Metabolic engineering strategies of de novo pathway for enhancing 2'-fucosyllactose synthesis in Escherichia coli

Mengli Li,¹ Chenchen Li,¹ Miaomiao Hu¹ and Tao Zhang¹,²
¹State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu 214122, China.
²International Joint Laboratory on Food Science and Safety, Jiangnan University, Wuxi, Jiangsu 214122, China.

Summary

2'-Fucosyllactose (2'-FL), one of the most abundant human milk oligosaccharides (HMOs), is used as a promising infant formula ingredient owing to its multiple health benefits for newborns. However, limited availability and high-cost preparation have restricted its extensive use and intensive research on its potential functions. In this work, a powerful Escherichia coli cell factory was developed to ulteriorly increase 2'-FL production. Initially, a modular pathway engineering was strengthened to balance the synthesis pathway through different plasmid combinations with a resulting maximum 2'-FL titre of 1.45 g l⁻¹. To further facilitate the metabolic flux from GDP-L-fucose towards 2'-FL, the CRISPR-Cas9 system was utilized to inactivate the genes including lacZ and wcaJ, increasing the titre by 6.59-fold. Notably, the co-introduction of NADPH and GTP regeneration pathways was confirmed to be more conducive to 2'-FL formation, achieving a 2'-FL titre of 2.24 g l⁻¹. Moreover, comparisons of various exogenous x1,2-fucosyltransferase candidates revealed that futC from Helicobacter pylori generated the highest titre of 2'-FL. Finally, the viability of scaled-up production of 2'-FL was evidenced in a 3 l bioreactor with a maximum titre of 22.3 g l⁻¹ 2'-FL and a yield of 0.53 mole 2'-FL mole⁻¹ lactose.

Introduction

Human milk oligosaccharides (HMOs), the third largest solid component of breast milk (5–15 g l⁻¹) after fat and lactose, play an irreplaceable role in the growth and development of neonatal (Gonia et al., 2015). Increasing animal experiments and clinical trials have confirmed the beneficial properties of HMOs, such as maintaining intestinal homeostasis as probiotics, resisting the adhesion of pathogenic bacteria, modulating immune responses and promoting the development and repair of the nervous system (Weichert et al., 2013; Bych et al., 2019). Notably, 2'-fucosyllactose (2'-FL) has been proved to be the most abundant oligosaccharide in HMOs (Castanyas-Muñoz et al., 2013). 2'-FL is approved for addition to infant formulas, dietary supplements and medical foods by U.S. Food and Drug Administration (FDA) and the European Food Safety Authority (Vandenplas et al., 2018). Studies have shown that infants fed formula with 2'-FL have the same variety of intestinal flora as breastfed infants (Marriage et al., 2015; Goehringer et al., 2016). Therefore, 2'-FL has received great attention as a functional food ingredient for nutritional health and medicinal purposes.

Generally, 2'-FL is produced by isolation from breast milk or chemical synthesis (Fernandez-Mayoralas and Martin-Lomas, 1986). However, the efficient production of 2'-FL could not be achieved due to the limited supply of breast milk and the chemical synthesis process requiring precise side-chain protection and deprotection (Bode et al., 2016; Petschacher and Nidetzky, 2016). The enzymatic synthesis of 2'-FL allows the screening of appropriate enzymes based on the configuration of the receptor and glycosyl donor (Ye et al., 2019; Li et al., 2020). Unfortunately, the nucleoside donor, GDP-L-fucose, is expensive and the catalytic activity of x1,2-fucosyltransferase is low, making it impossible to achieve large-scale industrial production (Lee et al., 2012; Baumgartner et al., 2013). Synthetic biology and metabolic engineering provide the most efficient method for the direct synthesis of 2'-FL from microorganisms (Chin et al., 2015, 2017). The biosynthetic pathway of

© 2021 The Authors. Microbial Biotechnology published by Society for Applied Microbiology and John Wiley & Sons Ltd.
2'-FL in E. coli is illustrated in Fig. 1. The de novo pathway is the main pathway for microbial synthesis of GDP-L-fucose, which transforms mannose-6-phosphate into GDP-L-fucose via four enzymes, phosphomannomutase (manB, E.C. 5.4.2.8), mannose-1-phosphate guanyltransferase (manC, E.C. 2.7.7.22), GDP-D-mannose-4,6-ehydratase (gmd, E.C. 4.2.1.47) and GDP-L-fucose synthetase (wcaG, E.C. 1.1.1.271) (Zhai et al., 2015; Huang et al., 2017).

Currently, the biosynthetic pathway constructing for 2'-FL in host bacteria such as E. coli (Parschat et al., 2020), Bacillus subtilis (Deng et al., 2019), Saccharomyces cerevisiae and Yarrowia lipolytica (Hollands et al., 2019) has become a research hotspot. Most researches were conducted on E. coli due to the simplicity of the culture conditions and the availability of genetic tools. As an example, a batch fermentation of E. coli JM109 (DE3) expressing H. pylori α1,2-fucosyltransferase resulted in a 3.4-fold increase in 2'-FL production compared with the original (FucT2) produced 1.23 g l⁻¹ 2'-FL, while the addition of a triple-Asp-tag to the N-terminus bacteria (Chin et al., 2015). WbgL, the gene encoding FucT2 in E. coli O126, has high expression activity and catalytic specificity for lactose in E. coli, allowing most of the GDP-L-fucose in the reaction system to be used in the catalytic process, with a titre of 20.1 g l⁻¹ of 2'-FL (Engels and Elling, 2014).

Baumgartner et al. (2013) integrated two GDP-L-fucose metabolic pathways into the genome of E. coli JM109, leading to 20.3 g l⁻¹ 2'-FL in fed-batch fermentation.

Cofactors (NADPH and GTP) play an essential role in achieving high productivity and high titres of metabolites (Chemler et al., 2010). In microbial cells, the pentose
phosphate pathway (PPP) and the tricarboxylic acid (TCA) cycle are the dominant sources of NADPH and GTP (Sauer et al., 2004). During 2'-FL synthesis, NADPH and GTP are involved in the formation of GDP-L-fucose. The researchers constructed a de novo pathway of GDP-fucose in E. coli (wcaJ') and further expressed zwf and gsk genes to enhance intracellular NADPH and GTP expression, and the production of GDP-L-fucose could be accumulated to a maximum titre of 106 mg l⁻¹ (Wan et al., 2020). Thus, improving the intracellular redox state can effectively improve the performance of the host bacteria and increase the yield of the target.

In this work, a modular metabolic engineering modification strategy was introduced in engineered E. coli, which regulates the metabolic flux between modules through different plasmid combinations based on the de novo pathway. To weaken branch bypass, 2'-FL synthesis was promoted by inactivating the β-galactosidase (lacZ) and UDP-glucose lipid carrier transferase (wcaJ) genes using the CRISPR-Cas9 system. The NADPH and GTP regeneration pathways were engineered as embedded modules to significantly improve the yield of 2'-FL. The best choices among several exogenous x1,2-fucosyltransferase (futC) for 2'-FL production performance were screened. Finally, a fed-batch fermentation was performed to further explore the production potential of the optimal strain BZW-24.

Results and discussion

Plasmid combinatorial optimization for 2'-FL production

To optimize the 2'-FL synthetic pathway, a modular metabolic engineering modification strategy was introduced in E. coli. The metabolic flux of the different modules was regulated by changing the transcriptional control factor (plasmid copy number) of each module to balance the synthetic pathway. Given that GDP-L-fucose is an essential precursor substance for the 2'-FL synthesis pathway, however, the yield of GDP-L-fucose is extremely low, thus requiring a fine modification of this synthetic pathway (Wan et al., 2020). The critical enzymes relation to the GDP-L-fucose pathway (manB, manC, gmd and wcaG) were used as module I responsible for the conversion of mannone-1-P to GDP-L-fucose. It has been reported that futC converting lactose and GDP-L-fucose into 2'-FL was a rate-limiting enzyme in the metabolic pathway due to its low activity (Drouillard et al., 2006). However, overexpression of lacY can effectively strengthen the efficiency of lactose transport. Therefore, futC and lacY were divided into module II to investigate the methods to alleviate this bottleneck (Fig. 2A).

The module expression intensity was determined by promoter strengths and gene copy numbers (Tolia and Joshua-Tor, 2006; Wu et al., 2014). In this study, five different copies of plasmids under the control of T7 promoter were used including pRSFDuet-1 (RSF ori), pETDuet-1 (pBR322 ori), pCDFDuet-1 (CDF ori), pACYCDuet-1 (p15A ori) and pCOLADuet-1 (ColA ori). Under different plasmid combinations, eighteen engineered host strains (BZW1–18) were constructed to achieve the de novo synthesis of 2'-FL (Fig. 2B). The fermentation products were detected by HPLC, and 2'-FL was validated by ESI-MS. The results of shake flask fermentation indicated that under the fixed expression of module II, the accumulation of products showed an upward tendency with increasing expression of module I. For example, the combination of downstream module II (pET-CY) and upstream module I with different copy numbers produced recombinant strains BZW-1, BZW-9, BZW-13 and BZW-16 and remarkably improved the titres of 2'-FL to 1.45, 1.02, 0.86 and 0.82 g l⁻¹, respectively (Fig. 2B). This suggested that the upstream module I at a higher expression level favours the accumulation of intracellular GDP-L-fucose. Similarly, the same trend was observed for fixation of the other modules. Interestingly, fixing the expression of module I, module II showed the highest titre at an expression slightly lower than that of module I. For example, the strains BZW-5, BZW-10 and BZW-15 had 8.4%, 5.1% and 6.0% higher 2'-FL production compared with the strains BZW-4, BZW-9 and BZW-14 respectively. Combinatorial optimization revealed that BZW-1 (carrying plasmids pRSF-CBGW and pET-CY) was the best fermenting strain with a 2'-FL concentration of 1.45 g l⁻¹. A plausible explanation was that the high-intensity expression of module II resulted in an inhibition of futC conversion, which may trigger metabolic burden and energy inefficiency in the host strain (Lee and Kim, 2015).

In order to compare the mRNA levels of 2'-FL biosynthetic enzymes (manB, manC, gmd, wcaG, futC and lacY) in different strains, we divided the eighteen engineered strains into five groups according to module I. The strains with high expression levels (BZW-1, BZW-5, BZW-10 and BZW-15) and low expression levels (BZW-4, BZW-8, BZW-12 and BZW-16) were selected for RT-qPCR assays (Fig. 5A). As expected, BZW-1 exhibited higher transcription levels in the above strains. In particular, the mRNA levels of manB and wcaG were 11.8- and 12-fold higher than those in BZW-16. This indicated that the increase of plasmid copy number in module I resulted in an increased expression level of the gene for 2'-FL synthesis in strain BZW-1, which effectively boosted the synthesis of the precursor metabolite GDP-L-fucose. For the mRNA levels of futC and lacY in module II, the expression of futC and lacY in the other strains was increased to varying degrees compared with strain BZW-16; however, the increase was not significant.

© 2021 The Authors. Microbial Biotechnology published by Society for Applied Microbiology and John Wiley & Sons Ltd., Microbial Biotechnology, 15, 1561–1573
Overall, lower downstream gene dosage and higher upstream gene dosage resulted in higher 2′-FL yield, and it is possible that this combination increased the accumulation of intermediates and promoted efficient conversion of futC.

Effects of strengthening the cofactors NADPH and GTP regeneration pathways on 2′-FL production

NADPH and GTP are essential cofactors involved in bioenergy and carbon metabolism, which help maintain intracellular redox balance (Sauer et al., 2004). Currently, enhancing endogenous cofactor pathways associated with metabolites has become an effective approach to improve the yield of industrial microbial chemicals. For this reason, we coupled cofactor and metabolic engineering to construct a cofactor regeneration system based on strain BWZ1.

Previous studies have shown that the overexpression of zwf in PPP to enhance NADPH regeneration and the overexpression of Gsk in the guanosine nucleotide biosynthesis pathway to facilitate GTP regeneration were both effective in increasing the production of the key precursor GDP-ß-fucose (Wan et al., 2020). Here, the cumulative effect of cofactor engineering on 2′-FL was assessed by overexpression of multiple genes.

The zwf, pntAB, UdhA, Gsk, GuaA, GuaB, GuaC, Gmk and Ndk were selected as candidate genes, of which the mechanisms for energy regeneration are shown in Fig. 3A. Single or combined genes were cloned into the vector pCDFDuet-1 and introduced into strain BWZ1. The performance of the resulting strain was explored to further improve its redox status.

The single gene overexpression experiments enhanced the yield of 2′-FL to varying degrees. The strains overexpressing zwf (BZW-19) and pntAB (BZW-20) achieved better performance in four expression systems including zwf (BZW-19), pntAB (BZW-20), UdhA (BZW-21) and Gsk (BZW-22). The increased NADPH availability contributed to the accumulation of maximum

![Fig. 2. Plasmid combinatorial optimization for 2′-FL production. A. Schematic design of different plasmid combinations. Module I includes manB, manC, gmd and wcaG. Module II contains futC and lacY. Both modules were expressed under the control of extremely strong (pRSFDuet-1, denoted by R), relatively strong (pETDuet-1, denoted by E), moderate (pCDFDuet-1, denoted by D), weak (pACYCDuet-1, denoted by A) or very weak (pCOLADuet-1, denoted by O) plasmids. B. Influences of modular engineering of 2′-FL. Triplicate experiments were conducted, and error bars denote the standard deviation.](image_url)
titres of 1.71 (BZW-19) and 1.62 g l\(^{-1}\) (BZW-20) of 2'-FL, respectively, which were 17.9% and 11.7% higher than the titres of the control strain (Fig. 3B). These results revealed that overexpression of the genes zwf and pntAB intensified the NADPH regeneration pathway, as they directed the strain to a highly reduced state to overproduce 2'-FL. Remarkably, the 2'-FL yield was significantly improved over that of the single gene in the E. coli strains co-expressing PntAB-UdhA, Gsk-zwf, Gsk-GuaBA-GuaC and Gsk-Gmk-Ndk. The 2'-FL titres of strains BZW-23, BZW-24, BZW-25 and BZW-26 were 2.06, 2.24, 1.81 and 1.87 g l\(^{-1}\), which were 42.1%, 53.8%, 24.8% and 29.0% higher than that of the control respectively. This demonstrates that NADPH and GTP regeneration has indeed effectively increased the yield of 2'-FL. Particularly, the optimal performance of the BZW-24 strain might be attributed to the fact that the increased carbon flux through the overexpression of zwf was directed to adequate NADPH supply and that the overexpression of Gsk enhanced the GTP biosynthetic pathway, promoting the biotransformation of inosine and guanosine to IMP and GMP (Ledesma-Amaro et al., 2013). Furthermore, manC requires GTP for the biocconversion of GDP-D-mannose, while wcaG requires NADPH for GDP-L-fucose biosynthesis in the 2'-FL de novo synthetic pathway. Efficient feeding of cofactors NADPH and GTP is essential for yield improvement. Previous studies have attempted to improve NADPH regeneration or enhance guanosine nucleotide biosynthesis through overexpression or evolution of genes corresponding to the rate-limiting steps in the target cofactor regeneration pathway (Wan et al., 2020).

**Effect of the deletion of lacZ and wcaJ on 2'-FL production**

To further maximize the flow of metabolites to the target pathway, the CRISPR-Cas9 gene editing system was implemented to knockout lacZ and wcaJ in E. coli BL21 (DE3). Deletion of the UDP-glucose lipid carrier transferase (wcaJ) blocks the metabolic flux from GDP-L-fucose to colanic acid, favouring the accumulation of GDP-L-fucose (Huang et al., 2017). ß-Galactosidase (lacZ) cleaves lactose to galactose and glucose, catalysing the first step of lactose metabolism (Chin et al., 2015). The intracellular availability of lactose is essential in the efficient biosynthesis of 2'-FL, thus inactivating the lacZ gene in engineered E. coli.

As shown in Fig. 4B, the deletion of wcaJ gene in E. coli strains BWS and BWZS resulted in an increase of 98.5% and 44.1% in intracellular GDP-L-fucose, compared with the original strain. The DCW and growth conditions of the knockout strain were not noticeably affected, which was consistent with previous findings (Wan et al., 2020). Furthermore, weakening of the lacZ branch bypass further facilitated the accumulation of 2'-FL. The titres of 2'-FL for strains BWD, BZD and BWZ-1 were 0.392, 1.09 and 1.45 g l\(^{-1}\), illustrating a 1.78-, 4.95- and 6.59-fold increase compared with BD (0.22 g l\(^{-1}\) at 60 h). Among them, strains BZD and BWZ-1 achieved yields of 0.59 and 0.61 mole 2'-FL mole\(^{-1}\) lactose, which were 8.4 and 8.8 times higher than that of strain BD (Fig. 4C). To validate the effect of wcaJ and lacZ deletion on metabolic pathways, several relevant genes (manC, manB, gmd, wcaG, lacY, lacZ

---

*Fig. 3. Effect of increased cofactor NADPH and GTP availability on 2'-FL production. A. Cofactor regeneration pathways associated with the de novo pathway. The genes involved in the cofactor engineering include zwf, pntAB, UdhA, Gsk, GuaA, GuaB, GuaC, Gmk and Ndk. B. Shake flask fermentation results of different engineered strains with single and multiple gene overexpression involving cofactor regeneration. Triplicate experiments were conducted, and error bars denote the standard deviation. * and ** mean \(P < 0.05\) and \(P < 0.01\) respectively.*
and wcaJ) in the metabolic pathway were selected. The mRNA levels of the above genes (manC, manB, gmd, wcaG and lacY) were significantly increased in the defective strains (BL21(DE3)ΔwcaJ, BL21(DE3)ΔlacZ and BL21(DE3)ΔlacZΔwcaJ) compared with those in the original BL21(DE3) strain (Fig. 5B). Obviously, the knockout of wcaJ and lacZ genes greatly reduced their transcription levels. These results indicated that deletion of wcaJ and lacZ effectively enhanced the titres of GDP-L-fucose and 20-FL and significantly improved the accumulation of the target pathway.

Improving 20-FL production by comparing various α1,2-fucosyltransferases

α1,2-Fucosyltransferase allows fucosylation modification of the substrate to achieve enzymatic conversion from the donor GDP-L-fucose and the acceptor lactose to the product 20-FL. Herein, four α1,2-fucosyltransferases from prokaryotes were selected, namely HpFutC1 (protein ID: KY499613), HpFutC2 (protein ID: AFV41312.1), Bfwcfb (protein ID: WP_005817145.1) and EcowbgL (protein ID: ADN43847.1). To evaluate the contribution of different α1,2-fucosyltransferases to 20-FL, batch cultivation of four strains (BZW-24, BZW-27, BZW-28 and BZW-29) was employed using a metabolic engineering strategy (Fig. 6). Overexpression of different sources of α1,2-fucosyltransferases may result in different levels of 20-FL production. The BZW-24 strain (containing HpFutC1) achieved an optimal 20-FL concentration of 2.24 g l⁻¹ from 20 g l⁻¹ glycerol and 14 g l⁻¹ lactose at 60 h, with the yield of 0.60 mole 20-FL mole⁻¹ lactose. (Fig. 6A). Compared with BZW-24, the remaining three strains exhibited slightly lower 20-FL titres, with BZW-27, BZW-28 and BZW-29 exhibiting 20-FL titres of 1.80, 1.69 and 1.95 g l⁻¹, respectively (Fig. 6B–D). The higher 20-FL concentration obtainable with HpFutC1 might be attributed to the higher affinity of HpFutC1 on lactose. Albermann et al. (2001) found that the Km value of HpFutC1 (168 μM) was lower than that of other sources of α1,2-fucosyltransferases, thus giving futC a higher affinity for the substrate. It should be noted that

Fig. 4. Effect of gene knockout on 20-FL biosynthesis. A. Overview of CRISPR/Cas9 genome editing strategies in E. coli. Genome editing involves three main components: (1) a pCas9 plasmid expressing the λ Red machinery and Cas9 endonuclease; (2) a pTargetF plasmid carrying the sgRNA gene; (3) a homologous repair template, donor-DNA. B. GDP-L-fucose titre and DCW of BWS, BZS, BWZS and the control host BS. C. 20-FL titre, DCW and 20-FL yield on lactose in BD, BWD, BZD and BWZ-1. Triplicate experiments were performed, and the error bars indicate the standard deviation. * and ** mean P < 0.05 and P < 0.01 respectively.
α1,2-fucosyltransferase itself exhibits weak enzymatic activity and low soluble expression, and future work will be directed towards the evolution of futC to improve the productivity of 2′-FL in the de novo synthetic pathway.

Production of 2′-FL in 3 l bioreactors

To achieve high titres of 2′-FL for the engineered strain under certain conditions, glycerol fed-batch fermentation...
of engineered E. coli BZW-24 was carried out with intermittent lactose addition. Here, glycerol was employed as a carbon source for cell growth and lactose was added as a fucose receptor during the fermentation process. The reason for this is that both glycerol and lactose are transported into the cell via the non-PTS system (Yazdani and Gonzalez, 2007). The growth of the strain is not inhibited, and the substrates are low cost and easily available for large-scale production. After the initial addition of glycerol was completely depleted, glycerol was fed in a pH-constant mode.

Fermentation results revealed that the product started to accumulate gradually with the addition of IPTG (0.2 mM) at OD_{600} of 20 (Fig. 6E). After the initial lactose was exhausted from the medium, lactose was supplemented to the culture broth to sustain 2'-FL biosynthesis. As a result, 22.3 g l\(^{-1}\) of 2'-FL was obtained at the end of fed-batch fermentation, which was 9.96 times of that in the shake flask. As shown in Table S3, the strain was assimilated by glycerol to maintain cell growth to a final cell dry weight of 43 g l\(^{-1}\). As calculated that the 2'-FL yield was 0.53 mole 2'-FL mole\(^{-1}\) lactose or 0.52 g 2'-FL g\(^{-1}\) DCW, which is basically consistent with the yield of the batch fermentations. These results demonstrated that continuous supplementation of carbon sources, efficient NADPH and
GTP regeneration and metabolic flux to the desired biosynthetic pathway significantly facilitated the biosynthesis of 2'-FL. Here, *E. coli* BL21 (DE3) rather than JM109 (DE3) was utilized as a cell factory to produce 2'-FL. This was attributed to the fact that the biofilms formed by JM109 cause serious problems during fermentation, whereas BL21 (DE3) readily exhibits active sugar metabolism and tolerance against metabolic pressure, making it relatively easier for BL21 (DE3) to grow to high cell densities (Lee et al., 2012; Teodósio et al., 2012).

Conclusions

In this work, a biosynthetic pathway for the efficient production of 2'-FL was constructed using low-cost glycerol as a carbon source and lactose as a precursor. The intracellular titre of 2'-FL in engineered *E. coli* was effectively increased by modular optimization of cellular metabolic pathways. Gene knockout of metabolic bypass, regeneration of cofactors (NADPH and GTP) and screening of different 1,2-fucosyltransferase significantly increased the yield of 2'-FL. The best engineered strain BZW-24 had a 2'-FL titre of 22.3 g l⁻¹ and a yield of 0.53 mole 2'-FL mole⁻¹ lactose. The work reported here indicates that this engineered strain is a promising cell factory for efficient production of 2'-FL. It should be noted that the activity and soluble expression levels of 1,2-fucosyltransferase are still lower. More efforts should be made in the future, such as establishing a high-throughput screening method for 1,2-fucosyltransferase and performing directed evolution studies on the enzyme.

Experimental procedures

**Bacterial strains, plasmids and medium**

All strains and plasmids used in this study are summarized in Tables 1 and S1 respectively. *E. coli* BL21 (DE3) was used as a host strain, and *E. coli* DH5α was employed for amplification and construction of plasmids. The vectors with different copy numbers, including pRSFDuet-1, pETDuet-1, pCDFDuet-1, pACYC-Duet-1 and pCOLADuet-1 at the Nco I site, generating the plasmids pRSF-CB, pET-CB, pCDF-CB, pCDYC-CB and pCOLA-CB respectively. Subsequently, the gmd-wcaG cluster was further inserted into the aforementioned plasmids containing manC-manB cluster at the Nde I site to construct pRSF-CBGW, pET-CBGW, pCDF-CBGW, pACYC-CBGW and pCOLA-CBGW. Similarly, the *futC* and *lacY* fragments and vectors with different copy number were ligated to obtain recombinant plasmids pRSF-CY, pET-CY, pCDF-CY, pACYC-CY and pCOLA-CY. Finally, recombinant expression vectors with different plasmid combinations (Fig. 2A) were transformed into *E. coli* competent cells to obtain the recombinant strains.

The overexpressed genes involved in NADPH regulation and the cofactors GTP and ATP regeneration (Fig. 3A) in this study were *zwf*, *pntAB*, *UdhA*, *Gsk*, *GuaB*, *GuaC*, *Gmk* and *Ndk*. The templates for genes cloning were derived from the *E. coli* K12 MG1655 genome. As constructed above, the vector and target fragments were ligated to obtain the plasmids pCDF-zwf, pCDF-pntAB, pCDF-UdhA, pCDF-Gsk, pCDF-pntAB-UdhA, pCDF-Gsk-zwf, pCDF-Gsk-GuaBA-GuaC and pCDF-Gsk-Gmk-Ndk.

**Gene knockout through CRISPR-Cas9**

The genes encoding *lacZ* and *wcaJ* were targeted for deletion from *E. coli* chromosome via CRISPR-Cas9 system (Fig. 4A). The two-plasmid-based CRISPR-Cas9 system was used for genome editing, in which the pCas9 plasmid encodes the Cas9 nuclease and the pTargetF plasmid carries single-guide RNA (sgRNA) with a certain N20 sequence (Jiang et al., 2015). pCas9 and pTargetF were deposited in Addgene, numbered 62225.
and 62226 respectively. Knockout gene targets were designed using online software at http://www.regenome.net/cas-offinder/. The sgRNA expression cassette with the N20 sequence was constructed using the pTargetF plasmid as a template. The successfully constructed knockout plasmids were named pTF-ΔlacZ and pTF-ΔwcaJ respectively. E. coli BL21 (DE3) competent cells harbouring pCas9 were prepared as described previously (Sharan et al., 2009). The repair templates targeting anti-selection marker genes were designed to remove sequences between the start codon and stop codon of the target gene. Long repair templates (500–1000 bp arms) were constructed by linking homology arms upstream and downstream of target genes (Zerbini et al., 2017). Arabinose (final concentration 10 mM) was supplemented to the culture for λ-red induction to facilitate homologous recombination. The knockout plasmid pTF-ΔlacZ or pTF-ΔwcaJ and the homologous repair template were electrophoresed into competent cells. Positive transformants were screened by colony PCR and DNA sequencing on LB solid plates containing kanamycin (50 μg ml⁻¹) and spectinomycin (50 μg ml⁻¹). After that, the double-plasmid system in the defective bacteria was eliminated by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for induction and overnight incubation of positive transformants at 42°C.

**Batch fermentation for Z-FL production**

For batch fermentation, the engineered strains were inoculated into 3 ml of LB medium and cultured at 37°C.

---

**Table 1. List of strains used in this study.**

| Strains | Relevant genotype or relevant properties | Reference |
|---------|----------------------------------------|-----------|
| E. coli DH5a | F⁻ endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR supE44 galK dcm lacZΔM15(T18)lacY1 Δ(lacZYA-argF)U169, hsdR17 (rK- mK+) λ Δ(hamI296 lacI977) hsdS2 rK30 [dcm] ΔlacS78ΔlacZ874::Tn10 [Δ(icsR) Δmbl] int [Δ(PlacUV5::T7 gene1) i21 Δnin5] | Novagen |
| E. coli BL21 (DE3) | ΔlacZΔwcaJ | This study |
| BW | E. coli BL21 (DE3) | This study |
| BZ | E. coli BL21 (DE3): ΔlacZ | This study |
| BZW | E. coli BL21 (DE3): ΔlacZΔwcaJ | This study |
| BS | BL21 (DE3) harboring plasmids pRSF-CBGW and pET-CY | This study |
| BD | BL21 (DE3) harboring plasmids pRSF-CBGW and pCDF-CY | This study |
| BWS | BW harboring plasmids pRSF-CBGW and pET-CY | This study |
| BWD | BW harboring plasmids pRSF-CBGW and pET-CY | This study |
| BZS | BZ harboring plasmids pRSF-CBGW and pET-CY | This study |
| BZZ | BZ harboring plasmids pRSF-CBGW and pET-CY | This study |
| BZW | BZW harboring plasmids pRSF-CBGW and pET-CY | This study |
| BZZW | BZW harboring plasmids pRSF-CBGW and pCDF-CY | This study |
| BZW-2 | BZW harboring plasmids pRSF-CBGW and pET-CY | This study |
| BZW-3 | BZW harboring plasmids pRSF-CBGW and pACYC-CY | This study |
| BZW-4 | BZW harboring plasmids pET-CBGW and pRSF-CY | This study |
| BZW-5 | BZW harboring plasmids pET-CBGW and pACYC-CY | This study |
| BZW-6 | BZW harboring plasmids pET-CBGW and pACYC-CY | This study |
| BZW-7 | BZW harboring plasmids pET-CBGW and pCOLA-CY | This study |
| BZW-8 | BZW harboring plasmids pCDF-CBGW and pRSF-CY | This study |
| BZW-9 | BZW harboring plasmids pCDF-CBGW and pET-CY | This study |
| BZW-10 | BZW harboring plasmids pCDF-CBGW and pACYC-CY | This study |
| BZW-11 | BZW harboring plasmids pCDF-CBGW and pACYC-CY | This study |
| BZW-12 | BZW harboring plasmids pACYC-CBGW and pET-CY | This study |
| BZW-13 | BZW harboring plasmids pACYC-CBGW and pET-CY | This study |
| BZW-14 | BZW harboring plasmids pACYC-CBGW and pETF-CY | This study |
| BZW-15 | BZW harboring plasmids pACYC-CBGW and pETF-CY | This study |
| BZW-16 | BZW harboring plasmids pACYC-CBGW and pET-CY | This study |
| BZW-17 | BZW harboring plasmids pACYC-CBGW and pET-CY | This study |
| BZW-18 | BZW harboring plasmids pACYC-CBGW and pET-CY | This study |
| BZW-19 | BZW-1 harboring plasmids pCDF-zwf | This study |
| BZW-20 | BZW-1 harboring plasmids pCDF-pniAB | This study |
| BZW-21 | BZW-1 harboring plasmids pCDF-pniAB | This study |
| BZW-22 | BZW-1 harboring plasmids pCDF-Gmk | This study |
| BZW-23 | BZW-1 harboring plasmids pCDF-Gmk | This study |
| BZW-24 | BZW-1 harboring plasmids pCDF-Gmk | This study |
| BZW-25 | BZW-1 harboring plasmids pCDF-Gmk-GsaBA-UsaC | This study |
| BZW-26 | BZW-1 harboring plasmids pCDF-Gmk-GsaBA-UsaC | This study |
| BZW-27 | BZW-1 harboring plasmids pCDF-Gmk-GsaBA-UsaC | This study |
| BZW-28 | BZW-1 harboring plasmids pCDF-Gmk-GsaBA-UsaC | This study |
| BZW-29 | BZW-1 harboring plasmids pCDF-Gmk-GsaBA-UsaC | This study |
and 200 rpm. Subsequently, 0.5 ml of seed solution was transferred into 50 ml of defined medium with 20 g l\(^{-1}\) glycerol to expand growth in 250 ml shake flasks. IPTG and lactose were supplemented at the final concentration of 0.2 mM and 20 g l\(^{-1}\) until the cells' optical density (OD\(_{600}\)) was approximately 0.6–0.8. After the IPTG induction, the cells were incubated at 25°C for efficient expression of proteins. If necessary, ampicillin (100 μg ml\(^{-1}\)), chloramphenicol (50 μg ml\(^{-1}\)), kanamycin (50 μg ml\(^{-1}\)) and streptomycin (50 μg ml\(^{-1}\)) were added to the medium.

**Fed-Batch fermentation in a 3 l bioreactor**

Fed-batch cultivations were performed in a 3 l bioreactor with 1 l working volume of the defined medium and 5% of inoculation. Main culture was employed at 37°C, and when OD\(_{600}\) reached 20, IPTG and lactose were added to a final concentration of 0.2 mM and 20 g l\(^{-1}\). During induction, the temperature was adjusted to 25°C and the pH of the medium was maintained at 6.8 by addition of 28% NH_4OH. The feeding solution comprising 600 g l\(^{-1}\) of glycerol and 20 g l\(^{-1}\) of MgSO_4·7H_2O was streamed into the fermentation broth to replenish the carbon source by pH-stat mode (Chin et al., 2015). The fermentation process was controlled by a cascade system that regulated the speed, aeration and oxygen to achieve 20% dissolved oxygen in the fermenter.

**Analytical methods**

During cultivation, the culture broth was sampled at intervals and growth conditions were determined by OD\(_{600}\) analysis using a spectrophotometer (P7 Double Beam UV-VIS Spectrophotometer, Mapada, China). Dry cell weight (g l\(^{-1}\)) was calculated from OD\(_{600}\) using conversion factor of 0.36 (Chin et al., 2013).

The concentrations of glycerol, lactose and 2'-FL in the fermentation broth were quantified with an HPLC system (Waters e2695) equipped with a refractive index (RI) detector and a Rezex ROA-Organic Acid H\(^+\) column (Phenomenex, Torrance, CA, USA). The column was eluted with 0.01 M H_2SO_4 at a flow rate of 0.6 ml min\(^{-1}\) at 50°C (Chin et al., 2016). To assay 2'-FL, culture broth samples (1 ml) were boiled for 10 min to break the cells completely and centrifuged at 12 000 g for 10 min. The supernatant was used to estimate the total oligosaccharide content. To identify the product of 2'-FL, 2'-FL standards and samples were analysed by electro-spray ionization-mass spectroscopy (ESI-MS) in negative ion mode under standard conditions on a Waters MALDI SYNAPT Q-TOF MS (Milford, MA, USA). The content of GDP-L-fucose in fermentation broth was determined according to Huang et al. (2017).

**Real-time quantitative PCR (RT-qPCR)**

To evaluate the mRNA levels of intracellular 2'-FL biosynthetic enzymes in engineered *E. coli*, RT-qPCR was employed for analysis. Total RNA of the strains was extracted during the mid-exponential phase using FastPure Cell/Tissue Total RNA Isolation Kit V2 (Vazyme, Nanjing, China) following the manufacturer’s protocol. The RNA was reverse transcribed to cDNA using the HiScript III RT SuperMix for qPCR (+gDNA wiper) kit (Vazyme). The RT-qPCR was performed using the CFX96 Real-Time system (BioRad, Pleasanton, CA, USA) and ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China). The housekeeping gene (16S rRNA) was used as a leading control. The relative transcript levels of target genes were analysed from three biological replicates by the 2^–ΔΔCt method (Livak and Schmittgen, 2001). The primer sequences for RT-qPCR are shown in Table S2.

**Statistical analysis**

All numerical results are the average of at least three independent replicates. The mean differences were determined by Tukey’s HSD test (P < 0.05) using analysis of variance (one-way ANOVA) in ORIGINPRO 9.0 software (Origin Lab, Northampton, MA, USA).

**Acknowledgements**

This research was financially supported by Major Science and Technology Innovation Project of Shandong Province (2020CXGC010601) and the National Nature Science Foundation of China (No. 32072151).

**Conflict of interest**

The authors declare no competing financial interest.

**References**

Albermann, C., Piepersberg, W., and Wehmeier, U.F. (2001) Synthesis of the milk oligosaccharide 2'-fucosyllactose using recombinant bacterial enzymes. *Carbohydr Res* **334**: 97–103.

Baumgartner, F., Seitz, L., Sprenger, G.A., and Albermann, C. (2013) Construction of *Escherichia coli* strains with chromosomally integrated expression cassettes for the synthesis of 2'-fucosyllactose. *Microb Cell Fact* **12**: 40.

Bode, L., Contractor, N., Barile, D., Pohl, N., Prudden, A.R., Boons, G.-J., *et al.* (2016) Overcoming the limited availability of human milk oligosaccharides: challenges and opportunities for research and application. *Nutr Rev* **74**: 635–644.

Bych, K., Mikš, M.H., Johanson, T., Hederos, M.J., Vignaes, L.K., and Becker, P. (2019) Production of HMOs...
using microbial hosts from cell engineering to large scale production. *Curr Opin Biotechnol* **56**: 130–137.

Castanys-Munoz, E., Martin, M.J., and Prieto, P.A. (2013) 2’-fucosyllactose: an abundant, genetically determined soluble glycan present in human milk. *Nutr Rev* **71**: 773–789.

Chemler, J.A., Fowler, Z.L., McHugh, K.P., and Koffas, M.A. (2010) Improving NADPH availability for natural product biosynthesis in *Escherichia coli* by metabolic engineering. *Metab Eng* **12**: 96–104.

Chin, Y.W., Kim, J.Y., Lee, W.H., and Seo, J.H. (2015) Enhanced production of 2’-fucosyllactose in engineered *Escherichia coli* BL21star(DE3) by modulation of lactose metabolism and fucosyltransferase. *J Biotechnol* **210**: 107–115.

Chin, Y.W., Kim, J.Y., Kim, J.H., Jung, S.M., and Seo, J.H. (2017) Improved production of 2’-fucosyllactose in engineered *Escherichia coli* by expressing putative α1,2-fucosyltransferase, WcfB from *Bacteroides fragilis*. *J Biotechnol* **257**: 192–198.

Chin, Y.W., Park, J.B., Park, Y.C., Kim, K.H., and Seo, J.H. (2013) Metabolic engineering of *Corynebacterium glutamicum* to produce GDP-L-fucose from glucose and mannose. *Bioprocess Biosyst Eng* **36**: 749–756.

Chin, Y.W., Seo, N., Kim, J.H., and Seo, J.H. (2016) Metabolic engineering of *Escherichia coli* to produce 2’-fucosyllactose via salvage pathway of guanosine 5’-diphosphate (GDP)-L-fucose. *Biotechnol Bioeng* **113**: 2443–2452.

DeLong, C.M., Bragg, R., and Simmons, J.A. (2008) Evidence for spatial representation of object shape by echolocating bats (*Eptesicus fuscus*). *J Acoust Soc Am* **123**: 4582–4598.

Deng, J., Gu, L., Chen, T., Huang, H., Yin, X., Lv, X., et al. (2019) Engineering the substrate transport and cofactor regeneration systems for enhancing 2’-fucosyllactose synthesis in *Bacillus subtilis*. *ACS Synth Biol* **8**: 2418–2427.

Drouillard, S., Driguez, H., and Samain, E. (2006) Large-scale synthesis of H-antigen oligosaccharides by expressing *Helicobacter pylori* α1,2-fucosyltransferase in metabolically engineered *Escherichia coli* cells. *Angew Chem* **118**: 1810–1812.

Engels, L., and Elling, L. (2014) WbgL: a novel bacterial alpha1,2-fucosyltransferase for the synthesis of 2’-fucosyllactose. *Glycobiology* **24**: 170–178.

Fernandez-Mayoralas, A., and Martin-Lomas, M. (1986) Synthesis of 3- and 2’-fucosyllactose and 3,2’-difucosyl lactose from partially benzylated lactose derivatives. *Carbohydr Res* **154**: 93–101.

Goehring, K.C., Marriage, B.J., Oliver, J.S., Wilder, J.A., Barrett, E.G., and Buck, R.H. (2016) Similar to those who are breastfed, infants fed a formula containing 2’-fucosyllactose have lower inflammatory cytokines in a randomized controlled trial. *J Nutr* **146**: 2559–2566.

Gonia, S., Tureper, M., Heisel, T., Autran, C., Bode, L., and Gale, C. (2015) Human milk oligosaccharides inhibit *Candida albicans* invasion of human premature intestinal epithelial cells. *J Nutr* **145**: 1992–1998.

Hollands, K., Baron, C.M., Gibson, K.J., Kelly, K.J., Krasley, E.A., Laffend, L.A., et al. (2019) Engineering two species of yeast as cell factories for 2’-fucosyllactose. *Metab Eng* **52**: 232–242.

Huang, D., Yang, K., Liu, J., Xu, Y., Wang, Y., Wang, R., et al. (2017) Metabolic engineering of *Escherichia coli* for the production of 2’-fucosyllactose and 3-fucosyllactose through modular pathway enhancement. *Metab Eng* **41**: 23–38.

Jiang, Y., Chen, B., Duan, C., Sun, B., Yang, J., and Yang, S. (2015) Multigene editing in the *Escherichia coli* genome via the CRISPR-Cas9 system. *Appl Environ Microbiol* **81**: 2506–2514.

Ledesma-Amaro, R., Jiménez, A., Santos, M.A., and Revuelta, J.L. (2013) Biotechnological production of feed nucleotides by microbial strain improvement. *Process Biochem* **48**: 1263–1270.

Lee, S.Y., and Kim, H.U. (2015) Systems strategies for developing industrial microbial strains. *Nat Biotechnol* **33**: 1061–1072.

Lee, W.H., Pathanibul, P., Quarterman, J., Jo, J.H., Han, N.S., Miller, M.J., et al. (2012) Whole cell biosynthesis of a functional oligosaccharide, 2’-fucosyllactose, using engineered *Escherichia coli*. *Microb Cell Fact* **11**: 48.

Li, C., Wu, M., Gao, X., Zhu, Z., Li, Y., Lu, F., and Qin, H.M. (2020) Efficient biosynthesis of 2’-fucosyllactose using an in vitro multienzyme cascade. *J Agric Food Chem* **68**: 10763–10771.

Livak, K.J., and Schmittgen, T. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. *Methods* **25**: 402–408.

Marriage, B.J., Buck, R.H., Goehring, K.C., Oliver, J.S., and Williams, J.A. (2015) Infants fed a lower calorie formula with 2’-FL show growth and 2’-FL uptake like breast-fed infants. *J Pediatr Gastroenterol Nutr* **61**: 649–658.

Parschat, K., Schreiber, S., Wartenberg, D., Engels, B., and Jennewein, S. (2020) High-titer de novo biosynthesis of the predominant human milk oligosaccharide 2’-fucosyllactose from sucrose in *Escherichia coli*. *ACS Synth Biol* **9**: 2784–2796.

Petschacher, B., and Nidetzky, B. (2016) Biotechnological production of fucosylated human milk oligosaccharides: Prokaryotic fucosyltransferases and their use in biocatalytic cascades or whole cell conversion systems. *J Biotechnol* **235**: 61–83.

Sauer, U., Canonaco, F., Heri, S., Perrenoud, A., and Fischer, E. (2004) The soluble and membrane-bound tranhydrogenases UdhA and PntAB have divergent functions in NADPH metabolism of *Escherichia coli*. *J Biol Chem* **279**: 6613–6619.

Sharan, S.K., Thomason, L.C., Kuznetsov, S.G., and Court, D.L. (2009) Recombineering: a homologous recombination-based method of genetic engineering. *Nat Protoc* **4**: 206–223.

Teodósio, J.S., Simões, M., and Mergulhão, F.J. (2012) The influence of nonconjugative *Escherichia coli* plasmids on biofilm formation and resistance. *J Appl Microbiol* **113**: 373–382.

Tolia, N.H., and Joshua-Tor, L. (2006) Strategies for protein coexpression in *Escherichia coli*. *Nat Methods* **3**: 55–64.

Vandenplas, Y., Berger, B., Caneill, V., Ksiazek, J., Lagström, H., Sanchez Luna, M., et al. (2016) Human milk oligosaccharides: 2’-fucosyllactose (2’-FL) and lacto-
n-neotetraose (LNnT) in infant formula. *Nutrients* **10**: E1161.

Wan, L., Zhu, Y., Li, W., Zhang, W., and Mu, W. (2020) Combinatorial modular pathway engineering for guanosine 5’-diphosphate-l-fucose production in recombinant *Escherichia coli*. *J Agric Food Chem* **68**: 5668–5675.

Weichert, S., Jennewein, S., Hufner, E., Weiss, C., Borkowski, J., Putze, J., and Schroten, H. (2013) Bioengineered 2’-fucosyllactose and 3-fucosyllactose inhibit the adhesion of *Pseudomonas aeruginosa* and enteric pathogens to human intestinal and respiratory cell lines. *Nutr Res* **33**: 831–838.

Wu, J., Zhou, T., Du, G., Zhou, J., and Chen, J. (2014) Modular optimization of heterologous pathways for de novo synthesis of (2S)-naringenin in *Escherichia coli*. *PLoS One* **9**: e101492.

Yazdani, S.S., and Gonzalez, R. (2007) Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry. *Curr Opin Biotechnol* **18**: 213–219.

Ye, J., Xia, H., Sun, N., Liu, C.-C., Sheng, A., Chi, L., *et al*. (2019) Reprogramming the enzymatic assembly line for site-specific fucosylation. *Nat Catal* **2**: 1–9.

Zerbini, F., Zanella, I., Fraccascia, D., König, E., Irene, C., Frattini, L.F., *et al*. (2017) Large scale validation of an efficient CRISPR/Cas-based multi gene editing protocol in *Escherichia coli*. *Microb Cell Fact* **16**: 68.

Zhai, Y., Han, D., Pan, Y., Wang, S., Fang, J., Wang, P., and Liu, X.W. (2015) Enhancing GDP-fucose production in recombinant *Escherichia coli* by metabolic pathway engineering. *Enzyme Microb Technol* **69**: 38–45.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** List of plasmids used in this study.

**Table S2.** Primers used in this study.

**Table S3.** Results on the batch and fed-batch fermentations of engineered *E. coli* strains for 2'-FL production.

**Fig. S1.** Identification of 2'-FL by HPLC and MS. (a) The overlaid HPLC spectra of fermentation sample, 2'-FL standard and control. (b) Analysis of 2'-FL standard by TOF-MS. (c) Analysis of 2'-FL from fermentation broth by TOF.