Fine and dynamic tuning the glycolytic flux ratio of an artificial carbon saving pathway for high yield of mevalonate in Escherichia coli

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Abstract

Background

In natural *Escherichia coli*, glucose is mainly metabolized via the Embden-Meyerhoff-Parnas (EMP) pathway. However, in the metabolic process of conversion of pyruvate to acetyl-CoA, one-third of the carbon is lost at CO\(_2\). To decrease the loss of glucose in the metabolic process and enhance the carbon conversion efficiency production of desired products by *E. coli*, we constructed a carbon saving pathway, EP-bifido pathway. As the balance of energy and reducing power was not optimal, we use synthetic biology methods to precisely and dynamically adjust the EMP pathway and pentose phosphate pathway (PPP) flux to improve the production of mevalonate (MVA) via the EP-bifido pathway.

Result

Here, we enhanced the MVA titer and yield in *E. coli* in two ways. First, the promoter of the first gene of the PPP, *zwf*, was replaced with a set of promoters of different strength to enhance PPP flux for NADPH supply. Compared with the previous EP-bifido strains, the *zwf*-modified strains showed obvious differences in NADPH, NADH, and ATP synthesis levels and production routes. Among them, strain BP10BF accumulated 11.2 g/L of MVA after 72 h of fermentation and the molar conversion rate from glucose reached 62.2%. Second, the expression of *pfkA* was suppressed at a certain time by the clustered regularly interspaced short palindromic repeats interference (CRISPRi) system to avoid the growth defect caused by *pfkA* direct knock-out. The resulting MVA yield of strain BiB1F was 8.53 g/L, and the conversion rate from glucose reached 68.7%.

Conclusion

This is the highest MVA conversion rate reported in shaken flask fermentation. The CRISPRi and promoter fine-tuning provided an effective strategy for metabolic flux redistribution in many metabolic pathways and promotes the chemicals production.

Background

It is an important challenge in metabolic engineering to reasonably allocate metabolic flux to achieve high yields of target products[1]. Traditional metabolic engineering methods modify and optimize an organism for production of chemicals by decreasing flow through competing pathways and introducing heterogeneous production pathways. As such, metabolic rewiring designs are necessary to increase flux towards essential metabolites, for example, overexpressing native pathways[2, 3], inhibition of competing pathways[4], increasing Coenzyme A (CoA) availability[5], and construction of pyruvate dehydrogenase bypass. Many strategies have been applied to improve production of chemicals [6].

A new strategy is to decrease the generation of harmful byproducts such as CO\(_2\) or increase the reuse of byproducts by constructing artificial synthetic pathways. With the rapid development of synthetic biology
and molecular biotechnology, scientists have made great efforts to maximize microbial chemical yields focusing on enhancing the efficiency of CO₂ fixation and decreasing CO₂ emission. Many unnatural metabolic pathways have been constructed, such as CETCH [7], MCG [8], NOG [9], MOG [10], and so on. These pathways provide a variety of new ideas to use CO₂ or one-carbon chemicals as carbon sources, and rewire metabolic pathways [11, 12].

In natural glycolysis, a variety of carbon sources are metabolized through the Embden-Meyerhoff-Parnas (EMP) pathway, which synthesizes C3 (pyruvate) and C2 (acetyl-CoA) metabolites. Acetyl-CoA is a precursor of almost all biosynthesis and energy metabolism pathways. It is normally produced via pyruvate decarboxylation, in which one-third of the carbon is lost as CO₂. To improve carbon conversion efficiency, Xu et al. constructed the “EP-bido pathway” in *E. coli* by introducing *fxpk* (encoding bifunctional phosphoketolase) and *fbp* (encoding fructose-1,6-bisphosphatase) to break down sugar phosphates into the theoretical maximum amount of acetyl-CoA from glucose with enough NADPH supply. The *edd* gene in the Entner-Doudoroff (ED) pathway and the key enzyme-encoding gene *pfkA* (phosphofructose kinase A) in the EMP pathway were knocked out to save more fructose 6-phosphate to break down into C2 metabolites; attenuate the flow of pyruvate to acetyl-CoA; and shift carbon flux from the EMP pathway to the pentose phosphate pathway (PPP) which supplies more NADPH. The EP-bido pathway achieved high yield in acetyl-CoA-derivative fermentation. The carbon yield of mevalonate (MVA) from glucose reached 64.3 mol%[13].

However, cells disrupted in *pfkA* in the EP-bido pathway cannot achieve a balance of reducing power and caused a growth defect of the cells as the native EMP pathway is used to generate pyruvate and other essential biosynthetic precursors. This situation significantly limits the utility of this pathway. Many reports have demonstrated this limitation of *pfkA* deficiency[14]. Therefore, the artificial pathway must be optimized to be robust and catabolize sugar at a reasonable rate.

The design of artificial synthetic pathways is usually static, blocking a competing pathway or introducing heterogeneous pathways permanently and continuously [15-17]. Sometimes this has a detrimental effect, for example in the early growth period when resources would ideally be dedicated to building biomass. Implementing flexible regulation would be valuable in engineering projects by rebalancing synthetic pathways to respond to the growth phase or the buildup of precursor metabolites [18-20].

The PPP is an important energy metabolism pathway in all organisms. The PPP, tricarboxylic acid (TCA) cycle, and hydrogen transfer provide 35%-45%, 20%-25%, and 30%-45% of the NADPH required during aerobic growth. The production/consumption balance of NADPH suggested that the oxidative PPP provided inadequate NADPH for higher MVA production [21]. The flux ratio between the EMP and oxPPP pathways affects the flux to MVA and acetate from acetyl-CoA[22]. Increase of the dehydrogenase reactions of the PPP is effective in increasing the yield of NADPH-dependent products [23-27].

To overcome the above challenges, here, we design and construct an *Escherichia coli* strain that relies on the EP-bifido pathway for carbon catabolism to support growth. The metabolic flow through the PPP was
enhanced to supply sufficient NADPH by introducing different strength promoters of zwf. And then to avoid the growth inhibition caused by deletion of pfkA, we used the clustered regularly interspaced short palindromic repeats interference (CRISPRi) system to suppress the expression of pfkA by designing three sgRNAs for suppression. The CRISPRi gene regulation system requires only two components, dCas9 protein and a gRNA, to achieve regulation of the transcription level of any gene in the genome. The degree of suppression of gene expression can be controlled by adjusting the binding position and expression amount of the gRNA. The system also has the advantages of strong applicability and no obvious off-target phenomenon. These characteristics mean CRISPRi is widely used in the field of metabolic engineering [28, 29]. After CRISPRi was applied, we obtained a titer of 8.53 g/L MVA and a yield of 68.7% (mol/mol). This is the highest MVA yield reported in shaken flask fermentation.

Results

Enhancement of PPP flux by increasing zwf expression

One of the essential purposes of the EP-bifido pathway is to lessen EMP pathway flux and improve PPP flux to enhance the NADPH supply. Suitable flux distribution between the EMP pathway and PPP has a positive effect on the production of target compounds. In the EP-bifido pathway, the theoretical optimal carbon split to the EMP pathway and PPP in the EP-bifido route for MVA production is 1:6[13]; therefore, the maximum carbon theoretical conversion rate of the EP-bifido pathway is 86% (mol/mol). In our previously constructed EP-bifido pathway, the carbon split ratio was 0.43:0.57. To further increase the shunt to the PPP, we aimed to enhance the expression of the first key gene of the PPP, zwf, by replacing its promoter with a high strength promoter.

We selected five constitutive promoters with different strengths from the Anderson promoter library. The theoretical strength of each promoter is shown in Table 3. We compared the actual expression strength of these five synthetic promoters with the original zwf promoter by placing a green fluorescent protein gene downstream of the promoters and detecting fluorescence intensity. The fluorescence/OD$_{600}$ at 16 h is shown in Fig. 1A. The strength of the promoters was relatively consistent with that stated by the Anderson promoter library. The strength of promoters BBa-J23100 and BBa-J23104 was relatively high, and BBa-J23100 was the strongest. The strength of the original (native) zwf promoter is between that of BBa-J23108 and BBa-J23114, and is relatively weak. Strain BW-P (pfkA deletion in BW25113) grew slightly poorer than that of the parental strain BW25113.

To detect the effect of PPP enhancement on MVA production, plasmids pBSA (expressing three enzymes catalysis acetyl-CoA to mevalonate) and pFF (carrying fbp and fxpk gene) were transformed into the five zwf-enhanced strains and cultivated with the control strain BW-P/pBSA pFF (abbreviated to BW-P BF). Strain BW-P10 BF showed almost the same growth and glucose consumption as the others, while the conversion rate of MVA was far higher than that in the control strain due to production of less byproducts. Promoters BBa-J23100 and BBa-J23108 resulted in the highest yield of MVA, 64.3% (mol/mol) and 62.3% (mol/mol) respectively, although the strength of the promoters did not show an
obvious positive correlation with the MVA yield. This proved that enhancing expression of gene \textit{zwf} was effective for increasing the PPP flux.

\textbf{13 C-Metabolic flux analysis of changes in central carbon metabolism flux and energy metabolism}

Strains BW-P10 BF and BW-P08 BF and control strain BW-P BF were chosen for metabolic flux analysis. With the \textit{zwf} promoter replaced, the normalized data showed that the carbon flux through the oxidative part of the PPP was significantly increased, and the carbon flux through the TCA cycle was decreased, which was consistent with our expectations. More carbon flux moved towards the EP-bifido pathway. The two \textit{zwf}-expression-enhanced strains showed a large difference in TCA cycle flux, which may explain the growth difference between these strains (Fig. 1C).

In addition, the ATP, NADPH, and NADH synthesis capacity and glucose consumption of the three strains were compared based on the \textsuperscript{13}C-MFA data. After the EP-bifido pathway and the MVA synthetic pathway were introduced, the NADPH content and yield of the strain were significantly improved. \textit{pfkA} deficiency shunted carbon flux to the PPP and the expression level of \textit{zwf} was increased. Comparison of the \textit{zwf}-expression-enhanced strains showed that overexpression of \textit{zwf} enhanced NADPH synthesis, and the NADPH level was positively correlated with the promoter strength. Taking strains BW25113, BW-P BF, and BW-P10 BF as examples, the introduction of the EP-bifido pathway and overexpression of \textit{zwf} changed the main source of NADPH: The main NADPH generating pathway shifted from isocitrate dehydrogenase in the TCA cycle to glucose-6-phosphate dehydrogenase in the PPP. This further proved that we have redirected part of the carbon flux of the EMP pathway to the PPP.

In addition, the production of NADH also changed significantly, as shown in Figs. 3C and Figs. 3D. Through \textit{zwf} enhancement, the total amount and the yield of NADH decreased significantly. The NADH was produced distinctly in strain BW-P10 BF compared with wild-type strain BW25113: In strain BW25113, five dehydrogenases were the main source of NADH [glyceraldehyde-3-phosphate dehydrogenase (GAPDH), pyruvate dehydrogenase (PDH), malate dehydrogenase, \(\alpha\)-ketoglutarate dehydrogenase, and succinate dehydrogenase]; in strain BW-P10 BF, NADH was mainly formed via GAPDH and PDH.

The EP-bifido pathway and MVA synthetic pathway expression increased the ATP yield from glucose, but the total amount of ATP decreased (Fig. 3E). The \textit{pfkA} deficiency greatly impaired the EMP pathway and thereby blocked the three steps of substrate level phosphorylation absorption (glycerate-1, 3-diphosphate, phosphoenolpyruvate kinase, and succinyl CoA synthetase). This can also be verified from the origin ratio of ATP (Fig. 3F). Enhancing \textit{zwf} expression resulted in increased ATP production and yield in strain BW-P08 BF compared with BW-P BF.

\textbf{Down regulation of EMP pathway flux by targeting \textit{pfkA} using CRISPRi system}

To further rationally use the carbon source, we tried to suppress \textit{pfkA} in a time-controlled way through exogenous addition of inducers or self-induction to ensure sufficient growth and building block
production before activation of MVA synthesis. The CRISPRi system has shown a relatively good inhibition effect in single gene suppression. To further explore the optimal flux through the EMP pathway and improve the conversion rate of highly-NADPH-dependent products, we introduced the CRISPRi system to our EP-bido pathway (Fig. 4).

To avoid the growth inhibition that may be caused by dCas9 from the CRISPRi gene regulation system, we selected a relatively low strength promoter, BBa-J23134, to promote dcas9. In order to obtain a different repression range, three different sgRNAs targeting the promoter or coding region of pfkA were designed. sgRNA1 were designed on the promoter region of pfkA, sgRNA2 and sgRNA3 targeted the coding chain of pfkA, at the region of 100 bp and 200 bp downstream of the initial codon, which may cause different repression effect [30]. After dcas9 and the sgRNAs were incorporated into pFF and pBSA respectively, six CRISPRi-regulated strains were generated. The fermentation results showed that the introduction of CRISPRi significantly inhibited the growth of cells and the glucose consumption was also reduced compared with that of the control strain BW25113 zwf-23100 pFF pBSA (abbreviated to BBF). This may be caused by the toxicity or leaky expression of dCas9. The CRISPRi-regulated strains were induced at 12 h by adding IPTG. From the fermentation results, we can see that the three inhibitory sites we selected had different inhibitory effects in Fig. 5. sgRNA1 showed a better inhibition effect. Although strain BW25113 pFF-dCas9 pBSA-sgRNA1 produced only 8.53 g/L MVA, its MVA conversion rate reached 68.7%, which exceeded the previous best MVA conversion rate. Four control strains were also constructed to confirm the effect of the CRISPRi system on cell growth (strains BW25113 zwf-23100 pBSA-sgRNA1 pFF, BW25113 zwf-23100 pBSA pFF-dCas9, BW25113 zwf-23100 pBSA-sgRNA1-dCas9 pFF, and BW25113 zwf-23100 pBSA pFF, abbreviated to BB1F, BBFd, BB1dF, and BBF, respectively). The results proved our speculation that dCas9 had negative effective on cell growth in our engineered strains (Figure S1). The fermentation result showed that inhibitory effect of sgRNA1 on pfkA is suitable for enhancing MVA fermentation in the EP-bido system (Fig. 5). In the CRISPRi-regulated strains, we hardly detected any acetic acid, ethanol, or other byproducts during the fermentation process, which was in line with our expectations. The timely inhibition of pfkA reduces the flux of glycolysis, so that the carbon source cannot overflow excessively.

Discussion

Global warming is mainly due to excess CO₂ emission; it is urgently necessary to find sustainable solutions to address this issue. Moreover, this wasted carbon may have a major impact on the overall economy of biobased products derived from fermentable carbon sources. Scientists have explored the possibility of using microbial systems to optimize carbon conservation during metabolic processes. The pyruvate decarboxylation step of glycolysis releases CO₂ into the environment, resulting in 33% loss of carbon yield; this carbon loss has now been challenged by many scientists. We previously constructed an EP-bifid pathway in E. coli to reduce CO₂ emissions and successfully applied it to produce a series of acetyl-CoA-derived compounds such as PHA, PHB, and MVA. This provides a new approach for the efficient production of acetyl-CoA as a precursor in E. coli. However, in the EP-bifido pathway, the EMP
pathway was blocked by knocking out pfkA to attenuate the carbon flow from pyruvate to acetyl-CoA, which limits the growth of the engineered strains. Based on theoretical calculation and metabolic flux analysis, this EP-bifido pathway has great potential for further optimization.

It is possible to control the flux ratio between the EMP and oxPP pathways by fine-tuning and dynamic control of the expression of pfkA and the first key gene in the PPP, zwf, whose expression acts as a gateway into the EMP and PP pathways. Here, our fine-tuning strategy to improve NADPH availability was to enhance the expression of zwf by replacing its promoter, we used five promoters with different strengths. The engineered strains BW-P08 BF and BW-P10 BF produced higher MVA titers than the control strain BW-P BF, 9.12 and 11.2 g/L, respectively. The MVA production by these strains did not show an obvious positive correlation with the zwf promoter strength. Since the MVA yield did not represent the flux distribution between the EMP pathway and the PPP, $^{13}$C-MFA was performed to detected the metabolic flux distribution in strains BW-P08 BF and BW-P10 BF (which had high MVA titers) and the control strain BW-P BF. The flux ratio between the PPP and the EMP pathway in strains BW-P08 BF and BW-P10 BF was much higher than that in strain BW-P BF (Fig. 2), indicating an improved shunt to the PPP. Also, enhanced zwf expression increased the total amount and molar yield of NADPH (Fig. 3). The NADPH-generating pathway shifted from isocitrate dehydrogenase in the TCA cycle to glucose-6-phosphate dehydrogenase in the PPP, further proving that carbon flux was redirected from the EMP pathway to the PPP. The NADH level also showed a decreased TCA cycle activity. In terms of the ATP level, enhancement of zwf expression increased the ratio of substrate level phosphorylation (Fig. 3) and reduced the energy supply ratio of pyruvate kinase. Thus, we identified the metabolic flux distribution following the fine-tuning of central metabolic nodes, which helps us to understand the impact on metabolism.

In dynamic regulation of metabolic pathways, the CRISPRi system has recently been used to improve flux through different pathways [31, 32]. One benefit of using the CRISPRi system over promoter replacement methods is that it does not require genome editing of the target gene, which remains a challenge. The introduction of the CRISPRi system is achieved by adding an inducer at a certain time to start the CRISPRi system. Here, we used it to adjust the inhibition level of pfkA, so as to achieve timely adjustment of EMP pathway/PPP flux. To reduce the growth inhibition caused by pfkA knockout, considering that the strain itself already harbors two plasmids and the CRISPRi system, we integrated dcas9 and the sgRNA onto the two plasmids respectively. After introduction of the CRISPRi system, cell growth and sugar consumption of the engineered strains were significantly decreased. The introduction of dCas9 may also have an inhibitory effect on bacterial growth. The relevant limitation of the CRISPRi system is therefore the toxicity of dCas9 expression in certain hosts [33, 34] that would affect the growth of pathway-expressing cells that typically already suffer from growth defects. The three targeting sites played a role in fine-tuning of pfkA and sgRNA1 showed the best inhibition effect. The MVA yield of strain BW25113 pFF-dCas9 pBSA-sgRNA1 was only 8.53 g/L, its yield reached 68.7%, exceeding the previous best conversion rate.

**Conclusion**
This study showed glycolytic flux ratio fine-tuning strategies applying in an artificial carbon saving pathway for efficient MVA production. The strategies presented in this work serve as a guide to metabolic engineering projects requiring acetyl-CoA as a metabolic precursor.

**Materials And Methods**

**Media and Culture**

For plasmid preparation, *E. coli* strains were cultured at 37°C on a rotary shaker (220rpm) in test tubes containing 5mL Luria-Bertani (LB) medium. For MVA production, 50-mL shake flask cultures were started by 2% inoculation from the 5-mL LB culture. The 50-mL cultures contained M9 minimal medium with 0.2% yeast extract containing 20 g/L glucose and shaken at 37°C in a rotary shaker (120rpm) for 48h. Overnight cultures were shaken at 37°C in a rotary shaker (220rpm). Antibiotics were added as follows: ampicillin (Amp) 100 mg/mL, spectinomycin (Spc) 50 mg/mL and chloromycetin (Cm) 25mg/mL. For promoter integration and replacement procedure, strains were cultivated in SOB medium.

LB medium contains (g/L): tryptone(10), yeast extract(5) and NaCl(10). M9 medium contains (g/L): Na$_2$HPO$_4$·12H$_2$O (15.138), KH$_2$PO$_4$ (3), NaCl (0.5) and NH$_4$Cl (1). SOB medium contains (g/L): tryptone (20), yeast extract (5) and NaCl (5).

**Strains and plasmids**

All *E. coli* strains and plasmids used are listed in Table 1. Strain BW-P was used as the starting strain for further genetic manipulation [13]. All primers used for molecular manipulations are listed in Table S1. All promoter used for genetic manipulation are listed in Table 2.

**Plasmid construction for MVA production**

To replace the original tac promoter of pBSA plasmid, five promoters BBa-J 23119, BBa-J 23100, BBa-J 23102, BBa-J 23104, BBa-J 23118 was designed into primers to construct plasmids pBSA-23119, pBSA-23100, pBSA-23102, pBSA-23104, pBSA-23118. Two primers were designed in the opposite direction. Five PCR amplicons were obtained using the original plasmid as the template with primier pcr-23119-F/R, pcr-23100-F/R, pcr-23102-F/R, pcr-23104-F/R, pcr-23118-F/R. *DpnI* was added to the PCR system in 37°C for 1 hour to remove the methylated template. A mixture of 50 μL was transformed into competent cells using chemical transformation. Colony PCR was performed by picking monoclonal from resistance plate to eliminate false positives and template interference. Finally, the five plasmids were transformed into the BW-P 23100 strain with the plasmid pFF.

**Construction of CRISPRi suppression system**

To select the CRISPRi inhibition site, three different sgRNAs was designed by targeting *pfkB* promoter sequence, 100bp downstream of the promoter sequence, and 200bp downstream of the initiation codon. *dcas9* and sgRNAs were assembled into pFF and pBSA plasmids respectively downstream of an IPTG-
induced promoter. Primer dCas9FF-F/R, dCas9-F/R were used to amplify dCas9 sequence. Primer sgRNA-F1, sgRNA1-R and sgRNA-F2, sgRNA1-R were used to amplify sgRNA1 sequence by PCR. The two amplified sequences were overlapped from homology arms. Sequences sgRNA2 and sgRNA3 were amplified as above. All constructed plasmids were electro-transformed into *E. coli* strains. To accomplished the timely control of *pfkA* by CRISPRi system, cells was induced by 200 μM IPTG after 12h of fermentation.

**Promoter replacement on *E. coli* genome**

Promoter for replacing the promoter of *zwf* gene were selected from the Anderson promoter library (http://parts.igem.org/Promoters/Catalog/Anderson). Promoter replacement primers homoarm-F and homoarm-cm-R were designed using homology arms at about 300-500 bp at both ends of the target gene promoter, and plasmid pKD3 or pKD4 was used as a template to obtain recombinant fragments with kan or Cm resistance by using PKD3-cm-F/R or PKD4-cm-F/R. Three amplified sequences were overlapped from homology arms and resistance tag. All five replacing sequences were amplified as above.

The Red homologous recombination method was employed for gene integration. The pTKRED complementary plasmid was transformed into the target strain. The electrotansfection were performed by growing BW-P in 5 mL LB medium at 30°C and shaking at 220rpm for 12h. 5-mL shake flask cultures using SOB broth were started with a 1% inoculation from the overnight culture. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM to induce λ-prophage (bet, gam, and exo) gene expression. Cells were then incubated at 30°C and shaking at 220rpm until reaching an OD$_{600}$ of 0.5 to 0.6. Cell were collected (2 mL), pelleted, and washed three times with cold sterile water to make them electrocompetent. ssDNA mixture (1μM) was added to electrocompetent cells and electroporated at 2.5kV. Add 1 mL LB liquid medium and cultivate for 1 hour. After centrifuged, the collected bacteria were plated onto plates containing 25ug/ml kan or 18ug/ml spc for overnight incubation at 37°C. Transformed strains were selected by their kanR phenotype and were verified by PCR.

**Measurement of extracellular metabolites**

A spectrophotometer was used to measure the optical density at 600nm (OD$_{600}$) of the bacterial culture. For extracellular metabolite analysis, 1 mL of culture was centrifuged at12,000 ×g for 2 min. The supernatant was filtered through a 0.22-μm syringe filter for high-performance liquid chromatography analysis. Glucose, MVA, acetate, and pyruvate were measured on an ion exchange column (HPX-87H; Bio-Rad Labs) with a differential refractive index detector (Shimadzu RID-10A). A 0.5-mL/min mobile phase using a 5 mM H$_2$SO$_4$ solution was applied to the column. The column was operated at 65°C.

**$^{13}$C-MFA**

To investigate if the carbon flux was really redistributed to the newly constructed EP-bifido pathway, 13C-MFA was performed using 100% 1-$^{13}$C$_1$ glucose as the feeding substrate was added to a concentration of 10 g/L. Cells at the exponential growth phase were harvested by centrifugation at 7000g for 5 min at 4
°C. The cell pellet was then washed twice with chemical defined medium and hydrolyzed in 6 M HCl for 24 h at 120 °C (Schwender et al., 2006). The resulting proteinogenic acids were derivatized with N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide containing tert-butyldimethylchlorosilane in acetonitrile at 105 °C for 1 h, and then analyzed by a GC-MS [Agilent 7890 A GC and 5975 C Mass Selective Detector (Agilent Technologies, Santa Clara, USA)] equipped with a DB-1 column (Agilent Technologies). The data obtained from GC-MS were corrected by reduction of the natural abundance ratio of C, H, O, N, and Si isotopes [30]. Metabolic fluxes were estimated by minimizing the residual sum of squares between experimentally measured and model predicted $^{13}\text{C}$-enrichment using $^{13}\text{C}$-Flux software obtained from Dr. W. Wiechert [35].

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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**Contributions**

WQ and QQS designed the work. LY, XH and XY performed the experiments. SZJ and LY analyzed the $^{13}\text{C}$-MFA data. QQS encouraged this project. WQ and LY wrote the manuscript. All authors read and approved the final manuscript.

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**References**
1. Keasling JD. Manufacturing molecules through metabolic engineering. Science. 2010;330:1355–8.
2. Xu P, Ranganathan S, Fowler ZL, Maranas CD, Koffas MA. Genome-scale metabolic network modeling results in minimal interventions that cooperatively force carbon flux towards malonyl-CoA. Metab Eng. 2011;13:578–87.
3. Hirokawa Y, Kubo T, Soma Y, Saruta F, Hanai T. Enhancement of acetyl-CoA flux for photosynthetic chemical production by pyruvate dehydrogenase complex overexpression in Synechococcus elongatus PCC 7942. Metab Eng. 2020;57:23–30.
4. Centeno-Leija S, Huerta-Beristain G, Giles-Gomez M, Bolivar F, Gosset G, Martinez A. Improving poly-3-hydroxybutyrate production in Escherichia coli by combining the increase in the NADPH pool and acetyl-CoA availability. Antonie Van Leeuwenhoek. 2014;105:687–96.
5. Ogata Y, Chohnan S. Prokaryotic type III pantothenate kinase enhances coenzyme A biosynthesis in Escherichia coli. J Gen Appl Microbiol. 2015;61:266–9.
6. Ku JT, Chen AY, Lan EI. Metabolic Engineering Design Strategies for Increasing Acetyl-CoA Flux. Metabolites 2020, 10.
7. Schwander T, Schada von Borzyskowski L, Burgener S, Cortina NS, Erb TJ. A synthetic pathway for the fixation of carbon dioxide in vitro. Science. 2016;354:900–4.
8. Yu H, Li X, Duchoud F, Chuang DS, Liao JC: Augmenting the Calvin-Benson-Bassham cycle by a synthetic malyl-CoA-glycerate carbon fixation pathway. Nat Commun 2018, 9:2008.
9. Bogorad IW, Lin TS, Liao JC. Synthetic non-oxidative glycolysis enables complete carbon conservation. Nature. 2013;502:693–7.
10. Bar-Even A, Noor E, Lewis NE, Milo R. Design and analysis of synthetic carbon fixation pathways. Proc Natl Acad Sci U S A. 2010;107:8889–94.
11. Hu G, Li Y, Ye C, Liu L, Chen X. Engineering Microorganisms for Enhanced CO2 Sequestration. Trends Biotechnol. 2019;37:532–47.
12. Francois JM, Lachaux C, Morin N. Synthetic Biology Applied to Carbon Conservative and Carbon Dioxide Recycling Pathways. Front Bioeng Biotechnol. 2019;7:446.
13. Wang Q, Xu J, Sun Z, Luan Y, Li Y, Wang J, Liang Q, Qi Q. Engineering an in vivo EP-bifido pathway in Escherichia coli for high-yield acetyl-CoA generation with low CO2 emission. Metab Eng. 2019;51:79–87.
14. Hollinshead WD, Rodriguez S, Martin HG, Wang G, Baidoo EE, Sale KL, Keasling JD, Mukhopadhyay A, Tang YJ. Examining Escherichia coli glycolytic pathways, catabolite repression, and metabolite channeling using Deltapfk mutants. Biotechnol Biofuels. 2016;9:212.
15. Schellenberger J, Que R, Fleming RM, Thiele I, Orth JD, Feist AM, Zielinski DC, Bordbar A, Lewis NE, Rahmanian S, et al. Quantitative prediction of cellular metabolism with constraint-based models: the COBRA Toolbox v2.0. Nat Protoc. 2011;6:1290–307.
16. Zhang YX, Perry K, Vinci VA, Powell K, Stemmer WP, del Cardayre SB. Genome shuffling leads to rapid phenotypic improvement in bacteria. Nature. 2002;415:644–6.
17. Price ND, Reed JL, Palsson BO. Genome-scale models of microbial cells: evaluating the consequences of constraints. Nat Rev Microbiol. 2004;2:886–97.
18. Farmer WR, Liao JC. Improving lycopene production in Escherichia coli by engineering metabolic control. Nat Biotechnol. 2000;18:533–7.
19. Zhang F, Carothers JM, Keasling JD. Design of a dynamic sensor-regulator system for production of chemicals and fuels derived from fatty acids. Nat Biotechnol. 2012;30:535–8.
20. Xu P, Li L, Zhang F, Stephanopoulos G, Koffas M. Improving fatty acids production by engineering dynamic pathway regulation and metabolic control. Proc Natl Acad Sci U S A. 2014;111:11299–304.
21. Wada K, Toya Y, Banno S, Yoshikawa K, Matsuda F, Shimizu H. (13)C-metabolic flux analysis for mevalonate-producing strain of Escherichia coli. J Biosci Bioeng. 2017;123:177–82.
22. Kamata K, Toya Y, Shimizu H. Effect of precise control of flux ratio between the glycolytic pathways on mevalonate production in Escherichia coli. Biotechnol Bioeng. 2019;116:1080–8.
23. Siedler S, Lindner SN, Bringer S, Wendisch VF, Bott M. Reductive whole-cell biotransformation with Corynebacterium glutamicum: improvement of NADPH generation from glucose by a cyclized pentose phosphate pathway using pfkA and gapA deletion mutants. Appl Microbiol Biotechnol. 2013;97:143–52.
24. Chin JW, Cirino PC. Improved NADPH supply for xylitol production by engineered Escherichia coli with glycolytic mutations. Biotechnol Prog. 2011;27:333–41.
25. Chemler JA, Fowler ZL, McHugh KP, Koffas MA. Improving NADPH availability for natural product biosynthesis in Escherichia coli by metabolic engineering. Metab Eng. 2010;12:96–104.
26. Sundara Sekar B, Seol E, Park S. Co-production of hydrogen and ethanol from glucose in Escherichia coli by activation of pentose-phosphate pathway through deletion of phosphoglucone isomerase (pgi) and overexpression of glucose-6-phosphate dehydrogenase (zwf) and 6-phosphogluconate dehydrogenase (gnd). Biotechnol Biofuels. 2017;10:85.
27. Kwon D-H, Kim M-D, Lee T-H, Oh Y-J, Ryu Y-W, Seo J-H. Elevation of glucose 6-phosphate dehydrogenase activity increases xylitol production in recombinant Saccharomyces cerevisiae. Journal of Molecular Catalysis B: Enzymatic. 2006;43:86–9.
28. Cho S, Shin J, Cho BK. Applications of CRISPR/Cas System to Bacterial Metabolic Engineering. Int J Mol Sci 2018, 19.
29. Larson MH, Gilbert LA, Wang X, Lim WA, Weissman JS, Qi LS. CRISPR interference (CRISPRi) for sequence-specific control of gene expression. Nat Protoc. 2013;8:2180–96.
30. Bikard D, Jiang W, Samai P, Hochschild A, Zhang F, Marraffini LA. Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system. Nucleic Acids Res. 2013;41:7429–37.
31. Kim S, Hahn J-S. Efficient production of 2,3-butanediol in Saccharomyces cerevisiae by eliminating ethanol and glycerol production and redox rebalancing. Metab Eng. 2015;31:94–101.
Tables

Table 1 Strains and plasmids
| Strain and plasmids | Relevant properties | Sources |
|---------------------|---------------------|---------|
| **E. coli** BW25113 | F- Δ(araD-araB)567, ΔlacZ4787(::rmb-3), λ-, rph-1, Δ(rhaD-rhaB)568, hsdR514 | Lab stock |
| **BW-P** | BW25113 derivative, ΔpfkA::FRT | [13] |
| **BW-P 23100-zwf** | BW-P derived, zwf promoter:: BBa-J 23100, CmR | This study |
| **BW-P 23104-zwf** | BW-P derived, zwf promoter:: BBa-J 23104, CmR | This study |
| **BW-P 23105-zwf** | BW-P derived, zwf promoter:: BBa-J 23105, CmR | This study |
| **BW-P 23108-zwf** | BW-P derived, zwf promoter:: BBa-J 23108, CmR | This study |
| **BW-P 23114-zwf** | BW-P derived, zwf promoter:: BBa-J 23114, CmR | This study |
| **BW-P10 BF** | BW-P 23100-zwf carrying pBSA pFF | This study |
| **BW-P04 BF** | BW-P 23104-zwf carrying pBSA pFF | This study |
| **BW-P 05 BF** | BW-P 23105-zwf carrying pBSA pFF | This study |
| **BW-P 08 BF** | BW-P 23108-zwf carrying pBSA pFF | This study |
| **BW-P 14 BF** | BW-P 23114-zwf carrying pBSA pFF | This study |
| **BPB10F** | BW-P 23100-zwf carrying pBSA-23100 pBSA | This study |
| **BPB02F** | BW-P 23100-zwf carrying pBSA-23102 pBSA | This study |
| **BPB04F** | BW-P 23100-zwf carrying pBSA-23104 pBSA | This study |
| **BPB18F** | BW-P 23100-zwf carrying pBSA-23118 pBSA | This study |
| **BPB19F** | BW-P 23100-zwf carrying pBSA-23119 pBSA | This study |
| **BW25113 23100-zwf** | BW25113 derived, zwf promoter:: BBa-J 23100 | This study |
| BiBF1 | BW25113 23100-zwf carrying pBSA-dCas9 pFF-sgRNA1 | This study |
|-------|-----------------------------------------------|------------|
| BiBF2 | BW25113 23100-zwf carrying pBSA-dCas9 pFF-sgRNA2 | This study |
| BiBF3 | BW25113 23100-zwf carrying pBSA-dCas9 pFF-sgRNA3 | This study |
| BiB1F | BW25113 23100-zwf carrying pBSA-sgRNA1 pFF- dCas9 | This study |
| BiB2F | BW25113 23100-zwf carrying pBSA-sgRNA2 pFF- dCas9 | This study |
| BiB3F | BW25113 23100-zwf carrying pBSA-sgRNA3 pFF- dCas9 | This study |
| BB1F  | BW25113 23100-zwf carrying pBSA-sgRNA1 pFF     | This study |
| BBFd  | BW25113 23100-zwf carrying pBSA pFF- dCas9     | This study |
| BiBd1F| BW25113 23100-zwf carrying pBSA-sgRNA1-dCas9 pFF | This study |
| pBSA  | pTrc99a with *atoB* from *E. coli*, mvaS and mvaA from *L. casei*. tac promoter. Amp<sup>R</sup> | Lab stock |
| pTKRED| ParaBAD promoter containing plasmid, Spe<sup>R</sup> | [36] |
| pCP20 | Helper plasmid expressing FLP recombinase, ts-rep, Amp<sup>R</sup>, Cm<sup>R</sup> | [37] |
| pKD3  | Template plasmid with Cm<sup>R</sup> gene and FLP recognition target | [37] |
| P23100-GFP | pTrc99a with BBa-J 23100 promoter and green fluorescent protein sequence, Amp<sup>R</sup> | This study |
| P23104-GFP | pTrc99a with BBa-J 23104 promoter and green fluorescent protein sequence, Amp<sup>R</sup> | This study |
| P23105-GFP | pTrc99a with BBa-J 23105 promoter and green fluorescent protein sequence, Amp<sup>R</sup> | This study |
| P23108-GFP | pTrc99a with BBa-J 23108 promoter and green fluorescent protein sequence, Amp<sup>R</sup> | This study |
| P23114-GFP | pTrc99a with BBa-J 23114 promoter and green fluorescent protein sequence, Amp<sup>R</sup> | This study |
| Pzwf-GFP  | pTrc99a with zwf promoter from *E. coli* BW25113 and green fluorescent protein sequence, Amp<sup>R</sup> | This study |
| pBSA-23119 | pBSA with BBa-J 23119 promoter | This study |
| PBSA-23100 | pBSA with BBa-J 23100 promoter | This study |
| PBSA-23102 | pBSA with BBa-J 23102 promoter | This study |
| PBSA-23104 | pBSA with BBa-J 23104 promoter | This study |
| PBSA-23118 | pBSA with BBa-J 23118 promoter | This study |
| pFF-sgRNA1 | pFF containing pfkA sgRNA1, Spc<sup>R</sup> | This study |
| pFF-sgRNA2 | pFF containing pfkA sgRNA2, Spc<sup>R</sup> | This study |
| pFF-sgRNA3 | pFF containing pfkA sgRNA3, Spc<sup>R</sup> | This study |
| pBSA-dCas9 | pBSA containing Cas9 array, Amp<sup>R</sup> | This study |
| pBSA-sgRNA1 | pBSA containing pfkA sgRNA1, Amp<sup>R</sup> | This study |
| pBSA-sgRNA2 | pBSA containing pfkA sgRNA2, Amp<sup>R</sup> | This study |
| pBSA-sgRNA3 | pBSA containing pfkA sgRNA3, Amp<sup>R</sup> | This study |
| pFF-dCas9 | pFF containing Cas9 array, Amp<sup>R</sup> | This study |
| pFF-sgRNA1-dCas9 | pFF containing Cas9 array and pfkA sgRNA1, Amp<sup>R</sup> | This study |
| PBSA-23102-sgRNA1 | pBSA-23102 containing pfkA sgRNA1, Amp<sup>R</sup> | This study |

Table 2 Relative strength of Promoters
| Promoter   | Relative strength |
|------------|-------------------|
| BBa-J23119 | -                 |
| BBa-J23100 | 1.0               |
| BBa-J23102 | 0.86              |
| BBa-J23104 | 0.72              |
| BBa-J23105 | 0.51              |
| BBa-J23108 | 0.24              |
| BBa-J23134 | 0.18              |
| BBa-J23114 | 0.10              |

**Figures**

*Figure 1*
The fermentation results of EP-bifido strains with modified zwf promoter replaced by different strength synthetic promoters. A: The characterization of the strength of a series of synthetic promoters and zwf promoter. The fluorescence/OD600 of strains was detected at 16h. MVA titre and molar conversion rate (B), cell growth (C) and glucose consumption (D) during 72h fermentation.

Figure 2

Metabolic flux diagram of zwf-strengthened EP-bifido strains. The metabolic flux shown for strains from top to bottom are BW-P10 BF, BW-P08 BF, BW-P BF.
Figure 3

NADH, NADPH and ATP level and its derivations. A: NADPH generation. B: NADPH derivations of BW25113, BW-P BF, BW-P08 BF. C: NADH generation. D: NADH derivations of BW25113 and BW-P10 BF. D: ATP generation. E: ATP derivations. G6PDH, Glucose-6-phosphate dehydrogenase; ICDH, Isolate dehydrogenase; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; ME, Malate enzyme; MDH, Malate dehydrogenase; PDH, Pyruvate dehydrogenase; KDH, α-Ketoglutarate dehydrogenase; SDH, Succinate dehydrogenase; IDH, Isolate dehydrogenase.
Figure 4

Schematic diagram of CRISPRi on pfkA suppression. A: Schematic diagram of pfkA transform. B: Abridged general view of metabolic flux change after CRISPRi regulation. The red arrow indicated decreased metabolic flux, the green arrow indicated increased metabolic flux.
Figure 5

Fermentation of CRISPRi-regulated EP-bifido strains. A: Glucose consumption of CRISPRi-regulated EP-bifido strains. B: OD600 of CRISPRi-regulated EP-bifido strains. C: MVA accumulation of CRISPRi-regulated EP-bifido strains. D: MVA titre and yield of CRISPRi-regulated EP-bifido strains.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- simulatedresults.xlsx
- suppfiguresandtables0713.docx