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ORIGINAL ARTICLE

Metabolic engineering of *Escherichia coli* for the production of Lacto-*N*-neotetraose (LNnT)

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Abstract
Lacto-*N*-neotetraose (LNnT), one of the most important human milk oligosaccharides, can be used as infants' food additives. Nowadays, extraction, chemical and biological synthesis were utilized to obtain LNnT, while these methods still face some problems such as low yield and high cost. The aim of current work is to construct a de novo biosynthesis pathway of LNnT in *E. coli* K12 MG1655. The *lgtA* and *lgtB* were first expressed by a plasmid, resulting in a LNnT titer of 0.04 g/L. To improve the yield of LNnT on substrate lactose, *lacZ* and *lacI* were knocked out, and *lacY* was over-expressed. As a result, the yield of LNnT on lactose increased from 0.01 to 0.09 mol/mol, and the titer of LNnT elevated to 0.41 g/L. In addition, the pathway was regulated using the titer of Lacto-N-triose II (LNTII) as a measure, and obtained a high titer strain of LNnT for 1.04 g/L. Finally, the gene expressions were fine-tuned, the titer of LNnT reached 1.2 g/L, which was 93% higher than the control strain, and the yield on lactose reached 0.28 mol/mol. The engineering strategy of pathway construction and modulation used in this study is applicable to facilitate the microbial production of other metabolites in *E. coli*.

Keywords Lacto-*N*-neotetraose · Lacto-*N*-triose II · Human milk oligosaccharides · *Escherichia coli* K12 MG1655

Introduction

Human milk has been recognized as the best food of the infants, supplying nutrition and energy to meet the daily need for infants in their first 6 months [1, 2]. Many researchers suggest that breast-feeding infants are less susceptible to certain diseases such as diabetes and obesity [3–5]. A significant difference between the milk of human and other animals is the milk oligosaccharides [6]. The addition of human milk oligosaccharides (HMOs) to infant formula brings it closer to breast milk, which was proved safe and beneficial to infant growth [7–9]. Human milk contains a great variety of HMOs, such as 2′-fucosyllactose (2′-FL) and 3′-fucosyllactose (3′-FL) [10–12]. Lacto-*N*-neotetraose (LNnT) is one of the high-content milk oligosaccharides in breast milk, which plays an important role in regulating intestinal flora, enhancing immunity, and promoting cell synthesis [13, 14]. As a complex HMOs, LNnT is composed of four *six-carbon* sugar units with D-glucose as the core [1, 12]. In particular, LNnT has been approved as an additive for infants' food by the U.S. FDA and EFSA [7, 8].

Currently, LNnT is produced by extraction, chemical synthesis and biological synthesis. LNnT can be isolated from breast milk, but the separation efficiency is low, and the yield was limited by the low concentrations of LNnT in human milk [7, 15]. At present, the process of chemical synthesis can produce LNnT, but there are problems like complex unit operation, which result in high production costs and is not applicable to large-scale industrial synthesis [16]. Moreover, the use of toxic reagent in chemical

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synthesis brings potential health issues [17]. Compared with chemical compounds, the biological methods can make use of inexpensive carbon sources and substrates to synthesize LNnT with less environmental impact. Therefore, increasing attention has been projected to the preparation of LNnT by biological methods [18, 19].

Previous study has constructed the synthesis of LNnT in *E. coli* JM109 by deleting *lacZ* gene and expressing the β-1,3-*N*-acetylglucosaminyltransferase and β-1,4-galactosyltransferase from *Neisseria meningitides* [20]. However, the lack of metabolic regulation results a low conversion rate of carbon [14, 21]. Recent research has constructed the pathway of LNnT in *Bacillus subtilis* [22, 23]. Engineering of modular pathway of role precursors has improved the titer of LNnT in some degree, but the metabolic regulation and the utilization of substrate still have difficulties.

UDP-glucose (UDP-Gal) and UDP-*N*-acetyglucosamine (UDP-GlcNAc) are considered the principal precursors among the biosynthesis pathway of LNnT in *E. coli*. In the metabolic pathway, glucose-6-phosphate and fructose-6-phosphate are converted to UDP-GlcNAc through Embden–Meyerhof–Parnas pathway (EMP) [22, 24]. In this study, *Escherichia coli* K12 MG1655, which is the most common biosynthesis host, was chosen as the original strain. Lactose served as the substrate for the production of intermediate Lacto-*N*-triose II (LNTII) with UDP-GlcNAc under the catalysis by *lgtA* (β-1,3-*N*-acetylglucosaminyltransferase). Then, LNTII and UDP-Gal were converted into LNnT by the catalysis of *lgtB* (β-1,4-galactosyltransferase). There are some crucial genes in the pathway include *pgi*, *glmS*, *glmM*, *glmU*, *manA*, *murA*, *wecB* and *nagB*, which may have effects on the accumulation of UDP-GlcNAc (Fig. 1). The de novo pathway of LNnT was first constructed by expressing *lgtA* and *lgtB* of *Neisseria meningitides*. Then, the lactose permease *lacY* was over-expressed, which allowed the substrate lactose transport from extracellular to intracellular [25]. The β-galactosidase *lacZ* was knocked out to prevent the degradation of lactose. LNTII is the precursor of LNnT; LNTII synthetic pathway was fine-tuned to improve the accumulation of LNTII via the optimizing protein expression levels and the eliminating the inhibition. This study revealed that the modulation of the pathway improved the titer of LNnT in engineered *E. coli*, and engineering strategy used here may be useful in other biosynthesis processes.

Materials and methods

Strains and plasmids

All the strains used in this study are listed in Table 1. *E. coli* JM109 was used as the host strain of the construction of the plasmid. *E. coli* K12 MG1655 was used as the original strain to produce LNnT.

Culture media and culture conditions

The *E. coli* JM109 and *E. coli* K12 MG1655 were cultured at 37 °C in shake flask with Luria–Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) or Terrific Broth (TB) medium (20 g/L tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 17 mM KH_2PO_4, and 72 mM K_2HPO_4). The concentration of lactose in the culture medium was 5 g/L. To select the plasmid-transformed strains from the wild type, 100 μg/mL spectinomycin was added into medium for recombinant *E. coli* cultivation. The promoter *tac* was induced with 0.2 mM isopropyl β-D-1-thiogalacto-pyranoside (IPTG).

The *E. coli* cells were cultured in 14-mL shake-tubes with 1-mL LB medium for 12 h at 37 °C with shaking at 220 rpm to get the seed culture. Then, 2% (v/v) of the seed culture was added to a 250-mL shake flask with 25-mL TB medium and 5 g/L lactose, 0.2 mM IPTG was added at optical density at 600 nm (OD_{600}) of 0.6.

DNA manipulation techniques

The manipulation and isolation of DNA were performed by standard protocols [26]. The plasmids are listed in Supplementary Table S1 and the primers used in this study are listed in Supplementary Table S2. The plasmid pACYCDuet-tac, pCDFDuet-tac, pRSFDuet-tac, and pETDuet-tac were obtained by replacing the initiation with *tac* promoter by plasmid pACYCDuet-1, pCDFDuet-1, pRSFDuet-1, and pETDuet-1. *lgtA* (Genbank ID: 12393808) and *lgtB* (Genbank ID: 12393807) were cloned into plasmid pACYCDuet-tac, pCDFDuet-tac, pRSFDuet-tac, resulting pACYCDuet-lgtAB, pCDFDuet-lgtAB, pRSFDuet-lgtAB. Both *lgtA* and *lgtB* were optimized for the expression of β-1,3-*N*-acetylglucosaminyltransferase and β-1,4-galactosyltransferase in *E. coli* and were synthesized by Nanjing Genscript Biotech Company (Nanjing, china). The plasmid pCDFDuet-*lgtA* and pETDuet-*lgtB* were constructed by cloning the *lgtA* and *lgtB* into plasmid pCDFDuet-tac and pETDuet-tac, respectively.

Gene knockout and over-expression

To enhance LNnT synthesis, the role genes were over-expressed and the inhibition genes were knocked out by the CRISPR-Cas9 system. The method to construct knockout mutations in *E. coli* was as described by Chung et al. [27, 28]. The DNA recombination fragment was obtained by overlap extension-PCR, and was transformed into *E. coli* by electronic transformation.

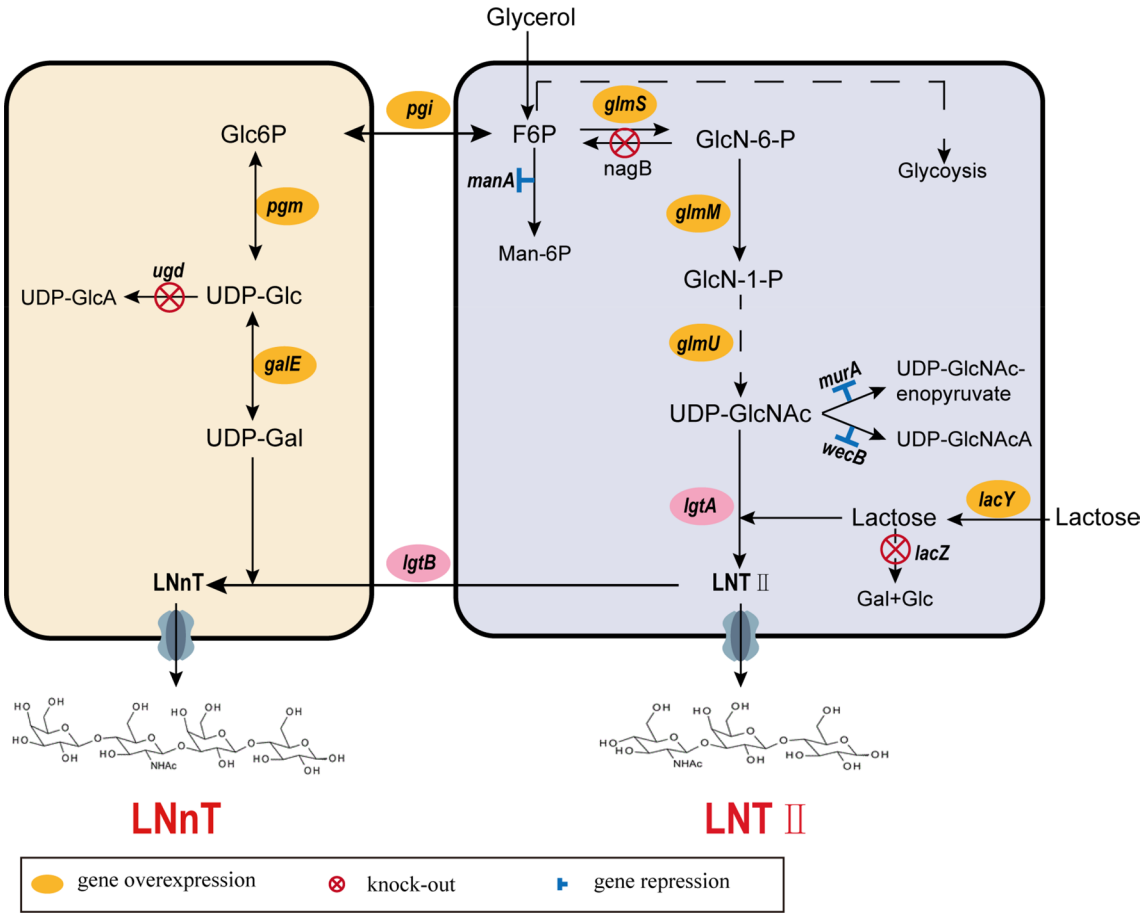

Fig. 1 The construction of the biosynthesis pathway of LNnT in *E. coli* K12 MG1655. The abbreviation of metabolites were as follows: *Gal* galactose, *Glc* glucose, *F6P* fructose-6-phosphate, *Glc6P* glucose-6-phosphate, *UDP-Glc* UDP-glucose, *UDP-Gal* UDP-galactose, *GlcN-6-P* glucosamine-6-phosphate, *GlcN-1-P* glucosamine-1-phosphate, *UDP-GlcNAc* UDP-*N*-acetylglucosamine, *UDP-GlcA* UDP-glucuronate, *Man-6P* mannose-6-phosphate, *LNTII* Lacto-*N*-triose II, *LNnT* Lacto-*N*-neotetraose. *glmS* encoding glucosamine-6-phosphate synthase, *glmM* encoding phosphoglucosamine mutase, *glmU* encoding *N*-acetylglucosamine-1-phosphate uridyltransferase/glucosamine-1-phosphate acetyltransferase, *pgi* encoding glucose-6-phosphate isomerase, *pgm* encoding phosphoglucomutase, *galE* encoding UDP-glucose 4-epimerase, *lacZ* encoding β-D-galactosidase, *ugd* encoding UDP-glucose 6-dehydrogenase, *nagB* encoding glucosamine-6-phosphate deaminase, *manA* encoding mannose-6-phosphate isomerase, *murA* encoding UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase, *wecB* encoding UDP-*N*-acetyl glucosamine-2-epimerase, *lgtA* β-1,3-*N*-acetylglucosaminyltransferase, *lgtB* β-1,4-galactosyltransferase

The locus we chose had little effect on strains' growth, as was reported in previous studies [29–32]. *lacY*, *pgi*, *glmS*, *glmM*, *glmU*, *galE*, and *pgm* were integrated into *fliT*, *motA*, *poxB*, *recA*, *arsB*, *cheY*, and *flhE* locus, respectively, and were over-expressed under *tac* promoter. *nagB* and *ugd* were knocked out via the same method. The diagrams of knockout and integration have shown in Fig. 4a, b.

Repression gene expression

Since the deletion of some genes may affect the growth of bacteria, the CRISPRi (CRISPR-mediated interference) system was used to inhibit the expression of those genes. The plasmid P-sgRNA constructed in this study consists of dcas9 and sgRNA, which could repression the specific genes [33]. To target the template DNA strand, 20 nucleotides were chosen as sgRNA and the primary sequence were replaced with these in plasmid P-sgRNA, resulting P-sgRNA-*manA*, P-sgRNA-*murA*, and P-sgRNA-*wecB*. The working principle of the CRISPRi system is illustrated in Fig. 4c.

Real-time quantitative PCR (RT-qPCR)

RNAprep Pure Kit (Tiangen Biotech, Beijing, China) was used to obtain total RNA. Then, the RNA was reverse transcript to cDNA with PrimeScript™ RT-PCR-Kit (Takara, Dalian, China). The RT-qPCR reactions were performed on a 96-well plate with total volume of 20 μL. The qPCR reaction was conducted with a LightCycler 480 II Real-time PCR instrument (Roche Applied Science, Mannheim, Germany)

Table 1 Strains used in this study

Strains	Characteristics	Resource
E. coli JM109	Cloning strain	Lab stock
E. coli K12 MG1655	Expression strain	Lab stock
K12-PAB	*E. coli* K12 MG1655/pCDFDuet-lgtAB	This work
Z-PAB	*E. coli* K12 MG1655△*lacZ*/pCDFDuet-lgtAB	This work
ZY-PAB	*E. coli* K12 MG1655△*lacZ*△*fliK*::*lacY*/pCDFDuet-lgtAB	This work
M0	*E. coli* K12 MG1655△*lacZ*△*lacI*△*fliK*::*lacY*	This work
M0-PAB	M0 derivate/pCDFDuet-lgtAB	This work
M0-PA/B	M0 derivate/pCDFDuet-*lgtA*/pETDuet-*lgtB*	This work
M0-PAB1	M0 derivate/pACYCDuet-lgtAB	This work
M0-PAB2	M0 derivate/pRSFDuet-lgtAB	This work
M01	M0 derivate/pCDFDuet-lgtA	This work
M02	M0 derivate, △*motA*::*pgi*/pCDFDuet-lgtA	This work
M03	M0 derivate, △*poxB*::*glmS*/pCDFDuet-lgtA	This work
M04	M0 derivate, △*flhE*::*glmM*/pCDFDuet-lgtA	This work
M05	M0 derivate, △*arsB*::*glmU*/pCDFDuet-lgtA	This work
M06	M0 derivate, P-sgRNA-*manA*/pCDFDuet-lgtA	This work
M07	M0 derivate, P-sgRNA-*murA*/pCDFDuet-lgtA	This work
M08	M0 derivate, P-sgRNA-*wecB*/pCDFDuet-lgtA	This work
M09	M0 derivate, △*nagB*/pCDFDuet-lgtA	This work
M10	M03 derivate, △*arsB*::*glmU*/pCDFDuet-lgtA	This work
M11	M03 derivate, P-sgRNA-*wecB*/pCDFDuet-lgtA	This work
M12	M03 derivate, △*nagB*/pCDFDuet-lgtA	This work
M13	M05 derivate, P-sgRNA-*wecB*/pCDFDuet-lgtA	This work
M14	M05 derivate, △*nagB*/pCDFDuet-lgtA	This work
M15	M14 derivate, △*poxB*::*glmS*/pCDFDuet-lgtA	This work
M16	M14 derivate, △*motA*::*pgi*/pCDFDuet-lgtA	This work
M17	M11 derivate, △*flhE*::*glmM*/pCDFDuet-lgtA	This work
M18	M15 derivate, P-sgRNA-*wecB*/pCDFDuet-lgtA	This work
M19	M10 derivate, △*nagB*/pCDFDuet-lgtA	This work
M20	M18 derivate, △*motA*::*pgi*/pCDFDuet-lgtA	This work
M15AB	M14 derivate, △*poxB*::*glmS*/pCDFDuet-lgtAB	This work
M18AB	M15 derivate, P-sgRNA-*wecB*/pCDFDuet-lgtAB	This work
M20AB	M18 derivate, △*motA*::*pgi*/pCDFDuet-lgtAB	This work
M21AB	M15 derivate, △cheY::galE/pCDFDuet-lgtAB	This work
M22AB	M15 derivate, △flhE::pgm/pCDFDuet-lgtAB	This work
M23AB	M15 derivate, △ugd/pCDFDuet-lgtAB	This work
M24AB	M18 derivate, △cheY::galE/pCDFDuet-lgtAB	This work
M25AB	M18 derivate, △flhE::pgm/pCDFDuet-lgtAB	This work
M26AB	M18 derivate, △ugd/pCDFDuet-lgtAB	This work
M27AB	M20 derivate, △cheY::galE/pCDFDuet-lgtAB	This work
M28AB	M20 derivate, △flhE::pgm/pCDFDuet-lgtAB	This work
M29AB	M20 derivate, △ugd/pCDFDuet-lgtAB	This work
M30AB	M29 derivate, △cheY::galE/pCDFDuet-lgtAB	This work
M31AB	M29 derivate, △flhE::pgm/pCDFDuet-lgtAB	This work
M32AB	M23 derivate, △cheY::galE/pCDFDuet-lgtAB	This work
M33AB	M23 derivate, △flhE::pgm/pCDFDuet-lgtAB	This work

and the primers used were listed in Supplementary Table S3. The 16S ribosomal RNA was chosen as the reference gene for relative quantification [10, 34].

Analytic methods

We used spectrophotometer (UVmini-1240, Shimadzu, Japan) to detect the optical density at 600 nm for growth

of *E. coli*. The dry cell weight (DCW) were calculated using the equation: DCW (g/L) = 0.4442 × OD_{600}-0.021 [35]. For SDS-PAGE analysis, the cell was diluted to OD_{600} of 5, and then the cells were boiled at 100 °C for 10 min.

The concentrations of lactose, LNTII and LNnT in the fermentation were measured with high-performance anion exchange chromatography-pulsed amperometric detection (HPEAC-PAD) [22, 36]. The column was CarboPac PA10 (4 × 250 mm) and the column temperature was 30 °C. The flow phase was 36 mM NaOH at a flow rate of 1.00 mL/min. Before the detection, the samples were preprocessed under the extracting with a solvent of chloroform/*n*-butanol (4:1 v/v) in a 1 mL tube for five times [22]. All experiments were repeated at least three times independently.

Results and discussion

Construction of the biosynthesis pathway of LNnT

Escherichia coli K12 MG1655 was chosen as the original strain and constructed de novo biosynthesis pathway for LNnT production in this study. Glycerol and lactose were used as the carbon resource and substrate. The precursors UDP-Gal and UDP-GlcNAc already exist in *E. coli*. To produce LNnT, two key exogenous genes are necessary, *lgtA* and *lgtB*. These two genes from *Neisseria meningitides* were codon optimized and over-expressed in *E. coli* K12 MG1655 using pCDFDuet-tac plasmid, resulting in recombinant strain, K12-PAB. SDS-PAGE analysis showed that recombinant strains successfully expressed a ~39-kDa protein encoded by *lgtA* and ~30-kDa protein encoded by *lgtB* (Fig. 2c).

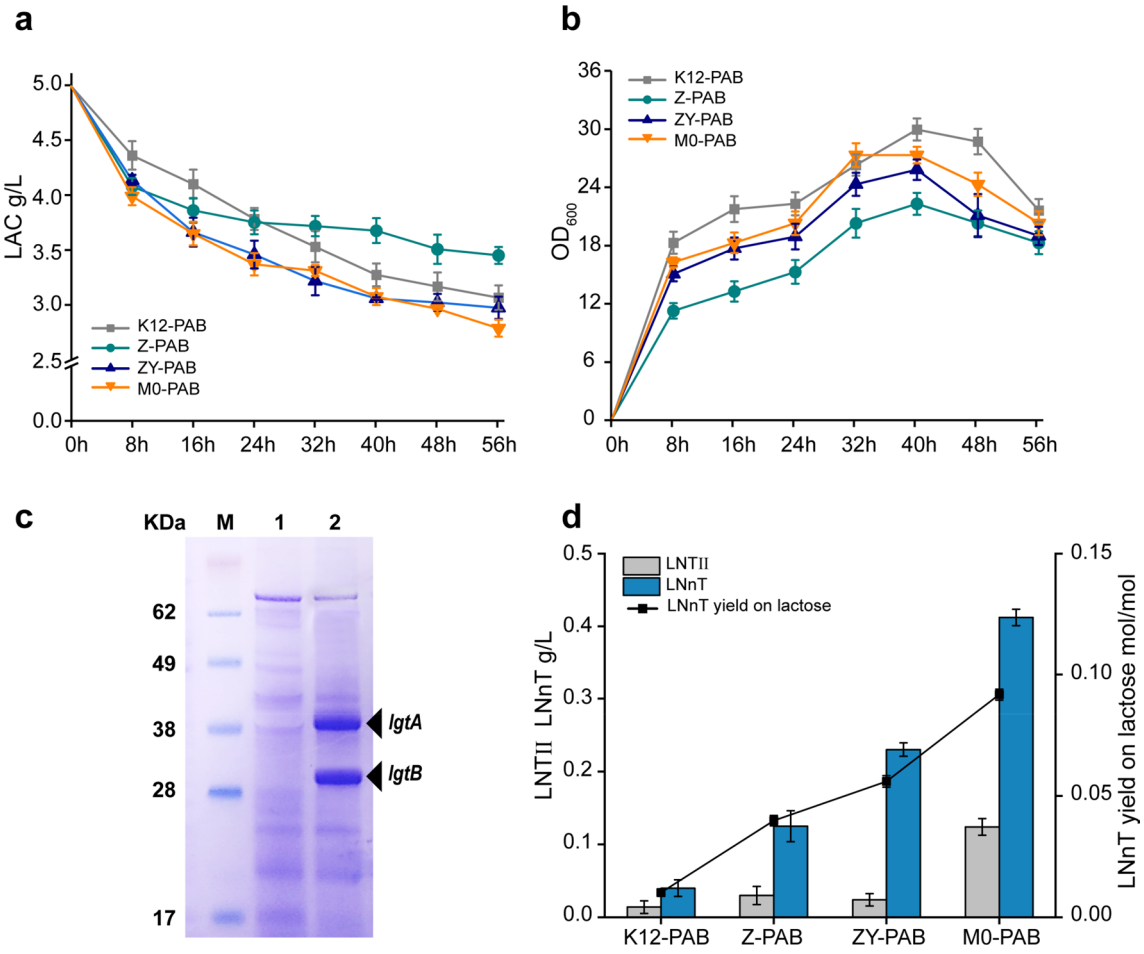

Fig. 2 Enhancing the supply of substrate lactose in the LNnT synthesis pathway. **a** The consumption of lactose in different recombinant *E. coli* K12 MG1655. **b** The cell growth of different recombinant *E. coli* K12 MG1655. **c** SDS- PAGE of recombinant *E. coli*. Lane M, protein marker; Lane 1, *E. coli* K12 MG1655 harboring pCDFDuet-tac; Lane 2, *E. coli* K12 MG1655 harboring pCDFDuet-lgtAB (*lgtA* first-arrow, *lgtB* first-arrow). **d** The production of LNnT and LNTII, the yield on lactose in different recombinant *E. coli* K12 MG1655

The K12-PAB strain was cultivated in TB medium with lactose as the substrate. After 60 h of fermentation, 0.04 g/L LNnT was obtained, which was transported to the medium (Fig. 2d). This indicated that the de novo pathway of LNnT from glycerol and lactose was successfully constructed in *E. coli* K12 MG1655. However, the concentration of the remaining lactose was 3.1 g/L, while the primary concentration was 5 g/L. This may be attributed to the blocking of lactose transport in *E. coli*, resulting in insufficient supply of substrate lactose.

As for the substrate, the utilization of lactose would affect LNnT production directly [11]. Without the optimizing of the pathway, most of lactose flows into the carbon metabolic pathway in *E. coli* K12 MG1655, while little flows to the synthesis of LNnT. Therefore, to block the consumption of lactose and to promote the intracellular mass transfer would be helpful [37].

Lactose permeate into the cell under the control of β-galactoside permease (encoded by *lacY*), and can be hydrolyzed into glucose and galactose by β-galactosidase (encoded by *lacZ*) in *E. coli* [10]. Reducing the degradation of lactose can elevate the flow to the destination path. Therefore, *lacZ* gene was first knocked out to prevent the decomposition of lactose, resulting in recombination strain Z-PAB (Table 1), so almost all of lactose can flow into the target pathway. The results showed that the consumption of lactose increased to 1.5 g/L (Fig. 2a) and the titer of LNnT increased from 0.04 to 0.125 g/L (Fig. 2d). To improve the transport efficiency of lactose, *lacY* gene was over-expressed by changing the original promoter to *tac* promoter to release the inhibition and enhance the intensity of expression. Meanwhile, one copy of *lacY* gene was integrated in the genome of *E. coli* K12 MG1655 under the control of *tac* promoter, resulting in ZY-PAB strain. The fermentation results showed that ZY-PAB strain consumed approximately 1.99 g/L lactose. The titer of LNnT increased to 0.23 g/L (Fig. 2d), which was resulted from the improved availability of intracellular lactose. Furthermore, to release the inhibition of the *lacY* gene on the genome, *lacI* gene was knocked out, resulting in M0-PAB strain. The titer of LNnT in M0-PAB increased to 0.41 g/L, and the consumption of lactose was 2.17 g/L, which increased by 42.8% as compared to the strain Z-PAB. These four strains consumed lactose in a faster rate in the first 24 h (Fig. 2a), indicating that the modification only increased the consumption of lactose, and did not change the consumption trend. The growth curve showed that the knockout of *lacZ* slightly inhibited the growth of strains due to the strains cannot use lactose as the carbon resource [38]. While the growth curve changed little compared to K12-PAB after over-expressing *lacY* and knockout *lacI* (Fig. 2b). This may be due to the increasing flow of lactose on target pathway contributes to cell growth. Furthermore, LNnT yield on lactose has increased from 0.01 mol/mol (K12-PAB) to 0.09 mol/mol (M0-PAB) (Fig. 2d). The expression of key genes would influence the titer of the product mostly, so the study on the expression of *lgtA* and *lgtB* may be helpful.

Increase LNnT yield by optimizing the expression of *lgtA* and lgtB

To optimize the expression of *lgtA* and *lgtB*, different expression cassettes were constructed. The *lgtA* and *lgtB* were cloned in pCDFDuet-tac and pETDuet-tac, respectively, resulting in pCDFDuet-*lgtA* and pETDuet-*lgtB*. Then, these two plasmids were transformed into M0 strain simultaneously, resulting in M0-PA/B strain. Subsequently, strain M0-PA/B and M0-PAB were fermented at the same time. The result indicated that the LNnT titer of M0-PAB (0.41 g/L) was higher than M0-PA/B (0.35 g/L). Furthermore, the yield of lactose on M0-PAB was 0.09 mol/mol and the DCW was 10.64 g/L (Fig. 3a). By comparing M0-PAB and M0-PA/B two strains, we found that when *lgtA* and *lgtB* were expressed on the same plasmid, the strain grew better and produced more LNnT than expressed on two plasmids. This may be attributed to the fact that the presence of two plasmids at the same time brought too much burden on the growth of the strains, resulting in the titer of the product was not as good as the single plasmid.

Then, the effects of single plasmid with different copy numbers on *lgtA* and *lgtB* genes expression were investigated. The *lgtA* and *lgtB* were cloned to pACYCDuet-1 (10–12 copy number), pCDFDuet-1 (20–40 copy number) and pRSFDuet-1 (> 100 copy number), resulting in pACYCDuet-lgtAB, pCDFDuet-lgtAB and pRSFDuet-lgtAB, respectively. Next, these plasmids were transformed into M0 strain, resulting M0-PAB1, M0-PAB and M0-PAB2. As shown in Fig. 3b, the titer of LNnT was 0.18 g/L, 0.41 g/L, and 0.36 g/L, respectively. Strain M0-PAB2 showed the highest titer in intermediate LNTII production (0.20 g/L), while the yield on lactose was lower than M0-PAB (0.08 mol/mol). This result showed that the plasmids with low or high copy numbers were not suitable for expression of *lgtA* and *lgtB*. When using the plasmid with high copy numbers, the intermediate LNTII was accumulate a lot (M0-PAB2, 0.2 g/L), but the titer of LNnT on M0-PAB2 (0.36 g/L) was lower than M0-PAB (0.41 g/L). Therefore, pCDFDuet-lgtAB was used for the production of LNnT in the subsequent optimizations. However, the titer of LNnT was still low, which may be due to the low flux in the pathway we constructed.

Improved the accumulation of intermediate product LNTII

LNTII is the key intermediate product of LNnT [14, 21]. Therefore, the titer of LNTII was used as a measure of the metabolic regulation to analyze the metabolic pathway of LNTII in *E. coli* K12 MG1655.

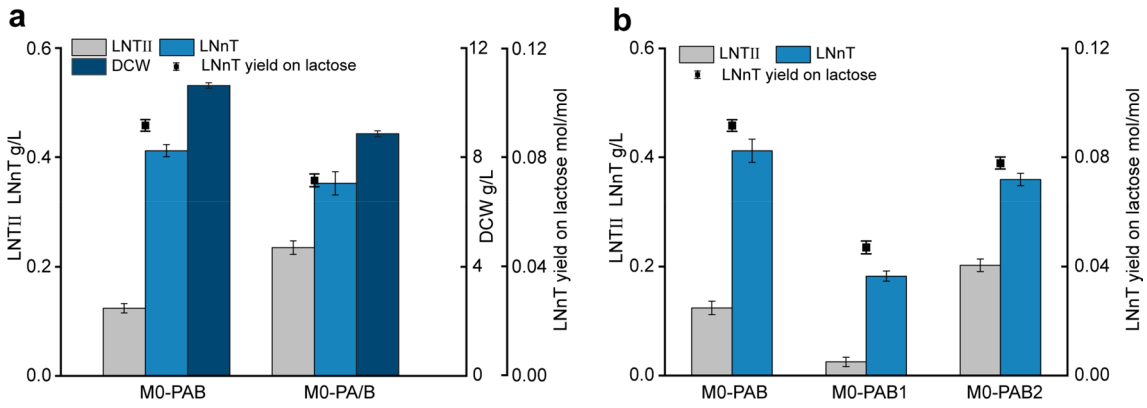

Fig. 3 Optimized expression of *lgtA* and *lgtB* in the LNnT synthesis pathway. **a** The production of LNnT and LNTII, and the yield on lactose and DCW. M0-PAB, *lgtA* and *lgtB* were co-expressed on the same plasmid; M0-PA/B, *lgtA* and *lgtB* were expressed separately. **b** The production of LNnT and LNTII and the yield on lactose when *lgtA* and *lgtB* were co-expressed in different copy numbers of plasmids. *M0-PAB* harboring pCDFDuet-lgtAB, *M0-PAB1* harboring pACYCDuet-lgtAB, *M0-PAB2* harboring pRSFDuet-lgtAB

To understand the effects of the key pathway genes on LNnT production, *pgi*, *glmS*, *glmM*, *glmU*, *manA*, *murA*, *wecB* and *nagB* were investigated separately based on strain M01. In addition, *pgi*, *glmS*, *glmM*, and *glmU* were over-expressed, *nagB* was knocked out, and *manA*, *murA*, and *wecB* were repressed, respectively, resulting recombination strains M02–M09.

After 60 h's fermentation, the titers of LNTII increased from 1.1 g/L (M01) to 1.2 g/L (M02), 2.2 g/L (M03), 1.5 g/L (M04), 2 g/L (M05), 1.2 g/L (M06), 1.3 g/L (M07), 1.6 g/L (M08) and 2.5 g/L (M09), respectively (Fig. 4d). Among these eight recombination strains, the strains over-expressed *glmS* (M03) and knocked out *nagB* (M09) gave the best results, where the titers of LNTII was 2.04-fold and 2.39-fold that of the control strain, respectively. These results suggested that the speed limit step in the LNnT metabolic pathway was likely between F6P and GlcN6P.

The over-expression or inhibition of individual genes has a different degree of positive effect on the production of LNTII, but the increments were very limited. Therefore, multiple genes were expressed in combination by selecting genes that have shown good performance, resulting in the new recombination strains, M10–M20. The titers of LNTII of these strains have increased by different degrees. The increase in LNTII production of M15, M18 and M20 was most significant, which were 2.95 g/L, 3.62 g/L and 3.82 g/L, respectively (Fig. 4d). Therefore, it is an efficient approach to improve the accumulation of intermediate product LNTII by combining genetic modification.

Then, these three high-yielding strains (M15, M18 and M20) were selected to determine the impact of production of LNnT. The plasmid pCDFDuet-lgtAB was transformed into these three strains, resulting in recombinant strains M15AB, M18AB, and M20AB, respectively. The results showed that the period of rapid consumption of lactose in M0-PAB was mainly in the last 16 h, while M15AB and M20AB were mainly in the first 16 h and M18AB was mainly between 16 and 40 h (Fig. 4e). The difference could be caused by the reformation of *wecB* and *pgi*, which changed the production period of LNnT. Little change in the growth curve indicated that the growth of the strains M0-PAB, M15AB, M18AB, M20AB was almost unaffected (Fig. 4f). The titer of LNnT in M15AB, M18AB and M20AB reached 0.98 g/L, 0.80 g/L and 1.04 g/L, respectively. The yield of LNnT on lactose of these three strains reached 0.19 mol/mol, 0.26 mol/mol and 0.24 mol/mol (Fig. 4g), which was higher than those of the control strain M0-PAB. After the modifications, the titer of LNnT on strain M20AB was 2.5-fold that of the control strain M0-PAB. The effects of *pgi*, *glmS*, *glmU* and *nagB* on LNnT production were consistent with the results of a previous study [22]. Some gene expressions that may affect the production of LNnT remain to be explored in subsequent metabolic pathways.

Improved the synthesis of LNnT by the fine-tuning of the gene expression

To further increase the titer of LNnT, the metabolic pathway of another important precursor UDP-Gal was investigated. In this pathway, *pgm*, *ugd* and *galE* were considered the possible genes influencing the LNnT titers. Through the aforementioned results, M15AB and M20AB had a higher titer on LNnT, and M18AB have the highest yield of LNnT on lactose. To obtain the higher titer strains, these three strains were selected to carry out further modifications. Further, *ugd* was knocked out and *pgm*, *galE* was over-expressed on these three high-yielding strains, respectively, resulting nine different recombination strains M21AB–M29AB.

The result showed that the deletion of *ugd* has a significant effect on the improvement of LNnT production. When

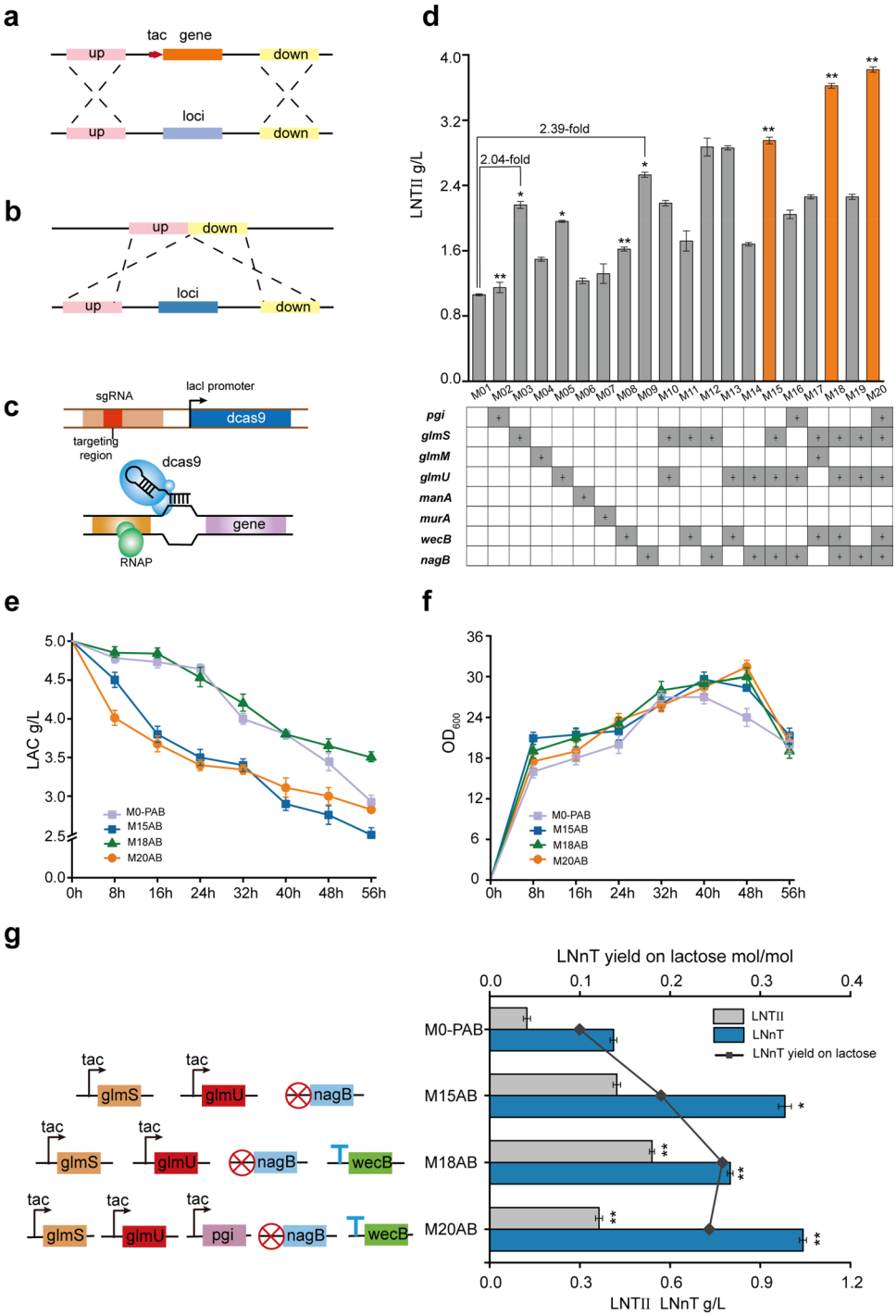

Fig. 4 Effects of regulating key genes in the LNnT synthesis pathway on intermediate product LNTII accumulation. **a** Schematic for integrating genes in different locus under *tac* promoter in *E. coli*. **b** Schematic for knocking out genes in *E. coli*. **c** Schematic for CRISPRi system in *E. coli*. **d** The production of LNTII of M02–M20 and the control strain M01. **e** The consumption of lactose of M0-PAB, M15AB, M18AB, and M20AB. **f** The cell growth of M0-PAB, M15AB, M18AB, and M20AB. **g** Effects of multiple gene expression on the titer of LNnT and LNTII, and the yield on lactose. * and ** mean $p < 0.05$ and $p < 0.01$, respectively

the *ugd* gene was deleted, the titer of LNnT increased from 1.04 g/L (M20AB) to 1.2 g/L (M29AB), a 93% increase over the control strain M0-PAB (Fig. 5b). The yield on lactose reached 0.28 mol/mol, which was 3.1-fold that of control strain M0-PAB. The deletion of *ugd* also had a good performance in strain M18AB, and the LNnT titer of which reached 1.1 g/L. However, the effect was not obvious to improve LNnT by over-expressing *galE* and *pgm*. When *galE* was over-expressed, the titer of LNnT were 0.98 g/L (M21AB), 0.46 g/L (M24AB), and 1 g/L (M27AB), respectively (Fig. 5b). The titer decreased to 0.64 g/L (M22AB), 0.78 g/L (M25AB), and 0.68 g/L (M28AB) when *pgm* was over-expressed. Furthermore, the accumulation of intermediate product LNTII of M29AB was 0.29 g/L, while that of M23AB and M26AB was only 0.18 g/L and 0.23 g/L, respectively (Fig. 5a). It indicated that the deletion of *ugd* was conducive to the transformation from LNTII to LNnT.

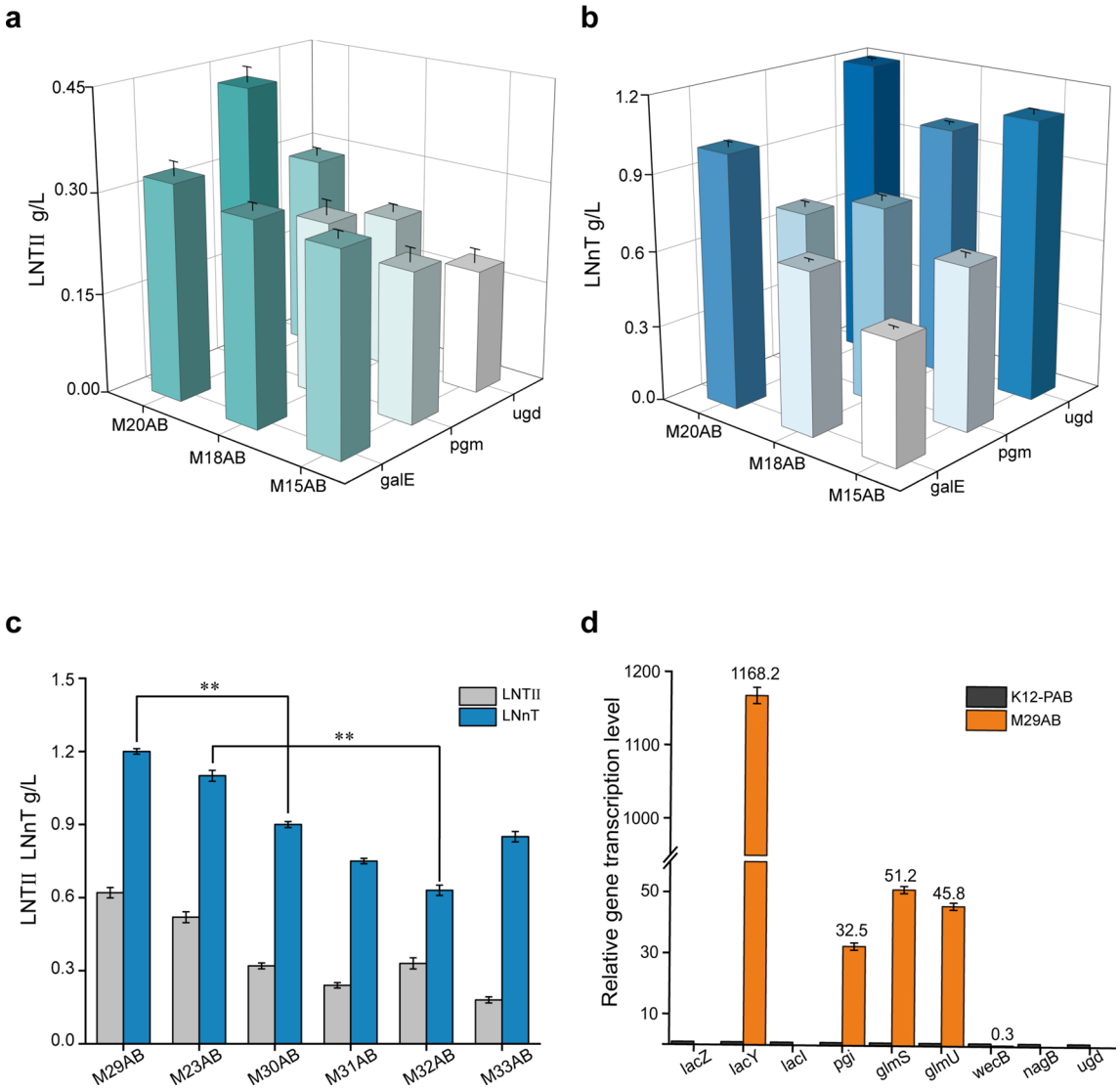

Fig. 5 Fine-tuning the gene expression in the LNnT synthesis pathway. **a** Effects of different recombinant *E. coli* of LNTII. **b** Effects of different recombinant *E. coli* of LNnT. **c** The production of LNnT and LNTII and the yield on lactose of M30AB–M33AB, the control strain were M29AB and M23AB. * and ** mean $p<0.05$ and $p<0.01$, respectively. **d** Comparison of transcription level of genes *lacZ*, *lacY*, *lacI*, *pgi*, *glmS*, *glmU*, *wecB*, *nagB* and *ugd* between strain K12-PAB and M29AB

To verify the synergy effects of multiple genes, *galE* and *pgm* were also over-expressed, respectively ,based on M29AB and M23AB, resulting in strains M30AB–M33AB. As shown in Fig. 5c, the titer were 0.9 g/L (M30AB), 0.75 g/L (M31AB), 0.63 g/L (M32AB), and 0.85 g/L (M33AB), respectively. The result indicated that the titer had a slight decrease when *pgm* and *galE* were over-expressed. Perhaps the over-expression of *pgm* and *galE* led to a feedback inhibition of the key metabolic flow by catalyzing the reverse reaction. Therefore, the highest titer, 1.2 g/L LNnT was achieved from the recombination strain M29AB.

The expression levels of key pathway genes were validated by RT-qPCR (Fig. 5d). The relative transcription levels of the *lacY*, *pgi*, *glmS* and *glmU* were all enhanced, while that of *wecB* gene was decreased significantly. In particular, *lacY*, *pgi*, *glmS* and *glmU* were improved 1168.2-, 32.5-, 51.2-, and 45.8-fold, respectively, compared to the control strain (strain K12-PAB), indicating that increased carbon flux was directed toward the target product pool to improve the production of LNnT.

Conclusion

In this study, the metabolic pathway of LNnT was constructed with wild strain *E. coli* K12 MG1655 as the starting strain, the initial titer of LNnT was 0.04 g/L. By improving the utilization rate of substrate lactose and optimizing the metabolic pathways, the titer of LNnT reached 1.2 g/L, which is a 93% increase over the control strain M0-PAB. The recombination strains and the fermentation conditions in this study can be used for industrial production. In addition, the yield on lactose has increased significantly from 0.01 mol/mol to 0.28 mol/mol, and the production process is environmental-friendly. Yet, the production of LNnT can be improved by further investigations, possibly by filtering *lgtB* gene from diverse sources.

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Author contributions WZ has a major contribution to the study, and writing of the manuscript. ZML, GMY, NL and XQL analyzed the experimental data. XMD, LYF, LJH and DGC revised the manuscript. LL conceived and supervised the project and contributed to the writing of the manuscript. All the authors read and approved the manuscript.

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Data availability All data generated or analyzed during this study are included in this published article and its supplementary information files.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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