Original Research Article

Modular engineering of E. coli coculture for efficient production of resveratrol from glucose and arabinose mixture

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RESVERATROL IS A VALUABLE PLANT- DERIVED POLYPHENOLIC COMPOUND WITH VARIOUS BIOACTIVITIES, HAS BEEN WIDELY USED IN NUTRACEUTICAL INDUSTRIES. MICROBIAL PRODUCTION OF RESVERATROL SUFFERS FROM METABOLIC BURDEN AND LOW MALONYL-COA AVAILABILITY, WHICH IS A BIG CHALLENGE FOR SYNTHETIC BIOLOGY. HEREIN, WE TOOK ADVANTAGE OF COCULTURE ENGINEERING AND DIVIDED THE BIOSYNTHETIC PATHWAY OF RESVERATROL INTO THE UPSTREAM AND DOWNSTREAM STRAINS. BY ENHANCING THE SUPPLY OF MALONYL-COA VIA CRISPRi SYSTEM AND FINE-TUNING THE EXPRESSION INTENSITY OF THE SYNTHETIC PATHWAY GENES, WE SIGNIFICANTLY IMPROVED THE RESVERATROL PRODUCTIVITY OF THE DOWNSTREAM STRAIN. FURTHERMORE, WE DEVELOPED A RESVERATROL ADDICTION CIRCUIT THAT COUPLED THE GROWTH OF THE UPSTREAM STRAIN AND THE RESVERATROL PRODUCTION OF THE DOWNSTREAM STRAIN. THE BIDIRECTIONAL INTERACTION STABILIZED THE COCULTURE SYSTEM AND INCREASED THE PRODUCTION OF RESVERATROL BY 74%. MOREOVER, CO-UTILIZATION OF GLUCOSE AND ARABINOSE BY THE COCULTURE SYSTEM MAINTAINED THE GROWTH ADVANTAGE OF THE DOWNSTREAM STRAIN FOR PRODUCTION OF RESVERATROL THROUGHOUT THE FERMENTATION PROCESS. UNDER OPTIMIZED CONDITIONS, THE ENGINEERED E. coli COCULTURE SYSTEM PRODUCED 204.80 mg/L OF RESVERATROL, 12.8-FOLD IMPROVEMENT OVER MONOCULTURE SYSTEM. THIS STUDY DEMONSTRATES THE PROMISING POTENTIAL OF COCULTURE ENGINEERING FOR EFFICIENT PRODUCTION OF NATURAL PRODUCTS FROM BIOMASS.

1. Introduction

Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a natural plant polyphenolic compound [1]. It is well known that resveratrol has anti-oxidant, anti-inflammatory, and anticancer activities [2] which is beneficial to human health of cardiovascular and neurological systems. Resveratrol also shows positive metabolic effects on anti-aging, anti-diabetes and elongates the survival of aging yeast [3], drosophila [4], and mice [5]. Recently, the novel target of resveratrol on caloric restriction was discovered, and it directly inhibited CAMP-dependent phosphodiesterase, triggering a cascade of energy-sensing metabolic events [6]. Because of its broad physiological and pharmacological properties, resveratrol become an attractive ingredient for the pharmaceutical, food supplement, nutraceutical, and cosmetic industries [7].

Production of resveratrol is mainly extracted from knotweed roots or grape seeds, which suffers the low content of resveratrol and the geographic dependence for good plant growing practice. Considering shortfall of resveratrol supply from natural sources, microbial production of resveratrol via synthetic biology and metabolic engineering has attracted worldwide interest [8]. The tyrosine ammonia lyase (TAL), p-coumarate: CoA ligase (4CL), and stilbene synthase (STS) consists of the heterologous biosynthetic pathway of resveratrol from the endogenous metabolite l-tyrosine and malonyl-CoA in host microbes. The E. coli monoculture was firstly explored, and the heterologous expression of the TAL, 4CL, and STS genes led to biosynthesis of resveratrol using l-tyrosine or p-coumaric acid as precursor [9–13]. The de novo bioproduction of resveratrol from sugar is more attractive than from precursor. However, minor amounts of resveratrol were synthesized from glucose by engineered E. coli [14,15]. Recently, using glucose and malonate as carbon sources, the resveratrol titers were improved in engineered E. coli monoculture via increasing the precursor malonyl-CoA supply as well as optimizing expression of the TAL mRNA secondary structure [16]. Alternatively, the synthetic microbial coculture engineering, as the next generation strategy of synthetic biology,
has been proven to be a more promising route to produce valuable biofuels [17,18], chemicals [19,20], and natural products [21,22]. For the case of resveratrol, the E. coli coculture produced 22.58 mg/L of resveratrol using glycerol as carbon source [23]. Further metabolic engineering of the pentose phosphate pathway for improving the biosynthesis of p-coumaric acid and the glycolytic pathway for enhancing biosynthesis of malonyl-CoA increased the production of resveratrol to 55.7 mg/L using glucose as carbon source [24]. Despite the more efforts made by researchers, resveratrol production in coculture is needed to improve.

In this coculture engineering, we divided the de novo biosynthetic pathway of resveratrol into two E. coli strains (Fig. 1). The upstream strain harbored the TAL gene to synthesize p-coumaric acid from glucose, and the downstream strain harbored the 4CL and STS genes for production of resveratrol from p-coumaric acid. To increase the intracellular availability of malonyl-CoA, we applied the RppA biosensor [25] for screening CRISPRi inhibitory targets. The upstream strain was engineered of the pentose phosphate pathway for improving the biosynthesis and the glycolytic pathway for enhancing resveratrol production in coculture is needed to improve.

2. Methods and materials

2.1. Chemicals and reagents

Resveratrol standard (≥98%, HPLC) was purchased from Solarbio (Beijing, China), and p-coumaric acid standard (≥98%, HPLC) was purchased from HEOWNS (Tianjin, China). Acetonitrile (HPLC grade) was purchased from Concord Tech (Tianjin, China). The ClonExpress II One-Step Cloning Kit was obtained from Biomed (Beijing, China) and DNA Polymerase of Phanta Super Fidelity and Rapid Taq Master Mix was purchased from Concord Tech (Tianjin, China). Acetonitrile (HPLC grade) was purchased from Concord Tech (Tianjin, China). The ClonExpress II One-Step Cloning Kit was obtained from Biomed (Beijing, China) and DNA Polymerase of Phanta Super Fidelity and Rapid Taq Master Mix was purchased from Vazyme (Nanjing, China). Restriction enzymes and T4 DNA ligase were purchased from Thermo Scientific (USA). Purification of DNA, gel extraction, and plasmid preparation were conducted using kits from Tiangen Biotech Co., Ltd. (Beijing, China).

2.2. Construction of plasmids and strains

All strains and plasmids used in this study were listed in Table 1 and Table 2, respectively. All primers used in this study were listed in Supplementary Table S1. E. coli DH5α was used for propagation and cloning. For gene integration and deletion, E. coli genome were edited by CRISPR-Cas9 mediated method [28]. The T7 RNA polymerase gene was inserted into the locus between the yhbB and yhbC genes on E. coli MG1655 chromosome to obtain E. coli MG1655(DE3), followed by sequential deletion of the endA and recA genes to generate strains LJM1 and LJM2, respectively. By knocking out the serA gene on the chromosome of BAK11(DE3) which derived from E. coli BW25113, generating serine-deficient strain BAK21(DE3).

Genes SypppA from Streptomyces griseus [25] (GenBank: BAE07216) and Nasaro_0803 from Novosphingobium aromaticivorans DSM 12444 [29] (GenBank: AB025248.1) were synthesized by GenScript (Nanjing, China). The resveratrol biosynthetic pathway genes were amplified from pCDF-T7-PcTAL-T7-4CL and pET-T7-VvSTS [27], respectively. The CgaccBC and CgdtsR1 genes were amplified from pRSF-acc [30]. Endogenous molecular chaperone genes (groEL-groES, dnaK-dnaJ, ihpA/B, tig, and clpB) were amplified from E. coli MG1655 chromosome by PCR. Plasmids for gene expression were constructed by PCR amplification and homologous recombination methods. For example, the VsSTS fragment was amplified using primers VsSTS-F and VsSTS-R, and an At4CL-carrying vector fragment was amplified from pCDF-T7-At4CL with primers VsSTS-ZT-F and VsSTS-ZT-R, then they were assembled to plasmid pLJ1 by 2 × Seamless Cloning Mix (Biomed, Beijing, China). Similarly, plasmids pLJ2 to pLJ14 were constructed, respectively.

To construct plasmid for expressing sgRNAs, the CRISPRi system backbone (with two BsaI restriction sites) and the dCas9 gene were assembled into the plasmid pRSFDuet-1 by homologous recombination method to obtain the starting vector pSGR. We used sgRNAcas9 (V3.0) [31] to design CRISPR sgRNA and evaluate potential off-target cleavage sites, and the design process conforms to the general sgRNA design workflow [32]. Specifically, the designed forward and reverse primers

Fig. 1. Schematic diagram of the E. coli-E. coli coculture to accommodate resveratrol biosynthetic pathway from glucose and arabinose mixture. TAL, tyrosine ammonia lyase; 4CL, p-coumarate: CoA ligase; STS, stilbene synthase; saro_0803, resveratrol biosensor gene; serA, phosphoglycerate dehydrogenase gene.
for each spacer were annealed to obtain a double-stranded inserted fragments, which could be cleaved by Bsd and ligated into plasmid pSGR by T4 DNA ligase, resulting in the desired sgRNA expression plasmids pSGR1 to pSGR33. All inhibitory target sequences in this study were listed in Supplementary Table S2.

### 2.3. Media and fermentation conditions

Lucia Bertani (LB) medium (5 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl) was used for plasmid propagation and seed preparation. M9 medium (6 g/L Na2HPO4, 3 g/L KH2PO4, 0.5 g/L NaCl, 1 g/L NH4Cl, 1 mM MgSO4, and 0.1 mM CaCl2) was used for resveratrol production. For monoculture, M9 medium supplemented with 1 g/L yeast extract and 10 g/L glucose. For coculture, when the resveratrol addiction circuit was introduced, the fermentation medium was changed to M9 medium supplemented with 50 mg/L phenylalanine and 10 g/L glucose (or mixture of glucose and arabinose with desired amounts). As required, antibiotics were added to the culture medium at a final concentration of 30 μg/mL chloramphenicol, 50 μg/mL ampicillin, 30 μg/L kanamycin, and 30 μg/mL streptomycin.

For biosynthesis of resveratrol, all engineered strains were cultivated overnight at 37 °C and 220 rpm in 5 mL of LB medium, and then 500 μL of culture was transferred into 50 mL of LB medium in 250 mL shake flask and incubated at 37 °C again with shaking at 220 rpm for 6–8 h. Cells were collected by centrifugation and resuspended into 250 mL shake flask with 25 ml fermentation medium with an initial optical density at 600 nm (OD600) of 1.0 (unless otherwise specified), and 0.2 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) was added for induction of gene expression. The fermentation was carried out at 30 °C with shaking at 220 rpm to produce resveratrol. Broth sample was removed at 48 h (unless otherwise specified) for analysis of products.

### 2.4. Coculture system analysis

To measure the subpopulation in coculture system, the upstream strain was engineered to possess red fluorescence protein gene mCherry. The fermentation broth was periodically withdrawn and centrifuged, and the cell pellets were washed twice, adjusted the to 1.0 of OD600 with phosphate buffered saline (PBS) for measuring mCherry expression. Red fluorescence intensity was detected using 96-well black polystyrene plate by the microplate reader (SpectraMax M2, Molecular Devices, USA), with an excitation wavelength of 587 nm and emission wavelength of 610 nm. Then the fluorescence intensity of each sample was corrected by subtracting its background (strain without the mCherry gene). The upstream strain subpopulation in the coculture system was estimated with the fluorescence intensity of the coculture divided by that of the upstream strain monoculture.

### 2.5. Biomass and metabolite analysis

Cell growth of strains was measured by detecting the OD600 value using a T6 spectrometer (Purkinje General, Beijing, China), and the residual glucose was measured with an S-10 biosensor (Siemantec Technology, Shenzhen, China). When using mixed carbon sources for fermentation, glucose and arabinose were measured by a Morphing™ Sugar-H column (300 × 7.8 mm, 5 μm) and a RI detector with a mobile
phase (5 mM H$_2$SO$_4$) at 0.6 mL/min, 65 °C. For analysis of resveratrol and p-coumaric acid, 5 ml of the fermentation broth was removed and extracted with 3 ml ethyl acetate by vortex mixer for 2 h. The top organic layer was evaporated to dryness, and then dissolved in 0.5 mL of ethanol, and the solution was filtered through a 0.22-μm syringe filter, followed by HPLC analysis on an Agilent C18 column (150 * 4.6 mm with a particle size of 5 μm) at 35 °C and a PDA detector (Agilent). Resveratrol and p-coumaric acid were analyzed at 303 and 277 nm, respectively, using a solution (30% acetonitrile, 70% water, 0.1% trifluoroacetic acid) as mobile phase and a flow rate of 1.0 mL/min. The amount was calculated using a five-point calibration curve with the R$^2$ coefficient higher than 0.99.

### 2.6. Malonyl-CoA biosensor characterization

For malonyl-CoA biosensor characterization, strain harboring the reporter gene $R_{ppA}$ was cultured in liquid media for 16 h, the broth was centrifuged for 10 min at 4,000 rpm, and 150 μL of supernatant was transferred to a 96-well polystyrene plate to measure its optical density at 340 nm using a microplate reader (SpectraMax M2, Molecular Devices, USA). The cell pellets were resuspended in phosphate buffered saline (PBS) to measure OD at 600 nm. The biosensor signal was defined as OD$_{600}$/OD$_{400}$.

### 3. Results

#### 3.1. Identification of target genes for enhancing malonyl-CoA using CRISPRi system

Three molecules of malonyl-CoA and one molecule of p-coumaroyl-CoA condenses to resveratrol. However, the limited supply of intracellular malonyl-CoA in E. coli significantly impedes the biosynthesis of resveratrol. The cellular malonyl-CoA derived from acetyl-CoA is used to synthesize fatty acids and phospholipids in microorganisms (Fig. 2a).

#### Table 2 (continued)

| Plasmids | Description | Source |
|----------|-------------|-------|
| pSGR23   | pSGR derivate, $P_{J23119}$ fabH-2-sgRNA scaffold | This study |
| pSGR24   | pSGR derivate, $P_{J23119}$ fabH-3-sgRNA scaffold | This study |
| pSGR25   | pSGR derivate, $P_{J23119}$ fabB-1-sgRNA scaffold | This study |
| pSGR26   | pSGR derivate, $P_{J23119}$ fabB-2-sgRNA scaffold | This study |
| pSGR27   | pSGR derivate, $P_{J23119}$ fabB-3-sgRNA scaffold | This study |
| pSGR28   | pSGR derivate, $P_{J23119}$ fabF-1-sgRNA scaffold | This study |
| pSGR29   | pSGR derivate, $P_{J23119}$ fabF-2-sgRNA scaffold | This study |
| pSGR30   | pSGR derivate, $P_{J23119}$ fabF-3-sgRNA scaffold | This study |
| pSGR31   | pSGR derivate, $P_{J23119}$ pabA-1-sgRNA scaffold | This study |
| pSGR32   | pSGR derivate, $P_{J23119}$ pabA-2-sgRNA scaffold | This study |
| pSGR33   | pSGR derivate, $P_{J23119}$ pabA-3-sgRNA scaffold | This study |
| pSGR34   | pSGR derivate, $P_{J23119}$ pabA-4-sgRNA scaffold, $P_{J23119}$ pabA-5-sgRNA scaffold | This study |
| pSGR35   | pSGR derivate, $P_{J23119}$ pabA-6-sgRNA scaffold | This study |
| pSGR36   | pSGR derivate, $P_{J23119}$ pabA-7-sgRNA scaffold and $P_{J23119}$ fabB-2-sgRNA scaffold | This study |
| pSGR37   | pSGR derivate, $P_{J23119}$ pabA-8-sgRNA scaffold and $P_{J23119}$ fabB-3-sgRNA scaffold | This study |
| pSGR38   | pSGR derivate, $P_{J23119}$ pabA-9-sgRNA scaffold and $P_{J23119}$ fabB-4-sgRNA scaffold | This study |
| pYG1     | pRSFDuet-1, $P_{T7}$-mCherry, Kan' | This study |
addition, acetyl-CoA is participated in tricarboxylic acid (TCA) cycle and formation of ethanol, resulting in the diversion of carbon flux away from malonyl-CoA (Fig. 2a). To enhance intracellular malonyl-CoA pool, we systematically adjusted the competition pathways of malonyl-CoA via CRISPR interference (CRISPRi) system. For increasing the availability of precursor acetyl-CoA, five genes (gltA, sucC, fumC, mdh, and aceB) in TCA circle and acetaldehyde dehydrogenase gene (adhE) were chosen as silencing targets. For preventing the diversion of malonyl-CoA to fatty acid biosynthesis, the fabD, fabH, fabB, and fabF genes were chosen as targets. In addition, knockdown of the pabA gene (encoding amino-benzoate synthetase) has been shown to be effective in increasing the pool of malonyl-CoA in E. coli for the increased titer of resveratrol [25].

In total, we chose 11 chromosomal genes that involves in the multiple pathways for modulation by CRISPRi (Fig. 2a). Targeting different sites in a gene allows the dCas9-sgRNA complex to exhibit different regulatory efficacy [34]. To obtain a better silence effect on each target gene, anti-sgRNAs sequences were designed by targeting the promoter region, 5′-untranslated region (5′-UTR), and/or 5′- terminal coding region (5′-TCR) (Supplementary Table S2). We constructed a library of 33 synthetic sgRNAs under controlled by J23119 promoter, and the dCas9 gene was driven by T7 promoter (Fig. 2b).

To identify target genes with enhanced malonyl-CoA pool, we employed RppA (a type III polyketide synthase) which converts five molecules of malonyl-CoA into red-colored flaviolin (Fig. 3a) as malonyl-CoA biosensor [25] for high-throughput screening. Plasmid pLJ4 expressing the RppA gene was transformed into strain LJM2 to generate strain LJF1. Then we introduced plasmid harboring each of the 33 synthetic sgRNAs individually into strain LJF1 to implement gene perturbation. Plasmid pSGR without the target site complementary sequence was introduced into strain LJF1 to generate control strain LJF2, which did not modulate any chromosomal genes. As shown in Fig. 3b, for five genes of the TAC circle, the sgRNAs targeting gltA and mdh had no effects on the malonyl-CoA level, while the sgRNAs targeting sucC-1, sucC-2, fumC-3, and aceB-3 increased 64%, 62%, 56%, 36% of malonyl-CoA level than the control, respectively. For silencing fatty acid pathway, the sgRNAs targeting fabH showed minor effect on malonyl-CoA level, while the sgRNAs targeting fabD-1 (and fabD-3), fabB-1 (and fabB-2), and fabF-1 (and fabF-2) produced a dramatic increase in malonyl-CoA level (from 71% to 142%) (Fig. 3c). In addition, the sgRNA targeting pabA-2 produced a 72% of increase in malonyl-CoA level, while sgRNAs targeting adhE had on effect (Fig. 3d).

Targeting different sites in the gene allows the dCas9-sgRNA complex to exhibit different regulatory effects. The inhibition of the gltA, mdh, fabH, and adhE genes by targeting three regions showed no effects on increasing the pool of malonyl-CoA, which were inconsistent with previous reports [16,35]. It might be due to targeting different sequences. Among the rest seven genes, the efficient inhibition was observed either in 5′-TCRs (fumC-3, aceB-3, fabD-1, fabD-3, fabB-2, fabF-1, fabF-2, and pabA-2), in 5′-UTRs (sucC-2 and fabB-1), or in promoter region (sucC-1), instead of all regions of target gene. One possible reason is that the target sequence might determine the inhibitory effect. Nevertheless, all inhibition of aforementioned seven genes showed no
significant difference in the final biomass. Hence, \textit{fabB-1, fabD-1, fabF-2, pabA-2, sucC-1, fumC-3, and aceB-3} were chosen as the optimal inhibitory targets to produce resveratrol.

3.2. Enhancing resveratrol production by regulating malonyl-CoA metabolism

We introduced plasmids harboring each of seven optimal sgRNAs individually into strain LJM3 to test changes in resveratrol production. Plasmid pSGR was introduced into the LJM3 strain to generate the control strain LJM4, which produced 78.32 mg/L resveratrol without modulation of any chromosomal genes. As shown in Fig. 4a, three inhibitory targets, \textit{fabB-1, fabF-2, and fumC-3}, were validated to be beneficial for production of resveratrol. Specifically, the corresponding strains expressing anti-\textit{fabB-1, anti-fabF-2, and anti-fumC-3} achieved resveratrol titers of 134.42, 113.49, and 89.75 mg/L, which were 72\%, 45\%, and 15\% higher than that of the control strain LJM4, respectively, without obvious biomass change (lower than 12\%). Furthermore, we explored the simultaneous interference of two genes by CRISPRi on resveratrol enrichment. However, combinatorial inhibition seemed unfavorable for resveratrol production, as all engineered strains exhibited lower titers of resveratrol and remarkably decrease in biomass (by over 24\%), compared with targeting single gene (Fig. 4b). The possible reason is that the availability of malonyl-CoA was insufficient to maintain cell physiology, as cell growth was greatly inhibited upon strong inhibition of two targeting genes.

Acetyl-CoA carboxylase (ACC) catalyzes acetyl-CoA to malonyl-CoA. \textit{E. coli} ACC consists of four subunits, and CgACC of \textit{C. glutamicum} has two subunits (AccBC and DtsR1) which has been proven to be effective in increasing the pool of malonyl-CoA in \textit{E. coli}, resulting in the increased titers of stilbenes and flavonoids [30]. As shown in Fig. 4b, when \textit{CgaccBC-CgdtsR1} was expressed, resulting strain produced 101.06 mg/L of resveratrol, 29\% increase compared with the strain LJM4, but an obvious decrease in biomass (by 39\%) was observed. Then we combined the ACC complex with the CRISPRi system. Unfortunately, the titer of resveratrol did not further increase (Fig. 4b), probably because of inhibiting cell growth (decreased biomass by over 51\%). Balanced allocation of malonyl-CoA between cell growth and heterologous molecule production is desirable to prevent the impairment of cell viability.

3.3. Combinatorial optimization of resveratrol production from p-coumaric acid in downstream strain

We previously showed that overexpression of molecular chaperone \textit{groEL-groES} gene was favored for production of resveratrol [27]. In order to screen best chaperone, the \textit{tig, cipB, ibpA-ibpB, and dnaK-dnaJ} genes [36] were overexpressed in strain LJM5, respectively. As shown in Fig. 5a, the overexpression of the \textit{tig} and \textit{cipB} genes greatly decreased the titer of resveratrol, while the overexpression of \textit{ibpA-ibpB} and \textit{dnaK-dnaJ} enhanced resveratrol production of 162.92 mg/L and 177.08 mg/L, respectively, but they were less efficient than the overexpression of \textit{groEL-groES}, which led to 186.66 mg/L of resveratrol, and was used in following optimization.

To further enhance the production efficiency of resveratrol from p-coumaric acid, it was necessary to regulate the expression intensities of
After resveratrol was efficiently produced from p-coumaric acid, we further extended the resveratrol biosynthesis from glucose. For producing p-coumaric acid from glucose, we used our previously reported l-tyrosine overproducing strain BAK11(DE3) [26] as chassis. For screening the expression pattern compatible with chassis, the PctAL gene from Phanerochaete chrysosporium [27] under the control of the trc promoter was cloned into various copy number plasmids pACYCDuet-1, pCDFDuet-1, and pETDuet-1, and transformed into strain BAK11(DE3) to generate strains BCR1, BCR2, and BCR3, respectively. As shown in Fig. 6a, strain BCR1 produced 298.86 mg/L of p-coumaric acid, while strains BCR2 and BCR3 gave the almost the same amounts of p-coumaric acid (596.53 mg/L and 612.25 mg/L, respectively), over 200% of strain BCR1. It indicated that strain BCR2 would be sufficient for production of p-coumaric acid. For de novo biosynthesis of resveratrol from glucose, plasmids pLJ2, pLJ10 and pSGR25 were transformed into strain BCR2, resulting in strain LJMB28. Resveratrol was successfully synthesized in strain LJMB28 from glucose with the titer of 14.87 mg/L (Fig. 6b).

However, cell growth was seriously retarded, and glucose consumption was very slow during the fermentation process (Fig. 6b). We speculated that the metabolic burden and conflicting metabolic goals were key factors leading to undesirable physiological changes, especially when multiple heterologous genes were overexpressed.

To reduce the metabolic burden and possible mutual interference between the biosynthesis of p-coumaric acid and resveratrol, we divided the biosynthetic pathway of resveratrol into two separate strains. The aforementioned strain LJMB24 harboring the downstream pathway was used for production of resveratrol from p-coumaric acid. For tracking population composition and balancing resistance between two strains, plasmids pYG1 (derived from pRS福德u特-1 backbone) expressing mCherry gene and pACYCDuet-1 were transformed into strain BCR3, resulting in the upstream strain BCR4 for producing p-coumaric acid from glucose (Fig. 7a). We first made efforts to balance the metabolic strength of two strains by adjusting the inoculation ratio of strains BCR4 to LJMB24 (defined as the BCR4/LJMB24 ratio) from 4:1 to 1:4 with an initial OD₆₀₀ at 1.0. When the BCR4/LJMB24 ratio was decreased, resveratrol production increased until the BCR4/LJMB24 ratio at 1:2, where the highest production of resveratrol was achieved with a titer of 41.07 mg/L (Fig. 7b), nearly 3-fold of the monoculture. Then we analyzed the changes of total cell density, sugar consumption and population percentage in the coculture system with time under the optimal inoculation ratio at 1:2. As shown in Fig. 7c, the cell growth in the coculture system was poor and glucose consumption was very slow. Remarkably, the population of strain BCR4 increased from 33% to 60% after 48 h of fermentation (Fig. 7c), while strain LJMB24 decreased from 67% to 40% (Fig. 7c), which indicates that two strains undergo competition and strain LJMB24 exhibited a competitive disadvantage against strain BCR4.

### 3.4. Comparison of resveratrol production capacity in monoculture and coculture systems

After resveratrol was efficiently produced from p-coumaric acid, we further extended the resveratrol biosynthesis from glucose. For producing p-coumaric acid from glucose, we used our previously reported l-tyrosine overproducing strain BAK11(DE3) [26] as chassis. For screening the expression pattern compatible with chassis, the PctAL gene from Phanerochaete chrysosporium [27] under the control of the trc promoter was cloned into various copy number plasmids pACYCDuet-1, pCDFDuet-1, and pETDuet-1, and transformed into strain BAK11(DE3) to generate strains BCR1, BCR2, and BCR3, respectively. As shown in Fig. 6a, strain BCR1 produced 298.86 mg/L of p-coumaric acid, while strains BCR2 and BCR3 gave the almost the same amounts of p-coumaric acid (596.53 mg/L and 612.25 mg/L, respectively), over 200% of strain BCR1. It indicated that strain BCR2 would be sufficient for production of p-coumaric acid. For de novo biosynthesis of resveratrol from glucose, plasmids pLJ2, pLJ10 and pSGR25 were transformed into strain BCR2, resulting in strain LJMB28. Resveratrol was successfully synthesized in strain LJMB28 from glucose with the titer of 14.87 mg/L (Fig. 6b). However, cell growth was seriously retarded, and glucose consumption was very slow during the fermentation process (Fig. 6b). We speculated that the metabolic burden and conflicting metabolic goals were key factors leading to undesirable physiological changes, especially when multiple heterologous genes were overexpressed.

To reduce the metabolic burden and possible mutual interference between the biosynthesis of p-coumaric acid and resveratrol, we divided the biosynthetic pathway of resveratrol into two separate strains. The aforementioned strain LJMB24 harboring the downstream pathway was used for production of resveratrol from p-coumaric acid. For tracking population composition and balancing resistance between two strains, plasmids pYG1 (derived from pRS福德u特-1 backbone) expressing mCherry gene and pACYCDuet-1 were transformed into strain BCR3, resulting in the upstream strain BCR4 for producing p-coumaric acid from glucose (Fig. 7a). We first made efforts to balance the metabolic strength of two strains by adjusting the inoculation ratio of strains BCR4 to LJMB24 (defined as the BCR4/LJMB24 ratio) from 4:1 to 1:4 with an initial OD₆₀₀ at 1.0. When the BCR4/LJMB24 ratio was decreased, resveratrol production increased until the BCR4/LJMB24 ratio at 1:2, where the highest production of resveratrol was achieved with a titer of 41.07 mg/L (Fig. 7b), nearly 3-fold of the monoculture. Then we analyzed the changes of total cell density, sugar consumption and population percentage in the coculture system with time under the optimal inoculation ratio at 1:2. As shown in Fig. 7c, the cell growth in the coculture system was poor and glucose consumption was very slow. Remarkably, the population of strain BCR4 increased from 33% to 60% after 48 h of fermentation (Fig. 7c), while strain LJMB24 decreased from 67% to 40% (Fig. 7c), which indicates that two strains undergo competition and strain LJMB24 exhibited a competitive disadvantage against strain BCR4.

### 3.5. Improving resveratrol production of coculture system by integrating resveratrol addiction circuit

In order to balance the subpopulations of two strains in coculture, we designed and constructed a resveratrol addiction circuit [29] in the upstream strain (Fig. 8a), wherein the growth of the upstream strain depended on resveratrol produced by the downstream strain. To validate the principle of resveratrol addiction circuit, we deleted the essential serA gene of strain BAK11(DE3) to generate the serine-auxotrophic strain BAK21(DE3), and introduced the resveratrol addiction circuit to construct strain BCR5. When cultivated in serine-deficient medium, strain BAK21(DE3) did not grow, and the expression of the serA gene under the control of resveratrol addiction circuit conferred the growth of serine-auxotrophic host BCR5 (Fig. 8b). Furthermore, adding 50 mg/L resveratrol to the medium enabled the cell growth of strain BCR5 to reach nearly 3-fold higher cell density (OD₆₀₀) than without resveratrol supply (Fig. 8b), which indicated that there was a coupling relationship between resveratrol formation and cell growth. The upstream strain BCR6 was eventually generated by introducing PctTAL and mCherry into strain BCR5. In the BCR6-LJMB24 coculture system, there might be a serine cross-feeding from strain LJMB24 to strain BCR6, causing a faulted crosstalk between the sub-populations. Thus, the coculture without IPTG induction was carried out. A time course of the population dynamics showed that strain BCR6 could grow slowly, and its proportion was always at an absolute disadvantage (lower than 15%) (Fig. 8c). Moreover, serine and resveratrol were not detected in fermentation broth, which demonstrated that strain LJMB24 without resveratrol biosynthesis had little effect on the growth of strain BCR6. Finally, the impact of the resveratrol addiction circuit on resveratrol production was evaluated in the BCR6-LJMB24 coculture system at varying inoculation ratios. The result showed that
71.32 mg/L resveratrol was achieved at the optimal ratio of 1:2, 74% higher than without the resveratrol addiction circuit (Fig. 8d). During the fermentation process, the subpopulations of strains BCR6 and LJM24 were fluctuated at early growth stage and then maintained at ratio of 1:2 (Fig. 8e). More glucose was consumed, and the final cell optical density of the BCR6-LJM24 coculture was 1.4-fold higher than that of the BCR4-LJM24 without resveratrol addiction circuit (Fig. 7c).
3.6. Utilizing mixed carbon sources for efficient production of resveratrol

Arabinose is the abundant sugar found in plant biomass and should therefore be potential attractive carbon source to produce valuable chemicals [37]. To enable strain LJM24 have a growth advantage in the coculture system, we tried to take arabinose with glucose as mixture carbon source to produce resveratrol. Especially, strain BCR6, derived from *E. coli* BW25113, is arabinose deficient as *araBAD* gene was deleted from the chromosome. We confirmed that strain BCR6 could not grow with arabinose as sole carbon source, while strain LJM24 could consume arabinose and smoothly grow (Fig. 9a).

We attempted to optimize the initial inoculation ratio of strains BCR6/LJM24 in the coculture at the glucose/arabinose ratio of 1/1 (5 g/L glucose and 5 g/L arabinose). As shown in Fig. 9b, when the inoculation ratio of BCR6/LJM24 was at 1:1, the highest production of resveratrol was achieved with a titer of 126.45 mg/L. Next, we attempted to balance the metabolic strength of two strains by varying the ratio of glucose to arabinose at the inoculation ratio of 1/1. As shown in Fig. 9c, the combinatorial profit of using the glucose and arabinose mixture was achieved at the ratio of 3/1 and the highest amount of resveratrol reached to 146.26 mg/L, which was 2.0-fold higher than glucose as the sole carbon source. Meanwhile the accumulation of intermediate p-coumaric acid was very low. To further improve the production of resveratrol, we optimized the initial inoculum size, and the maximum titer of 204.80 mg/L was achieved with an initial OD$_{600}$ of 2.0 (Fig. 9d).

During the fermentation process of the coculture at optimized conditions, the titer of resveratrol increased gradually to the maximum at 72 h, and the amount of p-coumaric acid accumulated at low level after 24 h, representing a high efficiency of bioprocess (Fig. 9e). The dynamic change of subpopulation ratio of strains BCR6/LJM24 was correlated to the sugar consumption and resveratrol titer (Fig. 9f). During the first 24 h fermentation, both strains in coculture system preferentially used glucose, and strain LJM24 showed an obvious growth advantage compared with strain BCR6 (Fig. 9f). Meanwhile glucose was depleting, arabinose was available for maintaining strain LJM24, and the BCR6/LJM24 ratio was maintained at 1:2.4 (Fig. 9f). Our results indicated that an additional carbon source to maintain growth of strain LJM24 at the later stage of fermentation, making the maintain at a high ratio throughout the fermentation process.

4. Discussion

Malonyl-CoA is an important precursor for the biosynthesis of resveratrol. However, intracellular malonyl-CoA concentration usually maintains at very low level, which severely limits the production of resveratrol. Enhancing the supply of malonyl-CoA has been proved to increase the production of resveratrol in both monoculture and coculture system. The efforts to improve malonyl-CoA availability were mainly focused on ways to increase revenue and reduce expenditure of malonyl-CoA, including overexpression of ACC from *C. glutamicum* [9] to increase the conversion of acetyl-CoA into malonyl-CoA, introducing the recombinant malonate assimilation pathway (*matB* and *matC*) of *Rhizobium trifolii* [12], fine-tuning the central metabolic pathways of malonyl-CoA metabolism [11,16,38]. Here, we took advantage of CRISPRi system with heterologous expression of ACC complex from *C. glutamicum* to increase the conversion of acetyl-CoA into malonyl-CoA, introducing the recombinant malonate assimilation pathway (*matB* and *matC*) of *Rhizobium trifolii* [12], fine-tuning the central metabolic pathways of malonyl-CoA metabolism [11,16,38]. Here, we took advantage of CRISPRi system with heterologous expression of ACC complex from *C. glutamicum* to increase the conversion of acetyl-CoA into malonyl-CoA, introducing the recombinant malonate assimilation pathway (*matB* and *matC*) of *Rhizobium trifolii* [12], fine-tuning the central metabolic pathways of malonyl-CoA metabolism [11,16,38]. Here, we took advantage of CRISPRi system with heterologous expression of ACC complex from *C. glutamicum* to increase the conversion of acetyl-CoA into malonyl-CoA, introducing the recombinant malonate assimilation pathway (*matB* and *matC*) of *Rhizobium trifolii* [12], fine-tuning the central metabolic pathways of malonyl-CoA metabolism [11,16,38]. Here, we took advantage of CRISPRi system with heterologous expression of ACC complex from *C. glutamicum* to increase the conversion of acetyl-CoA into malonyl-CoA, introducing the recombinant malonate assimilation pathway (*matB* and *matC*) of *Rhizobium trifolii* [12], fine-tuning the central metabolic pathways of malonyl-CoA metabolism [11,16,38]. Here, we took advantage of CRISPRi system with heterologous expression of ACC complex from *C. glutamicum* to increase the conversion of acetyl-CoA into malonyl-CoA, introducing the recombinant malonate assimilation pathway (*matB* and *matC*) of *Rhizobium trifolii* [12], fine-tuning the central metabolic pathways of malonyl-CoA metabolism [11,16,38]. Here, we took advantage of CRISPRi system with heterologous expression of ACC complex from *C. glutamicum* to increase the conversion of acetyl-CoA into malonyl-CoA, introducing the recombinant malonate assimilation pathway (*matB* and *matC*) of *Rhizobium trifolii* [12], fine-tuning the central metabolic pathways of malonyl-CoA metabolism [11,16,38].
When individually optimized strains are physically mixed and cocultivated, their subpopulations in coculture system often change dynamically, and the coexistence may collapse due to the competitive growth and metabolic stress [20]. One strategy is to engineer competitive growth advantage that links cell growth with product formation. Metabolic addiction to maintain the growth adaptability is underexplored in metabolic engineering. Biosynthesis of mevalonic acid in addicted E. coli was controlled by fine-tuning of essential genes using mevalonic acid responsive transcription factor, which allowed the engineered strain to retain the high production capacity of mevalonate [40]. The similar strategy was used to stabilize the naringenin production phenotype of Y. lipolytica [41]. In this study, we demonstrated that the resveratrol addiction circuit could balance the subpopulations of two strains in coculture system by coupling resveratrol formation in one subpopulation to the cell growth of the other subpopulation. Implementation of resveratrol addiction circuit in a resveratrol-producing coculture resulted in a 74% increase of titer over varying inoculation ratios. Moreover, glucose consumption and the final biomass of the BCR6-LJM24 coculture system were remarkably improved.

In conclusion, redirecting malonyl-CoA to the resveratrol biosynthesis pathway via CRISPRi significantly increased the resveratrol synthesis ability from p-coumaric acid. Furthermore, resveratrol addiction...
circuit and glucose-arabinose mixture utilization improved coculture system compatibility and resveratrol yield. Under the optimal conditions in shake-flask fermentation, the coculture produced 204.80 mg/L of resveratrol with low accumulation of intermediate p-coumaric acid. Hence, the associated findings lay a foundation for future studies aiming at using these strategies to improve the production capacity of existing microbial coculture systems. Furthermore, the accomplishment of this study marks an important progress of modular coculture engineering for advancing microbial biosynthesis of valuable natural products.

CRediT authorship contribution statement

Jia Li: Conceptualization, Investigation, Formal analysis, Visualization, Writing – original draft, Reviewing and Editing. Zetian Qiu: Investigation, Formal analysis, Writing – original draft. Guang-Rong Zhao: Conceptualization, Formal analysis, Visualization, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare no competing financial interest.
Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2022.03.001.

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