fragilis* NCTC 9343 was 28% identical to the *H. pylori* 26,695 FutC, while the percentage of amino acid sequence identity of Fut2 from *H. sapiens* to FutC was 25%. Amino acid sequence alignment of As2FT from *Acetobacter* sp. CAG:267, Te2FT from *Thermosynechococcus elongatus*, and Bt2FT from *Bifidobacterium thermacidophilum* revealed 26%–39%, 24%–31%, and 24%–28% homology, respectively, compared to previously characterized α1,2-FTs, such as FutC, WcfB, and Fut2.

Based on the results of multiple sequence alignment of protein sequences of known and candidate α1,2-FTs using ClustalW, a neighbor-joining tree was constructed (Fig. 1). As shown in Fig. 1, the protein sequence of As2FT from *Acetobacter* sp. CAG:267 was most closely related to known *H. pylori* FutC, while Te2FT from *T. elongatus* was more closely related to *B. fragilis* WcfB.

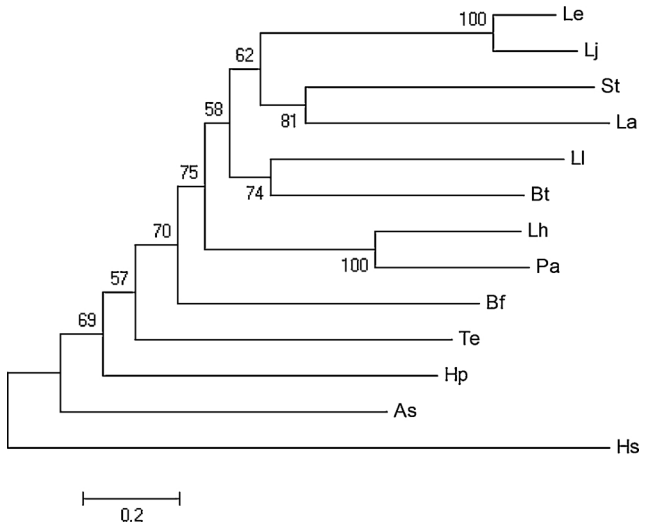

Fig. 1. Neighbor-joining tree of known and candidate α1,2-FTs from various sources based on protein sequence comparison. Abbreviations of α1,2-FT sources and NCBI accession numbers are as follows: Hs – *Homo sapiens* (AAC24453.1), Hp - *Helicobacter pylori* 26695 (ARB16053.1), Bf - *Bacteroides fragilis* NCTC 9343 (CAH06753.1), As - *Acetobacter* sp. CAG:267 (CDA17967.1), Te - *Thermosynechococcus elongatus* (WP_011056838.1), Lh - *Lactobacillus helveticus* (WP_072748791.1), Pa - *Propionispira arboris* (WP_091828428.1), Le - *Lactobacillus reuteri* (WP_086131601.1), Lj - *Lactobacillus johnsonii* (ABM21404.1), Ll - *Lactococcus lactis* (WP_010891358.1), St - *Streptococcus thermophiles* (WP_011226034.1), La - *Lactococcus raffinolactis* (WP_061773638.1), Bt - *Bifidobacterium thermacidophilum* (WP_033506803.1).

3.2. Features of the identified α1,2-FTs

Ten candidate α1,2-FTs identified by protein-BLAST searching were further analyzed for the presence of a signal sequence, which marks the protein for export from the cell. A signal sequence was not detected in these proteins, suggesting that they are intracellular (Table 2). For large-scale industrial 2′-FL production in bacterial hosts, such as *E. coli*, the availability of soluble fucosyltransferases is crucial. Previous studies showed that a TMD is common to eukaryotic fucosyltransferases and is typically located in the N-terminal region of the protein. This transmembrane segment renders mammalian fucosyltransferases insoluble (Petschacher and Nidetzky, 2016). Although some *E. coli* α1,2-FTs were reported to have a hydrophobic N-terminus (Engels and Elling, 2014) or short inner transmembrane domains (Li et al., 2008; Pettit et al., 2010), our results showed that 10 candidate α1,2-FTs do not contain a TMD (Table 2). Therefore, these proteins are likely soluble.

In most living organisms, disulfide bonds do not typically stabilize intracellular proteins and are mostly used to stabilize extracellular

Table 2
Main characteristics of candidate α1,2-FTs in comparison with known ones.

No	Source	Alias	Protein sequence length	Signal sequence	TMD	Cysteine residues	Proline residues
1	*Lactococcus raffinolactis*	La2FT	328 aa	–	–	3	8
2	*Acetobacter* sp. CAG:267	As2FT	308 aa	–	–	3	15
3	*Lactobacillus helveticus*	Lh2FT	301 aa	–	–	5	12
4	*Thermosynechococcus elongatus*	Te2FT	293 aa	–	–	5	16
5	*Streptococcus thermophiles*	St2FT	330 aa	–	–	6	5
6	*Bifidobacterium thermacidophilum*	Bt2FT	308 aa	–	–	6	15
7	*Lactococcus lactis*	Ll2FT	309 aa	–	–	7	8
8	*Lactobacillus reuteri*	Le2FT	313 aa	–	–	8	7
9	*Lactobacillus johnsonii*	Lj2FT	314 aa	–	–	8	8
10	*Propionispira arboris*	Pa2FT	300 aa	–	–	11	7
11	*Homo sapiens*	Fut2	343 aa	–	+	3	20
12	*Bacteroides fragilis* NCTC 9343	WcfB	287 aa	–	–	7	10
13	*Helicobacter pylori* 26,695	FutC	300 aa	–	–	7	16

Note: aa – amino acids; TMD – transmembrane domain. Symbol "+" stands for present, "-" stands for absent.

proteins. However, *E. coli* produces relatively fewer extracellular proteins and thus do not contain large numbers of disulfide bonds. As the sulfhydryl groups of two cysteines can form a disulfide bond, the number of cysteines in protein sequences of candidate α1,2-FTs was also considered. Table 2 shows that the protein sequences of La2FT from *Lactococcus raffinolactis* and As2FT from *Acetobacter* sp. CAG:267 contained the smallest number of cysteine residues (3), while the largest number of this amino acid (11) was found in the sequence of Pa2FT from *Propionispira arboris*.

Some heterologous proteins expressed in *E. coli* fail to fold into their native state, but rather accumulate in the form of biologically inactive inclusion bodies that must be converted to active proteins by a refolding process. No three-dimensional structure is available for α1,2-FTs; therefore, the folding behavior of these enzymes can only be predicted (Petschacher and Nidetzky, 2016). *Cis-trans* isomerization of Xaa-Pro peptide bonds (where Xaa is the preceding amino acid) in oligopeptides is a key rate-determining process during the folding pathway of many proteins (Ou et al., 2001; Yang et al., 1997). Therefore, the number of prolines in the protein sequences of candidate α1,2-FTs was also determined (Table 2). The amino acid sequence of Te2FT from *T. elongatus* contained the largest number of proline residues (16).

3.3. Performance evaluation of the synthesized α1,2-FT genes

Based on the homology search results and main characteristics of the 10 candidates listed in Table 2, the genes of four potential α1,2-FTs were selected and synthesized commercially after codon optimization to *E. coli*. The synthesized genes were sub-cloned into the pCOLADuet-1 expression vector (Fig. 2A). The plasmid containing the gene of interest was co-expressed with pBCGW, harboring genes for GDP-L-fucose biosynthetic enzymes.

Intracellular availability of lactose is critical for whole-cell biosynthesis of 2′-FL. Wild-type *E. coli*, such as BL21(DE3) type strains, contain a highly active β-galactosidase (encoded by *lacZ*), which hydrolyzes lactose into glucose and galactose. Therefore, most of the lactose is utilized for cell growth rather than for fucosyllactose production. Previously, *E. coli* JM109 strain, which has no detectable β-galactosidase activity, has been employed to ensure the complete conversion of the lactose, assimilated *via* lactose permease (LacY), to 2′-FL (Baumgärtner et al., 2013). Later, Chin et al. (2015) constructed *E. coli* ΔL M15 strain, derived from *E. coli* BL21Star (DE3). To improve 2′-FL production, the endogenous *lac* operon has been replaced with the modified *lac* operon bearing *lacZΔM15* for an alleviation of β-galactosidase activity. The results obtained by Huang et al. (2017) also confirmed that lactose was the key point in the 2′-FL biosynthetic pathway and that facilitation of lactose uptake and the blocking of lactose catabolism was an efficient approach to enrich 2′-FL production. To evaluate α1,2-FT activity in the present study, the production levels of extracellular 2′-FL were determined in the culture medium during batch fermentation of *E. coli* ΔL M15 expressing the synthesized genes (Fig. 2B–E). In accordance with the results reported by Chin et al. (2015), the *E. coli* ΔL M15 host strain used in our study showed very low lactose consumption rate (Fig. 2B–E).

It is common practice to use glucose and/or glycerol as the carbon and energy sources in microbial fermentation processes. Because acetic acid is overproduced using glucose as a carbon source during

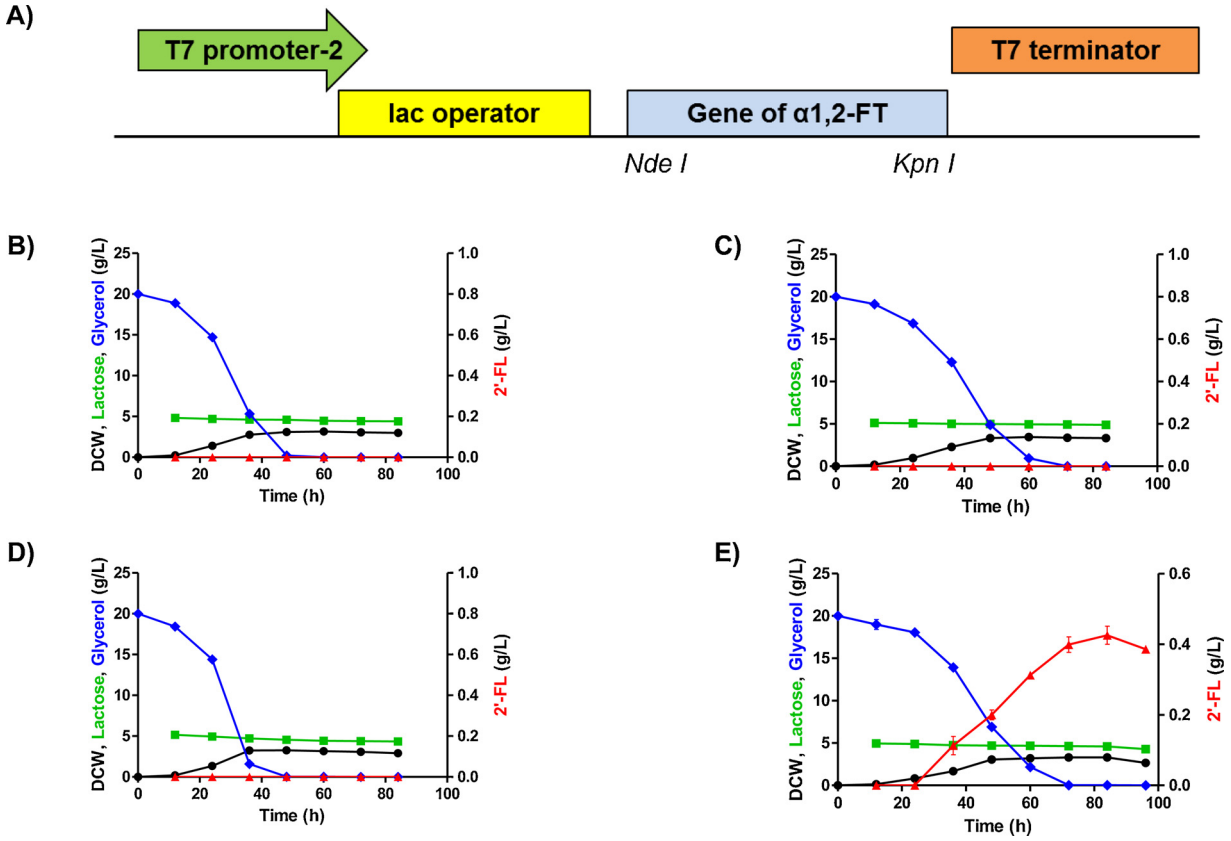

Fig. 2. Schematic of construction of pCOLADuet-1 expression vector with candidate α-1,2-fucosyltransferase gene (A) and fermentation profiles of extracellular 2′-FL production by *E. coli* ΔL M15 harboring plasmids: (B) pBCGW and pAs2FT, (C) pBCGW and pBt2FT, (D) pBCGW and pLa2FT, (E) pBCGW and pTe2FT. The important elements in the cloning region are shown and corresponding restriction sites are labeled, respectively. When OD_{600} reached approximately 0.5, IPTG (0.1 mM) and lactose (5 g/L) were added to initiate 2′-FL production. For *E. coli* ΔL M15 harboring pBCGW and pTe2FT, the highest concentration of extracellular 2′-FL was 0.426 ± 0.018 g/L. Symbols are denoted as follows: dry cell weight, ●; lactose, ■; glycerol, ◆; 2′-FL, ▲. Error bars represent standard deviations associated with three independent experiments ($p < 0.05$).

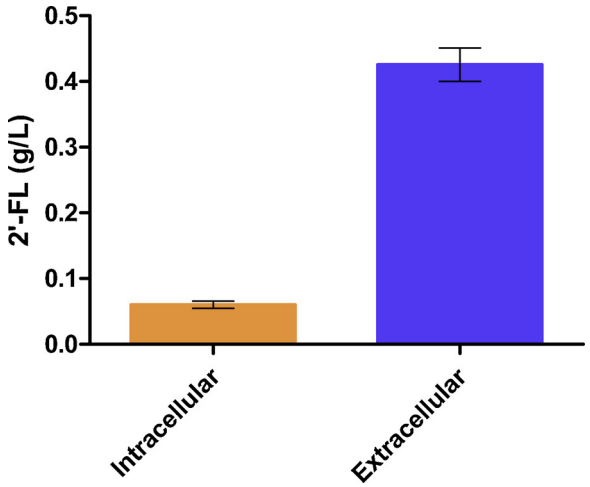

Fig. 3. Production of intracellular (0.060 ± 0.003 g/L) and extracellular (0.426 ± 0.018 g/L) 2′-FL by *E. coli* ΔL M15 co-expressing pBCGW and pTe2FT after 72 h of 0.1 mM IPTG induction. Error bars represent standard deviations associated with three independent experiments ($p < 0.05$).

fermentation the concentration of glucose should be regulated under a certain level to avoid acetic acid accumulation. Glucose can be considered as a carbon source when its feeding can be tightly controlled by using fermenter. Because glycerol does not evoke acetic acid accumulation it was used as carbon source in the flask cultures in this study. Furthermore, as glycerol is known to be an inexpensive substrate, and also a by-product generated from biodiesel production processes, usage of microbial systems capable to utilize glycerol for cell growth during formation of value-added products, can be advantageous and promising (Yazdani and Gonzalez, 2007). Utilizing cheaper raw materials in the medium might be essential for the production of 2′-FL in a cost-effective manner. Earlier, in order to demonstrate an upscale of the 2′-FL synthesis, fed-batch cultivation of the engineered *E. coli* JM109 strain with glycerol as carbon and energy source and lactose was conducted by Baumgärtner et al. (2013). As a result, 2′-FL concentration of 20.28 ± 0.83 g/L could be achieved. Our results demonstrated that *E. coli* ΔL M15 strain, used in this study, grew well on the defined medium that contains mostly simple salts and glycerol as a carbon source (Chin et al., 2015), which is advantageous.

During batch fermentation in our study, the concentration of 2′-FL in the culture medium was monitored by HPLC analysis. According to our results, extracellular 2′-FL was not synthesized in the control strain co-expressing pBCGW and pCOLADuet-1 plasmids. Among the α1,2-FT genes tested, 2′-FL was detected only in the culture expressing the Te2FT gene from *T. elongatus*. The highest production of extracellular 2′-FL of 0.426 ± 0.018 g/L was observed for *E. coli* ΔL M15 harboring pBCGW and pTe2FT after 72 h of induction with 0.1 mM IPTG. The results of HPLC analyses showed no peak for 2′-FL in cultures expressing the other synthesized genes. This may be because of low gene expression levels or the extracellular 2′-FL concentration may have been below the detection limit of the method used in this study. Furthermore, not all α1,2-FTs can utilize lactose as an acceptor of fucosyl residue for 2′-FL biosynthesis (McCoy et al., 2017). Notably, the biomass of the *E. coli* ΔL M15 expressing synthesized genes was similar to that of the control strain, indicating that the host strain was not influenced by the expression of these genes.

Next, 2′-FL production by the Te2FT gene in *E. coli* ΔL M15 was compared to that by known α1,2-FT genes. According to Chin et al. (2015), the same strain expressing *fut*C from *H. pylori* produced 0.160 g/L extracellular 2′-FL in batch fermentation using defined medium with glycerol a carbon source. This corresponds to a nearly 2.7-times lower 2′-FL level compared to that of Te2FT used in the present study. This large enhancement of 2′-FL biosynthesis by *E. coli* ΔL M15 may be related to the high activity of Te2FT. It is noteworthy that prior to Chin et al. (2015), with construction of the expression plasmid-free *E. coli* JM109 gwBC-F1 strain with only one *futC* copy on the chromosome, and the subsequent shake flask cultivations, Baumgärtner et al. (2013) achieved 0.206 g/g DCW 2′-FL from lactose in minimal medium with glycerol. After work of Chin et al. (2015), Huang et al. (2017) reported several functionally proven α1,2-FT genes. In their study, the batch fermentations of engineered *E. coli* JM109(DE3) transformed with pET-CBGF (carrying *man*C, *man*B, *gmd*, and *fcl*), pACYC-*man*A, and pCDFDuet-1 (carrying the gene of interest) was performed in LB medium using glucose as a carbon source, and the cells were treated with 0.1 mM IPTG to induce the expression of the heterologous genes. In accordance with their results, overexpression of *fut*C resulted in the highest total 2′-FL level (0.41 g/L), 51% of which was extracellular. Other candidates led to titers of 2′-FL equal to approximately 75% or less of the total level attained with *fut*C. Compared to *futC* reported by Huang et al. (2017), the amount of extracellular 2′-FL in *E. coli* ΔL M15 expressing the gene for Te2FT, which was used in our study, was 2-fold higher. Interestingly, the extracellular 2′-FL production level of WcfB reported by Huang et al. (2017) was nearly 8.5-fold lower than that reported by Chin et al. (2017). This is likely because different *E. coli* strains were used in their studies. Recently, to produce 2′-FL *via* the *salvage* pathway in engineered yeast, Yu et al. (2018) overexpressed in *S. cerevisiae* D452-2 three heterologous genes such as *fkp, fucT2, and LAC12* coding for *B. fragilis* 9343 FKP, *H. pylori* α1,2-FT, and *Kluyveromyces lactics* lactose permease, respectively. As a result, 0.092 g/L of 2′-FL was produced in flask cultures.

The amount of intracellular 2′-FL produced by *E. coli* ΔL M15 expressing synthesized α1,2-FT genes was also determined. 2′-FL was detected only in the culture expressing Te2FT from *T. elongatus*. As a result, 0.060 ± 0.003 g/L intracellular 2′-FL was obtained from the biomass of the host strain after 72 h of induction with 0.1 mM IPTG (Fig. 3). This amount was approximately 3.3-fold less than that of FutC (0.2 g/L) reported by Huang et al. (2017).

The total amount of 2′-FL produced by *E. coli* ΔL M15 expressing the gene for Te2FT was 0.486 g/L (Fig. 3), which is 1.2 fold higher than that of FutC (0.41 g/L) (Huang et al., 2017). Notably, only 12.3% of the total 2′-FL produced by Te2FT was intracellular, while in FutC this value reached 49% (Huang et al., 2017). Analysis and optimization of the downstream processing of fucosylated HMOs are important for improving the overall production, but have not been examined in detail. Final localization of the target product is considered as one of the major limitations affecting product recovery. Generally, synthesized HMOs are not only found in the culture supernatant, but also inside the cells (Petschacher and Nidetzky, 2016). Thus, the HMOs associated with the biomass must be released into the supernatant. In this case, using multiple steps for intracellular product recovery can be avoided to reduce overall production costs of the target metabolite. Our results show that very little portion of the total 2′-FL produced by *E. coli* ΔL M15 expressing Te2FT was intracellular, while 87.7% of the total 2′-FL was detected in the culture supernatant, which is advantageous for application.

3.4. Expression patterns of the selected α1,2-FTs in E. coli

To determine if 2′-FL production is related to the high expression levels of the synthesized α1,2-FT genes, the expression patterns of As2FT, Bt2FT, La2FT, and Te2FT were analyzed by SDS-PAGE. The *E. coli* BL21Star (DE3) harboring the pCOLADuet-1 plasmid was used as a negative control. Cells were harvested after 20 h of 0.1 mM IPTG induction at 16 °C. Fig. 4 demonstrates that the total expression levels of Bt2FT and Te2FT were considerably higher than those of As2FT and La2FT based on SDS-PAGE analysis of the crude extracts. Our results showed that although the bacterial α1,2-FTs selected in this study did not contain a transmembrane domain, overexpression of soluble protein in *E. coli* remains challenging. The expression level of Te2FT in the

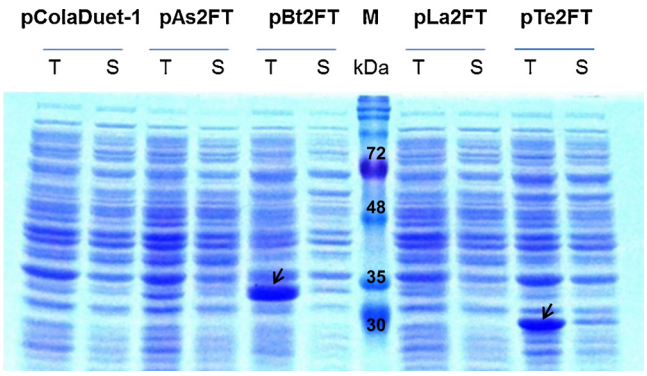

Fig. 4. SDS-PAGE analysis of the crude extracts of recombinant *E. coli* BL21Star (DE3) strains harboring pCOLADuet-1 (control), pAs2FT, pBt2FT, pLa2FT, and pTe2FT plasmids, respectively. The abbreviations were defined as follows: T, total protein fraction; S, soluble protein fraction; M, protein size marker. The arrows indicate the corresponding protein bands with the estimated molecular weights.

soluble fraction was relatively low, while most of the enzyme was found in the cell pellets (Fig. 4). Interestingly, although the total level of Bt2FT expression was as high as that of Te2FT, 2′-FL production in *E. coli* ΔL M15 cells expressing Bt2FT was not confirmed. This may be because of protein solubility and stability issues. Furthermore, acceptor specificities vary between α1,2-FTs from different sources. For instance, *H. pylori* α1,2-FTs accept a rather broad range of acceptor substrates (Wang et al., 1999). *Escherichia coli* α1,2-FTs (WbnK, WbwK, and WbiQ) show strict substrate specificity, while WbgL prefers lactose and lactulose over other substrates (Engels and Elling, 2014), which is more suitable for 2′-FL biosynthesis.

4. Conclusions

Previously, several research groups reported functionally proven α1,2-FTs. However, the bacterial sources of these genes are not regarded as safe, posing potential limitation to their use in the food industry depending on countries. In the present study, a gene screening approach was successfully utilized to identify 10 α1,2-FT candidate genes from safe bacterial sources. These enzymes were predicted to be intracellular proteins because they lack a transmembrane domain. The screening results showed that only *E. coli* ΔL M15 expressing genes for GDP-L-fucose biosynthetic enzymes and a gene for Te2FT from *T. elongatus* utilized synthesized GDP-L-fucose as a donor and available lactose as an acceptor of the fucosyl residue and produced both intracellular and extracellular 2′-FL with a total amount of 0.486 g/L. The amount of extracellular 2′-FL produced by this strain was 2.7-fold higher than that of previously characterized FutC from *H. pylori*. Only 12.3% of the total 2′-FL produced by *E. coli* ΔL M15 expressing Te2FT was associated with the biomass, while 87.7% was extracellular. The efficiency of 2′-FL biosynthesis was supported by the high expression level of Te2FT. Improved soluble expression of Te2FT using known gene expression optimization strategies may lead to higher 2′-FL production levels. The amount of this HMO may also be increased by using different enzyme engineering approaches as well as through host strain improvement. Therefore, further studies are needed to confirm this. Furthermore, the gene screening approach described herein may be useful for identifying and analyzing genes encoding other enzymes, which is important for producing functional fucosyllactoses.

Declarations of interest

None.

Ethical approval

Not required.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2019.02.009.

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