Original Research Article

Multi-strategy engineering unusual sugar TDP-\(\alpha\)-mycarosylerythronolide B biosynthesis to improve the production of 3-O-\(\alpha\)-mycarosylerythronolide B in *Escherichia coli*

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**A R T I C L E   I N F O**

Keywords:
- TDP-\(\alpha\)-mycarosyl
- Glycosylated products
- 3-O-\(\alpha\)-mycarosylerythronolide B
- Metabolic engineering
- CRISPRi

**A B S T R A C T**

The insufficient supply of sugar units is the key limitation for the biosynthesis of glycosylated products. The unusual sugar TDP-\(\alpha\)-mycarosyl was initially attached to the C3 of the polyketide erythronolide B, resulting in 3-O-\(\alpha\)-mycarosylerythronolide B (MEB). Here, we present the de novo biosynthesis of MEB in *Escherichia coli* and improve its production using multi-strategy metabolic engineering. Firstly, by blocking precursor glucose-1-phosphate competing pathways, the MEB titer of triple knockout strain QC13 was significantly enhanced to 41.2 mg/L, 9.8-fold to that produced by parental strain BAF230. Subsequently, the MEB production was further increased to 48.3 mg/L through overexpression of *rfbA* and *rfB*. Moreover, the CRISPRi was implemented to promote the TDP-\(\alpha\)-mycarose biosynthesis via repressing the glycolysis and TDP-\(\alpha\)-rhamnose pathway. Our study paves the way for efficient production of erythromycins in *E. coli* and provides a promising platform that can be applied for biosynthesis of other glycosylated products with unusual sugars.

1. Introduction

Glycosylated natural products have diverse bioactivity and improved properties, which are closely associated with the structure and attachment pattern of the sugar units [1,2]. It has been well explored that the sugar moiety of many therapeutic agents participates in the interaction between the drug and cellular target which enables effective drug targeting and improved pharmacological properties [3–7].

Typically, the sugar units were originated from nucleoside diphosphate (NDP)-sugars, which can be categorized into distinct groups according to the complexity of biosynthetic pathway, namely common sugars and unusual sugars. Common sugars such as UDP-glucose and TDP-glucose are directly synthesized from sugar-1-phosphate (mainly glucose-1-phosphate, G1P) under the catalysis of nucleotidylyltransferase. Highly modified unusual sugars are generated through multi-step decorations including the epimerization, deoxygenation, ketoreduction and \(\alpha\), \(\beta\), or \(\gamma\)-methylations of common precursor TDP-4-keto-6-deoxy-\(\alpha\)-glucose (TKDG), the immediate product of TDP-glucose catalyzed by TDP-4,6-dehydratase, which contribute to the formation of structurally diverse natural glycosylated compounds. In recent years, pathway modification and/or combinatorial biosynthesis has emerged as a promising strategy to generate various unusual sugars and novel glycosylated compounds with enhanced activities [8–10]. For example, by the inactivation of native gene *dnrV* and the expression of heterologous gene *arrB* or *eryBIV*, the TDP-\(\alpha\)-daunosamine biosynthetic pathway of *Streptomyces peucetius* was modified to produce TDP-\(\alpha\)-epi-\(\alpha\)-daunosamine that was the epimer at C4′ hydroxyl group of TDP-\(\alpha\)-daunosamine, and the resulting glycosylated product epirubicin (4′-epidoxorubicin) possessed more effective antitumor property [11]. Besides, a series of undescribed deoxysugars were synthesized and attached to the anthracycline aglycones, resulting in the generation of unusual anthracycline analogues [12].

Nevertheless, the biosynthesis of complex glycosylated products is usually hampered attributable to the insufficient availability of sugar
units in the heterologous host [13–15]. Several metabolic engineering approaches including overexpression of endogenous genes [16], deletion of competitive pathways [17], the introduction of heterologous pathway genes [18], and reconstruction of independent pathways from other carbon resources [19,20] have been widely employed to enhance the production of glycosides conjugated with UDP-glucose. Though attempts have been made to increase the supply of two unusual sugars. It has been demonstrated that deletion of three genes (WeCE, ViaO, and RmlC) in the TKDG consumed pathway, the genetically modified strains strengthened the biosynthesis of TDP-α-mycarose and TDP-α-desosamine and was able to transform 6-dEB into erythromycin D, 60-fold to that of the original strain [28]. While the reported biocatalysts of 6-dEB necessitate further improvement to support the high production of erythromycin, there have been rare efforts to investigate and optimize the de novo microbial biosynthesis of 3-O-α-mycarosylethronolide B (MEB), the first glycosylated intermediate of erythromycin.

In this study, we establish the de novo MEB biosynthesis in E. coli and present a more comprehensive approach to promote the yield of MEB (Fig. 1). To achieve this, the bypass pathway of G1P was initially blocked (strategy 1). Then, key enzymes of the metabolic pathway were overexpressed to facilitate the biosynthesis of TKDG (strategy 2). Next, genes rmlC and rfbD were repressed to redirect the metabolic flux toward the TDP-α-mycarose via CRISPRi (strategy 3). Furthermore, repression of the glycolysis pathway was applied to enhance the endogenous TDP-α-mycarose pool and facilitate MEB concentration (strategy 4).
Table 1

| Plasmids/Strains | Description | Source |
|------------------|-------------|--------|
| PBF130 | pET21c-T7-DEBS2-DEBS3-T7 | 29 |
| PBF144 | pET28a-T7-pccb-rbs-pccA-T7-DEBS1-T7 | 29 |
| PBF26 | pET21c-T7-eryBVII-T7 | Lab stock |
| PBF27 | pET21c-T7-eryBIII-T7 | Lab stock |
| PBF28 | pET21c-T7-eryBVII-T7 | Lab stock |
| PBF29 | pET21c-T7-eryBIII-T7 | Lab stock |
| PBF30 | pET21c-T7-eryBVII-T7 | Lab stock |
| PBF31 | pET21c-T7-eryBIII-T7 | Lab stock |
| PBF32 | pET21c-T7-eryBIII-T7 | Lab stock |
| PBF33 | pET21c-T7-eryBIII-T7 | Lab stock |
| PBF34 | pET21c-T7-eryBIII-T7 | Lab stock |

Table 1 (continued)

| Plasmids/Strains | Description | Source |
|------------------|-------------|--------|
| QT2 | BAP1 carrying pBP130, pBP144 | This study |
| QT3 | BAP1 carrying pBP130, pBP144, pZF93 | This study |
| QT22 | BAP1 carrying pBP130, pBP144, pZF229 | This study |
| QT23 | BAP1 carrying pBP130, pBP144, pZF230 | This study |
| ZF1 | BAP1 Δpg | This study |
| ZF2 | BAP1 Δwef | This study |
| ZF3 | BAP1 ΔynX | This study |
| ZF7 | BAP1 ΔpgΔwef | This study |
| ZF8 | BAP1 ΔwefΔynX | This study |
| ZF9 | BAP1 ΔpgΔynX | This study |
| ZF13 | BAP1 ΔpgΔwefΔynX | This study |
| QC1 | ZF1 carrying pBP130, pBP144, pZF230 | This study |
| QC2 | ZF2 carrying pBP130, pBP144, pZF230 | This study |
| QC3 | ZF3 carrying pBP130, pBP144, pZF230 | This study |
| QC7 | ZF7 carrying pBP130, pBP144, pZF230 | This study |
| QC8 | ZF8 carrying pBP130, pBP144, pZF230 | This study |
| QC9 | ZF9 carrying pBP130, pBP144, pZF230 | This study |
| QC13 | ZF13 carrying pBP130, pBP144, pZF230 | This study |
| QC234 | ZF13 carrying pBP130, pBP144, pZF234 | This study |
| DTAC | ZF13 carrying pBP130, pBP144, pZF234, pACYC-dCas9-ter | This study |
| DT246 | ZF13 carrying pBP130, pBP144, pZF234, pZF246 | This study |
| DT239 | ZF13 carrying pBP130, pBP144, pZF234, pZF239 | This study |

2. Materials and methods

2.1. Strains, plasmids and chemicals

*E. coli* DH10B was used for plasmid construction and the previously reported BAP1 [29] was used for the biosynthesis of polylactide EB and MEB. The compatible vectors pET21c and pCDFDuet-1 (Novagen, Germany) were used to express the heterologous gene of the TDP-α-mycarose pathway (Table S1). Saccharopolyspora erythraea was cloned and assembled into pET21c yielding plasmid pZF90 and pZF91. Gene AceryBIII was then inserted into pZF91 between the SpeI/SacI to result in pZF92. The chaperone GroESL coding sequence was obtained through PCR with

F.ompT

Gal dcm (DE3) prpRBCD::T7-sfp, T7-prpE

29
Similarly, other plasmids pZF10 and pZF11 were constructed with pCB003_N20_pgi_F/R and its sequence was confirmed by sequencing.

2.3. CRISPR/Cas9-mediated knockout of chromosomal genes

SacI between generated by inserting the DNA fragment containing gene the primers 224_F/R (Table S2) and the template pZF223, and then inserted into pZF95 between SpeI and SacI to generate pZF227 and pZF228. Finally, the DNA fragment containing SaeryBII, SaeryBV and ermE was obtained after the digestion of pZF37 with restriction enzyme XbaI and SacI, and then constructed into pZF227 and pZF228, respectively, creating the corresponding pZF229 and pZF230 (Fig. S3).

2.3. CRISPR/Cas9-mediated knockout of chromosomal genes

The knockout of chromosomal genes in E. coli BAP1 was conducted by CRISPR/Cas9 system [30]. The sgRNA plasmid pZFP9 was obtained from pCB003 by inverse PCR utilizing primer pairs pCB003_N20_pgi_F/R and its sequence was confirmed by sequencing. Similarly, other plasmids pZF10 and pZF11 were constructed with primers pCB003_N20_zwf_F/R and pCB003_N20_yihF_X_F/R, respectively (Table S2). The upstream (h1) and downstream (h2) homologous arms of the target genes (pgi, awf, yihX) with the length of about 300-bp were separately amplified and then generated the donor DNA fragments by overlap PCR (Table S2). The PCR products were purified by gel electrophoresis before electroproporation. For the electroproporation, 100 μL of E. coli BAP1 competent cells harboring pCB006 were prepared and mixed with 1000 ng donor DNA and 200 ng sgRNA plasmid. Electroporator (Bio-Rad, USA) was used for electroporation (1 cm cuvette, 1.8 kV). Cells were resuspended in 1 mL Luria Broth (LB) medium and recovered at 30 °C for 2 h before being plated onto LB agar containing kanamycin (50 mg/L) and spectinomycin (50 mg/L). The recombinant colonies were verified by colony PCR and DNA sequencing after incubating at 30 °C overnight. The individual colony edited successfully was inoculated into 2 mL of LB medium containing kanamycin (50 mg/L) and IPTG (0.5 mM) to cure the sgRNA plasmid, and the pCB006 could be eliminated when cell cultures were cultivated at 42 °C for 12 h.

2.4. Creation of plasmids for genes rfbA and rfbB overexpression

The rfbA and rfbB genes were amplified from the genomic DNA of E. coli BAP1 with primer pairs 225_rfbA_F/R, 225_rfbB_F/R (Table S2) and then fused by overlap PCR to generate rfbAB. The purified rfbAB fragments were assembled with pEt21c treated with FseI_rfbB/PacI_rfbA and then inserted into pPET225. The rfbAB expression cassette was amplified from template pZF225 with primer pairs FseI_rfbB/PacI_rfbA and then inserted into pET22b+ treated with BamHI and EcoRI restriction enzyme. The resulting plasmids pZF237, pZF238, pZF243, and pZF244. To produce vector pZF246 which express multiple guides and dCas9 under the control of individual Pcm, the pZF243 and pZF244 were used as templates to obtain the fragments which contain BsaI restriction site using primers 246_rmlc_F/R and 246_rfbD_F/R. The BsaI sites of pACYC-dCas9-Ter backbone were introduced by PCR using primers ACYC-F/R. Then, all fragments were digested with BsaI (NEB, USA), and ligated to yield vector pZF246. The pZF237 and pZF238 were used as templates to obtain the fragments which contain BsaI restriction site using primers 239.pykA_F/R and 239.pykF_F/R, then all fragments were digested with BsaI (NEB, USA) and ligated to yield pZF239.

2.6. Media and culture conditions

Fermentation medium, LB (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) supplemented with 15 g/L glycerol, 100 mM 4-(2-hydroxyethyl)-1-piperazineneethanesulfonic acid (HEPES), was adjusted to pH 7.6 by NaOH before autoclaving and used to compare the production of engineering strains. For the biosynthesis of MEB, 100 μL of seed inoculum was inoculated into a 100 mL flask containing 10 mL fermentation medium supplemented with appropriate antibiotics (100 mg/L ampicillin, 50 mg/L kanamycin, 50 mg/L spectinomycin, and 34 mg/L chloramphenicol) and grown at 37 °C. Isopropyl β-D-thiogalactopyranoside (IPTG) and sodium pyruvate were added at a final concentration of 0.5 mM and 5 mM when OD600 reached 0.4. 1-α- arabinose at a final concentration of 10 mM was added to induce the CRISPRi system when OD600 = 2. Cell cultures were subsequently incubated at 22 °C for 7 days.

2.7. HPLC and LC-MS/MS analytic methods

All samples were analyzed by high-performance liquid chromatography (HPLC) on an Ultimate 3000 HPLC system (ThermoFisher Scientific) with ELSD detector (Alltech U3000, Agilent) and a SilGreen ODS column (ø 4.6 × 250 mm, 5-μm, Greenhers, Beijing, China) maintained at 30 °C. Compounds were separated by acetonitrile (solvent A) and water (containing 50 mM ammonium formate, solvent B) at a flow rate of 1.0 mL/min under the following conditions: 0 min: 100% B; 0–30 min: linear-gradient increase to 95% B; 30–31 min: linear-gradient increase to 100% B; 31–35 min: 100% B. The data shown in this study were generated from three independent experiments.

LC-MS/MS was performed on Q Exactive hybrid quadrupole–Orbitrap mass spectrometer (Thermo Scientific, MA, U.S.A.) equipped with Infinity Lab Poroshell 120 SB-AQ C18 column (ø 3.0 × 100 mm, 2.7 μm, Agilent, U.S.A.). The mobile phase was acetonitrile (A) and H2O with 0.1% formic acid (B). A linear gradient was set as follows: 5–95% solvent A for 10 min; 95% solvent A for 1 min; 95-5% solvent A for 5 min. The flow rate was 0.4 mL/min, and the injection volume was 2 μL. The mass acquisition was performed in positive ionization mode with a full scan (100–1000).

2.8. Purification and quantification of 3-O-acetylmycarosylerythronolide B

To obtain high purity MEB, collected samples were first separated by column chromatography over SiliaSphere C18 (50 μm, Silicycle, Québec, QC, Canada), and then purified by semi-preparative HPLC (Dionex UltiMate 3000 Semi-Preparative HPLC Systems, Thermo Scientific, MA, U.S.A.) with 40% acetonitrile in water (flow rate of 10 mL/min, detected at 205 nm) and a SilGreen ODS C18 column (ø 20 × 250 mm, 5 μm, Greenhers Co., Ltd., Beijing, China). 1H and 13C and 2D NMR spectra of MEB were recorded by an Avance DRX 400 (500 MHz for 1H, 125 MHz for 13C) spectrometer (Bruker, Germany). The standard curve of MEB was generated by calculating the peak area of MEB.
3. Results and discussion

3.1. Establishing the biosynthesis of 3-O-α-mycarosylethyronolide B in E. coli

To rebuild the biosynthesis of 3-O-α-mycarosylethyronolide B (MEB) in E. coli, the previously reported BAP1 harboring pBP130 and pBP144 was used as the starting strain (WT) to produce 6-dEB. Subsequently, we performed the expression analysis of the pathway genes of TDP-α-mycarose. As shown in Fig. S1, except for EryBIV and ErmE, the other six proteins could not be expressed. It has been demonstrated that pBP130, pBP144, and pZF229) and BAP230 (BAP1 harboring pBP130, pBP144, and pZF230). ST-6-DEB, 6-DEB standard; ST-EB, EB standard; 1, 6-DEB; 2, EB; 3, 3-O-(2′,6′-dideoxy-α-L-arabinofuranosyl) erythronolide B; 4: 3-O-(2′,6′-dideoxy-α-L-ribofuranosyl) erythronolide B; 5: MEB; b, The LC-MS/MS fragments of compound 3. c, The LC-MS/MS fragments of compound 4. d, The LC-MS/MS fragments of compound 5.

39.30 in 3, and 36.39 in 4), C4′ (δC 70.20 in 3, and 67.70 in 4) as well as H6′ to C4′ and C5′ (Figs. S8–S15). The large coupling constants (J = 9.6 Hz) of H4′, H5′ and H6′ in 3 indicated that the substituents of C3′-C5′ are equatorial, while the small values of $J_{H1′,H2′}$ (3.0 Hz) and $J_{H2′,H3′}$ (3.0 Hz) in 4 suggested that the substituents of C1′ and C3′ are axial. Therefore, compounds 3 and 4 were assigned as 3-O-(2′,6′-dideoxy-α-L-arabinofuranosyl) erythronolide B and 3-O-(2′,6′-dideoxy-α-L-ribofuranosyl) erythronolide B, respectively, which were reported previously in the mutant S. erythraea [31].

We speculated that the generation of MEB derivatives instead of MEB might be attributed to the low enzymatic activity of SaeryBIVII and SaeryBIII of plasmid pZF229, resulting in the fermentation media of E. coli BAP1 with the aid of chaperones GroEL/GroES. As we anticipated, with the exception of SaeryBIVII and SaeryBIII, all TDP-α-mycarose pathway enzymes could be highly expressed (Fig. S16), which indicated that the soluble expression of eryBIVII and eryBIII exerted an important effect on the biosynthesis of MEB.

To facilitate the creation of TDP-α-mycarose, the homologous AeeryBII and AeeryBIII originated from A. erythreum were selected and synthesized with optimized codons. Protein expression analysis showed that both AeeryBII and AeeryBIII gave distinct expression bands under the same cultivation condition (Fig. S17), which were utilized to replace the SaeryBIVII and SaeryBIII of plasmid pZF229, resulting in plasmid pZF230 (Fig. S18). HPLC analysis clearly revealed the presence of 6-deB (1) and a new compound 5 (Rt = 20.4 min) in the fermentation media of strain BAP230 generated by introducing pZF230 into strain WT (Fig. 2a). Compound 5 was subsequently identified as MEB by the characteristic ion peaks $m/z$ 529.3389 observed in the HPLC-MS/MS mass profile and NMR spectra (Fig. 2d, S19 and S20). This result indicated the feasibility to achieve the biosynthesis of MEB in E. coli by combining pathway enzymes with effective expression. Eventually, the MEB concentration of BAP230 was quantified to be 4.2 mg/L on the basis of the established standard curve of MEB (Fig. S21).
3.2. Enhancing the 3-O-α-mycarosylerythronolide B production via pathway disruption

Given that the unusual sugar TDP-L-mycarose is the crucial biosynthetic bottleneck of MEB [28], it is essential to improve the yield of MEB by enhancing the intracellular TDP-L-mycarose pool. Glucose-6-phosphate (G6P) is the common precursor of glycolysis, pentose phosphate pathway and TDP-L-mycarose biosynthetic pathway. To provide more G6P for the synthesis of TDP-L-mycarose, glycolysis and pentose phosphate pathway that consume G6P need to be blocked. It has been demonstrated that deletion of genes *pgi* (encoding phosphoglucose isomerase) and *zwf* (encoding glucose-6-phosphate dehydrogenase) could improve the level of UDP-glucose and elevate the production of the corresponding glycosylated products [32–34]. Moreover, the G1P hydrolase encoded by gene *yihX* was capable of selectively hydrolyzing G1P, which is the intermediate of TDP-L-mycarose biosynthesis [35]. To reinforce the TDP-L-mycarose biosynthetic pathway and increase the MEB production, we individually knocked out genes *pgi*, *zwf*, and *yihX* in *E. coli* BAP1, generating strains ZF1 (ZF1 harboring pBP130, pBP144, and pZF230), ZF2 (ZF2 harboring pBP130, pBP144, and pZF230), ZF3 (ZF3 harboring pBP130, pBP144, and pZF230), QC1 (ZF1 harboring pBP130, pBP144, and pZF230), QC2 (ZF2 harboring pBP130, pBP144, and pZF230), QC3 (ZF3 harboring pBP130, pBP144, and pZF230), QC7 (ZF7 harboring pBP130, pBP144, and pZF230), QC8 (ZF8 harboring pBP130, pBP144, and pZF230), QC9 (ZF9 harboring pBP130, pBP144, and pZF230) and QC13 (ZF13 harboring pBP130, pBP144, and pZF230).

Fig. 3. Effects of the gene knockout on the cell growth and MEB production. a, The strategy 1 to enhance the MEB production by genes deletion. b, The 6-dEB, EB, and MEB production and OD<sub>600</sub> of recombinant strains BAP230 (BAP1 harboring pBP130, pBP144, and pZF230), QC1 (ZF1 harboring pBP130, pBP144, and pZF230), QC2 (ZF2 harboring pBP130, pBP144, and pZF230), QC3 (ZF3 harboring pBP130, pBP144, and pZF230), QC7 (ZF7 harboring pBP130, pBP144, and pZF230), QC8 (ZF8 harboring pBP130, pBP144, and pZF230), QC9 (ZF9 harboring pBP130, pBP144, and pZF230) and QC13 (ZF13 harboring pBP130, pBP144, and pZF230).

Fig. 4. The effects of overexpressing *rfbA* and *rfbB* on MEB production. a, The strategy 2 to reinforce the MEB production. b, Schematic diagram of pZF234. c, Cell growth and 6-dEB and MEB production of QC13 (ZF13 harboring pBP130, pBP144, and pZF230) and QC234 (ZF13 harboring pBP130, pBP144, and pZF234).
of strain QC1, while QC8 (OD$_{600}$ = 13.1) showed no growth difference with wild-type strain BAP230. The 6-dEB and MEB concentrations in strain QC7 were 24.7 mg/L and 35.1 mg/L, increasing significantly by 190% and 740% compared to strain BAP230, respectively. The strain QC8 generated 12.9 mg/L 6-dEB and 14.0 mg/L MEB. Compared with strain BAP230, the titers of 6-dEB and MEB have no significant change accompanied with the accumulation of EB in strain QC9, suggesting that engineered *E. coli* drive more metabolic flux towards EB biosynthesis and the availability of endogenous TDP-L-mycarose was limited. Hence, we attempted to disrupt genes *pgi*, *zwf*, and *yihX* simultaneously and construct the recombinant strain ZF13 (BAP1 Δpgi Δzwf ΔyihX) (Fig. S22).

The corresponding fermentation strain QC13 afforded the highest MEB production with a titer of 41.2 mg/L, which is a 9.8-fold increase to that produced by BAP230. In addition, QC13 also achieved the highest production of 24.5 mg/L 6-dEB, which indicated the efficient downstream pathways utilizing 6-dEB as building unit or biosynthetic precursor might lead to the improved metabolic flux toward 6-dEB. These results suggested that increasing the carbon flux at the G1P node by blocking the competing pathway could drive the biosynthesis of TDP-L-mycarose and MEB. Therefore, strain ZF13 was chosen for further engineering.

### 3.3. Overexpression of *rfbA* and *rfbB* for 3-O-α-mycarosylerythronolide B production

Glucose-1-phosphate thymidylyltransferase (*rfbA*) and TDP-glucose-4,6-dehydratase (*rfbB*) are capable of converting G1P to TKDG, a crucial intermediate of TDP-L-mycarose (Fig. 4a). To further increase the MEB titer, we sought to reinforce the TDP-L-mycarose biosynthetic pathway via overexpression of *rfbA* and *rfbB*. Accordingly, the *rfbA*-*rfbB* module was cloned from *E. coli* and incorporated on the pZF230 as an independent operon, yielding plasmid pZF234 (Fig. 4b). The shake flask fermentation results showed that the MEB production of strain QC234 (ZF13 harboring pBP130, pBP144, and pZF234) was slightly increased and reached a maximum of 48.3 mg/L, a 17% increase relative to strain QC13 (41.2 mg/L). Intriguingly, strain QC234 produced 9.6 mg/L of 6-dEB, which is comparable to that of QC13 (Fig. 4c).

To verify whether the increase in MEB yield was due to the enhancement of TDP-L-mycarose, we measured the cellular concentration of TDP-L-mycarose in the engineered strains that exclusively synthesize the sugar skeleton. As shown in Fig. S23, the triple knockout strain sZF13 (pZF230) that was engineered to enhance the supply of G1P was capable of producing 107.2 mg/L 6-dEB, a 12.9-fold to that of the control strain BAP1 (pZF230) (8.3 mg/L), while the sZF13 (pZF234) which was created to enhance the supply of G1P and further convert the precursor G1P into the key intermediate TKDG achieved the highest titer of TDP-L-mycarose of 143.3 mg/L, a 16.3-fold increase to that produced by BAP1 (pZF230).

### 3.4. Regulating the metabolic pathway of *E. coli* with CRISPRi

Considering that genes overexpression involved in TDP-L-mycarose pathway led to a modest increase in MEB production, which might be due to the leakage of TKDG caused by TDP-L-rhamnose synthesis and the shortage of intracellular G1P, we next aim to improve MEB titer by addressing these problems. TDP-4-dehydrorhamnose-3,5-epimerase (*rmlC*) and TDP-L-rhamnose synthase (*rfbD*) were reported to catalyze TKDG to form TDP-L-rhamnose that played pivotal roles in membrane synthesis and cellular function [26]. To mitigate the leakage of TKDG, we implemented the CRISPRi system mediated by P$_{ara}$ promoter in the established strain QC234 to downregulate the expression of these two endogenous genes *rmlC* and *rfbD* (Fig. 5a). Consequently, pZF246 was constructed by inserting two sgRNA cassettes targeting *rmlC* and *rfbD* into the dCas9-expressing plasmid pACYC-dCas9-Ter (Fig. S24a).
Introduction of the pACYC-dCas9-Ter and pZF246 into the efficient MEB producer QC234 resulted in recombinant strain DTAC and DT246, respectively. Unexpectedly, the repression of TDP-\-L-rhamnose biosynthesis in strain DT246 ($\text{OD}_{600} = 2.8$) resulted in striking growth defect compared with strain QC234 ($\text{OD}_{600} = 10.1$) (Fig. 5b), whereas strain DTAC ($\text{OD}_{600} = 8.0$) showed slightly impaired growth. The reason that the titers of MEB in strains DTAC (44.4 mg/L) and DT246 (46.9 mg/L) were decreased could be ascribed to the compromised cell growth. Despite the application of CRISPRi exhibited no beneficial effects on MEB production, the MEB concentration per $\text{OD}_{600}$ of DT246 was 3.5-fold to that of QC234.

Pyruvate kinases II ($\text{pykA}$) and I ($\text{pykF}$) are indispensable enzymes in the glycolysis pathway which have been widely investigated and engineered to rewire the carbon metabolism and facilitate the generation of nucleotide-activated sugar donor [34,37]. To further promote the formation of G1P, we thus reconstructed the CRISPRi system to reduce the expression of $\text{pykA}$ and $\text{pykF}$ by replacing the sgRNAs of pZF246, yielding pZF239 (Fig. S24b), which was transformed into strain QC234 to obtain DT239 (Fig. 6a). Although a slightly recovered cell growth was observed in DT239 ($\text{OD}_{600} = 3.7$) in comparison with DT246, simultaneous inhibition of $\text{pykA}$ and $\text{pykF}$ failed to accomplish the improvement of MEB production in strain DT239 (48.2 mg/L), nearly equal to the titer of QC234 (48.3 mg/L) (Fig. 6b). This might largely be attributable to the metabolite burden caused by the expression of multiple pathway genes and transcriptional regulators. In spite of the fact that the implementation of the CRISPRi resulted in no remarkable increase in the concentration of MEB, the higher biomass specific rate of MEB still demonstrated the functionality of CRISPRi system in driving carbon flux from G1P to TDP-\-L-mycarose.

4. Conclusions

In summary, the multi-level metabolic engineering approach including gene disruption, gene overexpression and CRISPRi was successfully performed to achieve high-level TDP-\-L-mycarose and boost MEB production. Notably, the strain QC234 producing 48.3 mg/L MEB and 9.6 mg/L 6-dEB, was constructed by deletion of $\text{pgi}$, $\text{zwf}$, and $\text{yihX}$ and overexpression of $\text{rfbA}$ and $\text{rfbB}$. The CRISPRi system was employed to repress bypass pathways that consume precursors, leading to a 250% increase in the titer of MEB per $\text{OD}_{600}$ in DT246 compared with QC234. This study lays the foundation for de novo biosynthesis of erythromycin and other glycosylated products decorated by unusual sugar.

CRediT authorship contribution statement

Zhifeng Liu: Conceptualization, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing.
Jianlin Xu: Methodology, Software, Validation, Writing – original draft, Writing – review & editing.
Zhuanguang Feng: Methodology, Software.
Yong Wang: Project administration, Funding acquisition, Supervision.

Declaration of competing interest

The authors declare no competing financial interest.

Acknowledgements

This study was financially supported by the National Key R&D Program of China (2018YFA0900600), the Program of Shanghai Academic Research Leader (20XD1404400), the Strategic Priority Research...
Program “Molecular mechanism of Plant Growth and Development” of CAS (XDB27020202), the National Natural Science Foundation of China (31670099), the Construction of the Registry and Database of Bioparts for Synthetic Biology of the Chinese Academy of Science (No. ZSYS-016), the International Partnership Program of Chinese Academy of Science (No. 153D31YKSB20170121) and the National Key Laboratory of Plant Molecular Genetics, SIPPE, CAS.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2022.03.002.

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