A CRISPRi mediated self-inducible system for dynamic regulation of TCA cycle and improvement of itaconic acid production in *Escherichia coli*

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**ABSTRACT**

Itaconic acid (ITA), an effective alternative fossil fuel, derives from the bypass pathway of the tricarboxylic acid (TCA) cycle. Therefore, the imbalance of metabolic flux between TCA cycle and ITA biosynthetic pathway seriously limits the production of ITA. The optimization of flux distribution between biomass and production has the potential to the productivity of ITA. Based on the previously constructed strain *Escherichia coli* MG1655 Δ1-SAS-3 (ITA titer: 1.87 g/L), a CRISPRi-mediated self-inducible system (CiMS), which contained a responsive module based on the ITA biosensor YphtcR/P4P and a regulative CRISPRi-mediated interferential module, was developed to regulate the flux of the TCA cycle and to enhance the capacity of the strain to produce ITA. First, a higher ITA-yielding strain, Δ4-P*mod*-SAS-3 (ITA titer: 3.20 g/L), derived from Δ1-SAS-3, was constructed by replacing the promoter P*J23100* Δ1-SAS-3 under the dynamic regulation of the CiMS, showed a 23% increase in the ITA titer, which reached 3.93 g/L. This study indicated that CiMS was a practical strategy to dynamically and precisely regulated the metabolic flux in microbial cell factories.

1. Introduction

With the rapid development of synthetic biology, conventional chemical and biological extraction technologies to produce valuable compounds are gradually being replaced by microbial fermentation [1–4]. However, owing to the complex metabolic circuits and regulatory networks in living microorganisms, it is difficult to precisely balance the central metabolism for biomass and the metabolite synthesis [5–7]. To accumulate several types of organic acids, it is necessary to inhibit the precursor-consuming pathways, most of which are a part of the basic metabolic pathway, such as glycolysis and the tricarboxylic acid (TCA) cycle. However, the disruption of these pathways would be harmful to cell growth. Therefore, a novel approach is urgently needed to balance the flux of metabolic bypass and product accumulation pathways.

Itaconic acid (ITA) is a C5 unsaturated dicarboxylic acid. As it possesses the methylene group and dicarboxylic acid, ITA has distinct active chemical properties [8]. ITA has garnered much interest, as it can participate in various polymerization reactions to produce promising and renewable polymer materials such as resins, rubbers, and fibers [9, 10]. Owing to its wide range of applications, it was considered one of the 12 high-added-value bio-based materials by the US Department of Energy in 2004. ITA has also been found in the metastatic tumor cell line, which indicates that it might also have a role in tumor biology [11]. With its broad utility, researchers have concentrated their efforts on enhancing the production of ITA. Currently, the most predominant way to produce ITA is microbial fermentation. Therefore, the development of metabolic engineering strategies to construct robust microbial cell factories will be critical to improve ITA production.

ITA is synthesized from cis-aconitate, the intermediate of the TCA cycle, by cis-aconitate decarboxylase (CAD). ITA synthesis competes for...
the cis-aconitate in the TCA cycle, therefore, balancing energy allocation between the TCA cycle and the ITA-producing pathway is important for the high-yielding production of ITA. Recently, a high-yield ITA strain *Escherichia coli* MG1655 ΔSAS-3, based on a sequential self-assembly system that enhances the cascade reaction among citrate synthase (glaA, GA), aconitase (acnA, AGN), and cis-aconitate decarboxylase (cadA, CAD), was reported, and this system could produce 1.87-g/L ITA in a shake flask within 100 h [12]. To further increase the production of ITA, a higher ITA-producing strain ΔΔSAS-3, which could produce 3.06-g/L ITA within 100 h, was engineered by combining downstream genes with the corresponding designed sgRNAs to regulate the distribution of metabolic flux between biomass and ITA production. Using the combination of different types of sgRNAs, an optimized sgRNA-array was constructed and verified to be effective in significantly improving ITA production by the *E. coli* cell factory.

2. Materials and methods

2.1. Bacteria, plasmids, and materials

The bacterial strains and plasmids employed in this study are presented in Supplementary Tables S1 and S2, respectively. Maps of plasmids employed in this study are presented in Supplementary Fig. S1. *Escherichia coli* DH5α and MG1655 were used for plasmid propagation and CIMS assays, respectively. *E. coli* MG1655 ΔΔSAS-3, which was constructed in our previous research [12], was adopted as the original host and used for ITA production in this study. The vector pACYCDuet-1 was used to construct the responsive module and regulated module. Antibiotic and standard compounds were purchased from Aladdin (Shanghai, China). Restriction enzymes, DNA polymerase, ligase, and other DNA-modifying enzymes were purchased from TaKaRa (Dalian, China). In addition, the Gibson assembly cloning kit was purchased from Yesen (Shanghai, China).

2.2. Media, culture conditions, and ITA analysis

The transformants of *E. coli* were cultured in Luria Bertani (LB) broth containing appropriate antibiotics for cell growth and protein expression. Then, the reconstructed strains for ITA production were grown at 30 °C on a rotary shaker at 200 rpm in minimal media (MM) broth [25]. To quantify ITA production, samples were collected from 1-mL of culture and filtered using a 0.45-μm filtration membrane. A high-performance liquid chromatography (Agilent 1200) system equipped with an Agilent ZORBAX SB-Aq C18 column (5 μm, 4.6 mm × 250 mm) and a UV/VIS detector were utilized to quantify ITA levels at a wavelength of 210 nm at 40 °C. The injection volume was 10 μL per sample and the mobile phase was 0.1 M NH₄H₂PO₄ (pH 2.6) at a flow rate of 1.0 mL/min.

2.3. Itaconic acid biosensor construction and functional testing

The sequence of the ITA biosensor, YpItcR/Ppdcas9, was synthesized by Tsingke Biotech (Beijing, China) and cloned into pACYCDuet-1, which contains a *mrfp* gene constructed by the Gibson assembly method, to yield pYpItcR/Ppdcas9. The primers used in this study are presented in Supplementary Table S3. To precisely measure the red fluorescence intensity, the MG1655 cells containing pYpItcR/Ppdcas9 were recultivated in fresh LB medium with a different concentration of ITA at 37 °C. Subsequently, the cells were diluted to an optical density of 600 nm (OD₆₀₀) of 0.5, and the red fluorescence intensity was measured in *vivo* using the Ascent (Thermo, Waltham, MA, USA) at excitation and emission wavelengths of 580 nm and 610 nm, respectively [27].

2.4. Regulation module construction

The gene *dcas9* was adopted as the template and amplified with pdCas9-Ppdcas9-F/pdCas9-Ppdcas9-R, and then, the amplified fragment was cyclized with the plasmid skeleton pYPhtcR/Ppdcas9 via the Gibson assembly method to generate pYPhtcR/Ppdcas9-dcas9. In addition, the sgRNAs (N20) were designed online (https://zlab.bio/guide-design-resources). The sequences of the sgRNAs are presented in the Supplementary Table S4. A single sgRNA expression cassette was constructed, via the Gibson assembly method, into the Sal I/Xba I restriction sites of pYPhtcR/Ppdcas9-dcas9. sgRNA expression cassettes were amplified with primers Srclc-F/sh611-c-R. The sgRNA arrays were assembled via the Gibson assembly method (Supplementary Fig. S2).

2.5. Real-time quantitative PCR (RT-qPCR) analysis

Cells were grown at 30 °C in MM broth and harvested at 24 and 48 h.
The total RNA was extracted from the cells using the Bacterial RNA Kit purchased from Omega Bio-tek (Georgia, USA), according to the manufacturer’s instructions. In addition, the 16s rRNA gene was adopted as the housekeeping gene. RT-qPCR was conducted with FastKing RT and SuperReal PreMix Plus kits purchased from Tiangen (Beijing, China) following the manufacturer’s instructions. The obtained results were analyzed via the \(2^{-\Delta\Delta Ct}\) method.

2.6. Statistical analysis

All quantitative data were measured based on at least three independent experiments. The experimental data were statistically processed using Origin 8.0 software, and the data results were presented as the mean ± standard error (mean ± SE). The data were considered statistically significant at \(P < 0.05\).

3. Results and discussion

3.1. Design of CiMS system

The TCA cycle provides most of the energy for biomass accumulation. When cells enter the stationary phase, the stored energy used for metabolites production is what we expect. ITA synthesis competes for the cis-aconitate in the TCA cycle. Therefore, during the ITA accumulated phase, balancing energy allocation between the TCA cycle and the ITA-producing pathway is essential for the high-yielding production of ITA. Here, we designed a CRISPRi-mediated self-inducible system (CiMS), which functioned as a dynamic regulator to continually optimize the flux distributions between biomass and metabolites production during the fermentation stage. The CiMS contained a regulative module based on the CRISPRi system and a responsive module based on a specific biosensor activated by the target product. (Fig. 1). At the biomass-accumulation stage, ITA was barely accumulated and the CRISPRi system was nearly silent (Fig. 1A and B). Therefore, the metabolic flux could be fully applied for cell growth. At the fermentation stage, ITA rapidly accumulated. The special biosensor was gradually launched which strengthened the CRISPRi to regulate the metabolic flux (Fig. 1A and C). Hence, the metabolic flux transformed from the TCA cycle to the ITA synthesis. To dynamically self-regulate the metabolic flux during the ITA synthesized phase, an efficient ITA-specific biosensor was indispensable. The Yptlcr/Pcld comprising the ITA itaconate-inducible promoter Pcl and its corresponding transcriptional regulator ItcR from Yersinia pseudotuberculosis, has been verified to be a special ITA biosensor in E. coli [26] and was used to construct the responsive module of CiMS.

To better balance the metabolic flux between the TCA cycle and ITA biosynthetic pathway, several key genes should be dynamically regulated by CiMS to accumulate the precursor of ITA and facilitate ITA production. Knocking out key genes icd and sucCD in the TCA cycle limited the metabolic flux of TCA cycle and accumulated the precursor of ITA, which turned out to be an efficient intervention to increase ITA yield [12]. Instead of gene deletion, inhibition of the expression of these two genes would cause a minor effect on cell growth. Therefore, in this study, the genes icd and sucCD were repressed by the CiMS to control the metabolic flux of the TCA cycle and further accumulate the precursor of ITA.

However, inhibiting the expression of the essential genes icd and sucCD blocked the metabolic flux of the TCA cycle and decreased the production of oxaloacetate (OAA), which was also indispensable for ITA production. Phosphoenolpyruvate (PEP) can be catalyzed by pyruvate kinase I (encodes by gene pykA) and pyruvate kinase II (encodes by gene pykF) to produce pyruvate, and then to produce acetyl coenzyme A (acetyl CoA). PEP can also be catalyzed by phosphoenolpyruvate carboxykinase (encodes by gene ppc) to produce OAA. Acetyl CoA and OAA are the starting substrates for the TCA cycle and the precursors of ITA. Therefore, it is necessary to balance the metabolic flux between the synthesis of acetyl CoA and OAA. Compared with pyruvate kinase II, pyruvate kinase I contributes significantly low enzymatic activity [28, 29]. A study has indicated that deleting pykA gene had a positive effect on ITA production [14]. Therefore, to ensure the proper function of the TCA cycle and to accumulate the precursor of ITA, the expression of gene pykA also needed to be regulated by the CiMS.

3.2. Feasibility analysis of CiMS system

To verify the availability of Yptlcr/Pcld for the CiMS, we first constructed the recombinant strain Yptlcr/Pcld:mrfp/MG1655, which was utilized to detect the Yptlcr/Pcl property to regulate the expression of mRFP (Fig. 2A). To analyze the relationship between the ITA concentration and Yptlcr/Pcld strength, different concentrations of ITA were adopted to activate Yptlcr/Pcl. The obtained results indicated that the strength of Yptlcr/Pcld was extremely weak with 40 mg/L ITA, and significantly increased with >80 mg/L ITA (Fig. 2B). Considering that the ITA titer of the MG1655 Δ1-SAS-3 strain was no more than 50 mg/L at the logarithmic phase, modulating of the TCA cycle via the CiMS would not affect the cell growth.

To verify the efficiency of CRISPRi intervention under the regulation of Yptlcr/Pcld, the constructed gene circuit Yptlcr/Pcl-CRISPRi was applied to regulate the expression of mRFP (Fig. 2C). After adding 80 mg/L ITA, CRISPRi was activated and dCas9 targeted mrfp mRNA, directed by a sgRNA-mrfp. The expression of red fluorescence in strain pYptlcr/Pcld:mrfp/MG1655 was repressed at the fourth hour, and the inhibition efficiency increased significantly over time. The obtained
The results indicated that the expression of mRFP was decreased by 23% after 10 h under the regulation of \( \text{YpItcR/P}_{\text{ccl}} \) (Fig. 2D, Supplementary Fig. S3), clearly demonstrating that it was suitable for the modulation of target genes.

**Fig. 2.** Verification of the feasibility of the regulation module and the response module of the CRISPRi-mediated self-inducible system (CiMS). (A) Construction of the itaconic acid (ITA) biosensor \( \text{YpItcR/P}_{\text{ccl}} \). (B) Analysis of the RFP fluorescence intensity to verify the expression strength of \( \text{YpItcR/P}_{\text{ccl}} \) with different concentrations of ITA. (C) Construction of the regulative module and response module of the CiMS. (D) Analysis of the RFP fluorescence intensity to verify the efficiency of interference by the CiMS. Error bars show the standard deviation from three independent experiments.

**Fig. 3.** Construction of engineered strains to improve the itaconic acid (ITA) yield. (A) Overview of Escherichia coli MG1655 \( \Delta 1\)-SAS-3. The gene marked with a blue color represents gene knock-out in the genome of \( \Delta 1\)-SAS-3. CSI (CAD-SH3lig), GPS (GA-PDZ-SH3), and API (ACN-PDZlig) represent the over-expression cascade reaction for ITA production. (B) Improvement in the ITA yield in engineered strain via promoter optimization. \( P_{\text{J23100}} \) for the expression of the CSI, GPS, and API was replaced by \( P_{\text{lac}} \), \( P_{\text{tet}} \), and \( P_{\text{rmd}} \), respectively. (C) Overview of E. coli MG1655 \( \Delta 4\)-\( P_{\text{rmd}}\)-SAS-3. The genes marked with blue and red represent the genes knocked out in the genome of \( \Delta 4\)-\( P_{\text{rmd}}\)-SAS-3. (D) Relative ITA yield of each engineered strain. Error bars show the standard deviation from three independent experiments.
3.3. Improving the ITA production of engineered strains

In our previous study, we had knocked out gene aceA in the glyoxylate pathway in the genome of E. coli MG1655 and constructed a self-assembly system that enhanced the cascade reaction among citrate synthase (gltA, GA), aconitase (acnA, ACN), and cis-acconitate decarboxylase (cadA, CAD) to construct strain Δ1-SAS-3 (Fig. 3A). Evidently, the ITA titer of Δ1-SAS-3 (1.87 g/L) was lower than that reported with ITA production strains, such as ita23 (2.27 g/L) [14]. Therefore, before conducting CIMS regulation, we expected to further improve the ITA yield of Δ1-SAS-3 via conventional metabolic engineering strategies. First, three different high-strength promoters, P suc1, P rmd1, and P rmd4 derived from E. coli were applied to replace P J23100 for the expression of the self-assembly cascade reaction. The results indicated that the ITA yield of the recombinant strain Δ1-P rmd SAS-3 was the highest, which could reach 1.35 times that of Δ1-SAS-3 (Fig. 3B). Next, the engineered strain, Δ4-P rmd SAS-3 (Fig. 3C, Supplementary Fig. S4), derived from Δ1-P rmd SAS-3 was obtained by knocking out three genes, pfbB, poxB, and ldhA, in the pyruvate bypass pathway to accumulate the precursor of ITA and the ITA titer of Δ4-P rmd SAS-3 (3.2 g/L) reached 1.27 times that of Δ1-P rmd SAS-3 (Fig. 3D).

3.4. Effectiveness of CIMS for dynamic regulation of ITA production

To effectively inhibit the expression of icd, pykA, and sucCD, three different sgRNAs were designed for each gene (Fig. 4A). Consequently, nine regulated strains were constructed to verify the effectiveness of the CIMS to improve ITA production capability (Fig. 4B). Compared with the control strain E. coli MG1655 Δ4-P rmd SAS-3/sgRNA-0 (Δ4-P mde SAS-3 harboring a non-targeting CRISPRi plasmid), ITA production by the five regulated strains with single-targeting intervention (sgRNA-icd-1, sgRNA-icd-2, sgRNA-pykA-1, sgRNA-sucCD-1, sgRNA-sucCD-2) was significantly improved. To verify the superposition of effective inhibited sites, these five sgRNAs were combined into a sgRNA array. The results showed that the regulated strain E. coli MG1655 Δ4-P rmd SAS-3/sgRNA-array was optimal, which could increase the ITA yield to 1.23 times that of the control strains (Fig. 4B). Moreover, the growth of the engineered strain was similar to that of the control strain but exhibited significantly increased production of ITA at the anaphase of the platform period (100 h, Supplementary Fig. S5), which clearly demonstrated that the CIMS could dynamically and precisely regulate ITA production in the E. coli cell factory.

To verify that the ITA yield improvement in the engineered strain was the result of the dynamic regulation via the CIMS, the transcriptional level of each metabolic bypass gene, icd, pykA, and sucCD, was analyzed at 24 and 48 h. The results showed that the transcriptional level of the three genes were slightly decreased at 24 h, which was possibly due to activation of the regulation module by the small amounts of accumulated ITA (Fig. 5). Additionally, the transcriptional levels of these three genes were significantly decreased at 48 h, which respectively reached 66% (icd), 67% (pykA), and 38% (sucCD) of those in the unregulated strain (Fig. 5). These results fully demonstrated that the collateral ITA pathway was dynamically regulated by the CIMS during ITA production.

4. Discussion

The biosynthesis of ITA in heterogenous microorganisms has been...
extensively studied [30–32]. Meanwhile, researchers have also encountered challenges posed by the uneven distributions of the metabolic flux. Isocitrate dehydrogenase, which plays an important role in the TCA cycle, is encoded by the gene $icd$, and the intervention of $icd$ significantly affects the growth of host cells [33]. In our previous work, the gene $icd$ was completely deleted to accumulate the precursor of ITA, which required the addition of extra expensive $\alpha$-KG to maintain stability of the TCA cycle [12]. Therefore, it is important to dynamically regulate the metabolic flux between biomass accumulation and metabolite production.

With the development of synthetic biology, novel strategies, drawing from the natural dynamic regulatory mechanisms in microorganisms, have been developed for the dynamic regulation of metabolic pathways. A temperature-sensitive promoter, pH-responsive biosensor, and quorum sensing were applied to dynamically regulate metabolic flux and enhance the production of metabolites [34–36]. However, the artificially engineered Ntr regulon, which senses of acetyl phosphate, was constructed to control the expression of genes $ppp$ and $lbd$ in lycopene synthesis in response to flux dynamics [37]. To date, dynamic regulation has made significant progress in the synthesis of valuable products in a low-cost manner. However, the metabolic regulation by a single signal would not be suitable for a complicated metabolic network. Therefore, dynamic regulation, comprised of real-time responses to target metabolites, would probably sustain cellular homeostasis to increase the productivity of metabolites.

Hence, in this study, we constructed a self-inducible system, which contained the biosensor Yptcr/P$_{col}$ to specially respond to ITA and a CRISPRi-mediated interferential module regulated by the ITA biosensor. Combined with the designed sgRNAs targeting the vital genes in the critical metabolic pathway, the CIMS was applied to dynamically regulate metabolic flux between biomass accumulation and ITA production (Fig. 1). During the logarithmic growth phase, trace amounts of ITA could be produced and Yptcr/P$_{col}$ was almost silent; thus, the CRISPRi system could not be activated to inhibit the TCA cycle and the cells could grow well (Fig. 1A). After the logarithmic growth phase, sufficient ITA accumulation could activate the designed CIMS to inhibit the TCA cycle, while metabolic flux was continuously converted to ITA production (Fig. 1B).

The key role of the CIMS was as a specific biosensor that could respond to the target product. Recently, the ITA biosensor Yptcr/P$_{col}$ was obtained, and its effectiveness was verified in E. coli [26]. Therefore, Yptcr/P$_{col}$ could function effectively as an important element for the construction of a CIMS to dynamically regulate ITA production in E. coli. In this study, we also utilized Yptcr/P$_{col}$ to directly regulate the expression of mRFP (Fig. 2B) and inhibit the transcription of $mrfp$ by combining it with the CRISPRi system (Fig. 2D), which fully confirmed that Yptcr/P$_{col}$ was suitable for GMS application to the dynamic regulation of ITA production. In addition, various biosensors responding to organic acids, such as glutamate and cysteine, have been reported [38–40]. The CIMS exhibited the potential to produce several types of organic acids. Furthermore, Yptcr/P$_{col}$ can be reconstructed to respond to other organic acids via protein engineering and directed evolution strategies [26].

However, some issues must be further addressed in future studies. The expression level of dCas9 was barely detected under the regulation of Yptcr/P$_{col}$ such that it was difficult to optimize the expression of dCas9. In addition, the promoter for the transcription of sgRNA should also be optimized, which might further improve the ITA yield of engineered strains. Alternatively, it is worth considering the application of CRISPR/dCas9 systems in our further research. Numerous studies have demonstrated that the gene repression mediated by dCas9 is highly efficient and has no substantial off-target effects [41–44]. The Cas9-based gene-editing system requires both a crRNA and tracrRNA to mediate interference, whereas the CPl1 system only needs a pre-crRNA, which remarkably simplifies the construction of sgRNA arrays [45].

5. Conclusions

In conclusion, we designed a CIMS, for dynamic regulation of the TCA cycle and ITA production. This novel system employed the CRISPRi strategy instead of the knock-out strategy to inhibit the expression of vital genes in the TCA cycle. Therefore, extra amounts of expensive organic acids, such as $\alpha$-KG and $\gamma$-glutamic acid were not required in the medium, and this would remarkably minimize the costs of fermentation. In addition, the biosensor responding to the target product ITA was employed to regulate the expression of dCas9, and sufficient ITA accumulation to activate the biosensor should be realized during the ITA accumulation period. Therefore, the CIMS exerted a negligible effect on cell growth. Consequently, the CIMS turned out to be an effective method to increase the ITA titers, which provides a novel idea for organic acid production.

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