

Enhancing glucaric acid production from *myo*-inositol in *Escherichia coli* by eliminating cell-to-cell variation

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ABSTRACT Glucaric acid (GA) is a value-added chemical and can be used to manufacture food additives, anticancer drugs, and polymers. The non-genetic cell-to-cell variations in GA biosynthesis are naturally inherent, indicating the presence of both high- and low-performance cells in culture. Low-performance cells can lead to nutrient waste and inefficient production. Furthermore, *myo*-inositol oxygenase (MIOX) is a key rate-limiting enzyme with the problem of low stability and activity in GA production. Therefore, eliminating cell-to-cell variations and increasing MIOX stability can select high-performance cells and improve GA production. In this study, an *in vivo* GA bioselector was constructed based on GA biosensor and tetracycline efflux pump protein TetA to continuously select GA-efficient production strains. Additionally, the upper limit of the GA biosensor was improved to 40 g/L based on ribosome-binding site optimization, achieving efficient enrichment of GA high-performance cells. A small ubiquitin-like modifier (SUMO) enhanced MIOX stability and activity. Overall, we used the GA bioselector and SUMO-MIOX fusion in fed-batch GA production and achieved a 5.52-g/L titer in *Escherichia coli*, which was 17-fold higher than that of the original strain.

IMPORTANCE Glucaric acid is a non-toxic valuable product that was mainly synthesized by chemical methods. Due to the problems of non-selectivity, inefficiency, and environmental pollution, GA biosynthesis has attracted significant attention. The non-genetic cell-to-cell variations and MIOX stability were both critical factors for GA production. In addition, the high detection limit of the GA biosensor was a key condition for performing high-throughput screening of GA-efficient production strains. To increase GA titer, this work eliminated the cell-to-cell variations by GA bioselector constructed based on GA biosensor and TetA, and improved the stability and activity of MIOX in the GA biosynthetic pathway through fusing the SUMO to MIOX. Finally, these approaches improved the GA production by 17-fold to 5.52 g/L at 65 h. This study represents a significant step toward the industrial application of GA biosynthetic pathways in *E. coli*.

KEYWORDS glucaric acid, transcription factor-based biosensor, GA bioselector, detection limit, metabolic engineering

Glucaric acid (GA) is a non-toxic platform chemical derived from glucose (1). GA was identified as one of the “top value-added chemicals from biomass” by the United States Department of Energy in 2004 (2). GA can be used as raw material to manufacture food additives, anticancer drugs, biodegradable detergents, and polymers such as nylon and plastics (3–6). By 2024, the global GA market is expected to exceed \$440 million (7). Marketed GA is currently produced chemically by non-selective oxidation of glucose using nitric acid as solvent and oxidant (8). However, this method is inefficient and easily leads to environmental pollution. In contrast, the microbial biosynthesis of GA may be cost-effective and environmentally friendly (9, 10).

Editor Nicole R. Buan, University of Nebraska-Lincoln, Lincoln, Nebraska, USA

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Nana Ding, Lei Sun, and Xuan Zhou contributed equally to this article. Authors order was determined based on the individual contributions to the study.

The authors declare no conflict of interest.

See the funding table on p. 13.

Received 26 January 2024

Accepted 8 May 2024

Published 29 May 2024

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In *Escherichia coli*, the GA biosynthetic pathway starting from glucose includes three heterologous genes: *myo*-inositol-1-phosphate synthase (INO1) from *Saccharomyces cerevisiae*, *myo*-inositol oxygenase (MIOX) from *Mus musculus*, and urinate dehydrogenase (Udh) from *Pseudomonas syringae* (11, 12). In this pathway, glucose-6-phosphate is converted to *myo*-inositol-1-phosphate by INO1. Then, *myo*-inositol-1-phosphate is hydrolyzed and dephosphorylated by an endogenous phosphatase to produce *myo*-inositol. Subsequently, *myo*-inositol is converted to glucuronic acid by MIOX using molecular oxygen. Finally, glucuronic acid is oxidized to GA by Udh using NAD⁺ as a cofactor. MIOX is a key rate-limiting enzyme in GA biosynthesis and faces the challenge of low stability and activity.

Therefore, GA biosynthesis is possible in *E. coli* recombinants by metabolic engineering methods, such as fusing an N-terminal tag small ubiquitin-like modifier (SUMO) to improve the solubility of the key enzyme MIOX and to help the proper folding of the recombinant protein (11), direct evolution to enhance the catalytic activity or stability of MIOX in the logarithmic growth phase using *myo*-inositol as a substrate (11, 13), controlling the metabolic flow to GA biosynthetic pathway (14–16), constructing a pathway-independent quorum sensing system and *myo*-inositol biosensor to regulate the MIOX expression (17), and introducing NAD⁺ regeneration system and fine-tuning the MIOX activity (15). These methods had successfully improved the titer of GA, and the reaction conditions were milder than chemical production. The highest total titer of extracellular and intracellular GAs could reach 5.35 g/L by batch fermentation (15). However, the biosynthesis of GA in *E. coli* still faces the problem of low titer. In addition, the lack of efficient high-throughput screening methods *in vivo* is a bottleneck restricting the research of GA biosynthesis.

Fortunately, transcription factor-based biosensors (TFBs) are a major focus in the field of biotechnology (18–20), allowing real-time control of the fluorescent protein expression by responding to GA (21). This is an effective approach to solving the above problems and has high application value in metabolic engineering. Rogers et al. developed many TFB systems in *E. coli*, including GA biosensor (TFB-GA) based on transcription factor CdaR and super-folded green fluorescent protein (sfGFP) under the control of inducible promoter P_{gudP} (21). It is possible to measure changes in a single cell using high-throughput methods when GA concentrations *in vivo* are converted to sfGFP fluorescence intensities (TFB-GA output) (22, 23). However, an upper limit of 3.15 g/L was previously reported for TFB-GA (22), which may not be sufficient for large-scale production of GA. Many studies have shown that the ribosome-binding site (RBS) can regulate the response performance of TFB by controlling protein expression or protein folding (19, 20, 23–25). Therefore, RBS has great potential to improve the detection limit of TFB-GA.

Additionally, some poorly behaved cells may not synthesize the target metabolite despite consuming nutrients (26), and this remains a major challenge. Therefore, to identify and eliminate low-performing cells and enrich well-behaved cells, a population quality control (PopQC) system has been successfully employed to improve biosynthetic efficiency (27). The PopQC approach consists of a biosensor for product response and a tetracycline (Tc) efflux pump protein TetA for pumping Tc out of the cells. Using this method, studies have shown that the titer of both free fatty acid and tyrosine was increased threefold (27). These results indicate that the construction of PopQC based on TFB-GA and TetA may also be worth investigating for GA biosynthesis. It has great potential to increase GA production.

Herein, we have established an efficient high-throughput screening method based on the PopQC system for enhancing GA biosynthesis. First, the detection limit of TFB-GA was optimized by manipulating the RBS. Then, to increase GA production, we randomly mutated the *miox* gene and screened high-titer GA strains based on TFB-GA output in a high-throughput manner. Furthermore, we constructed a GA bioselector based on TFB-GA and TetA and fused the SUMO to MIOX to further increase the GA titer. Finally,

this approach resulted in GA titer at 5.52 g/L by fed-batch fermentation. This represents a significant step toward the industrial application of GA biosynthetic pathways.

RESULTS

Design of the RBS to improve the glucaric acid biosensor detection limit

The work principle of glucaric acid biosensor (TFB-GA) shows that in the absence of GA, CdaR is inactive, resulting in the “OFF” state of TFB-GA and in the presence of GA, CdaR is activated and simultaneously increases the expression of its own and P_{gudP} -controlled genes, resulting in the “ON” state of TFB-GA, accompanied by an increase in the TFB-GA output (Fig. 1A).

We assumed that the strength of the RBS should be directly correlated with the upper limit of the TFB-GA. To explore the effect of the RBS on the upper limit, we used G10 from the plasmid pJKR-H-*cdaR* (TFB-GA-G10) and designed six RBSs with gradient translation initiation rate (Table S1) through an RBS calculator (28) to regulate the sfGFP expression level. Six TFBs-GA (pJKR-H-*cdaR*-RBS1–6) were then successfully constructed and separately transformed into *E. coli* BL21 (DE3). The transformants were cultured in a 24-well plate and supplemented with 0, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 g/L GA, respectively. As shown in Fig. 1B, the normalized TFB-GA output under the control of G10 and RBS3 was extremely significantly higher than for the other five RBSs (Table S2). It indicated that RBS was a key factor in increasing the detection limit of TFB-GA. Moreover, only the appropriate intensity of RBS was conducive to regulating the detection limit of TFB-GA (Table S1; Fig. 1B). In addition, we found that TFB-GA controlled by RBS3 had lower

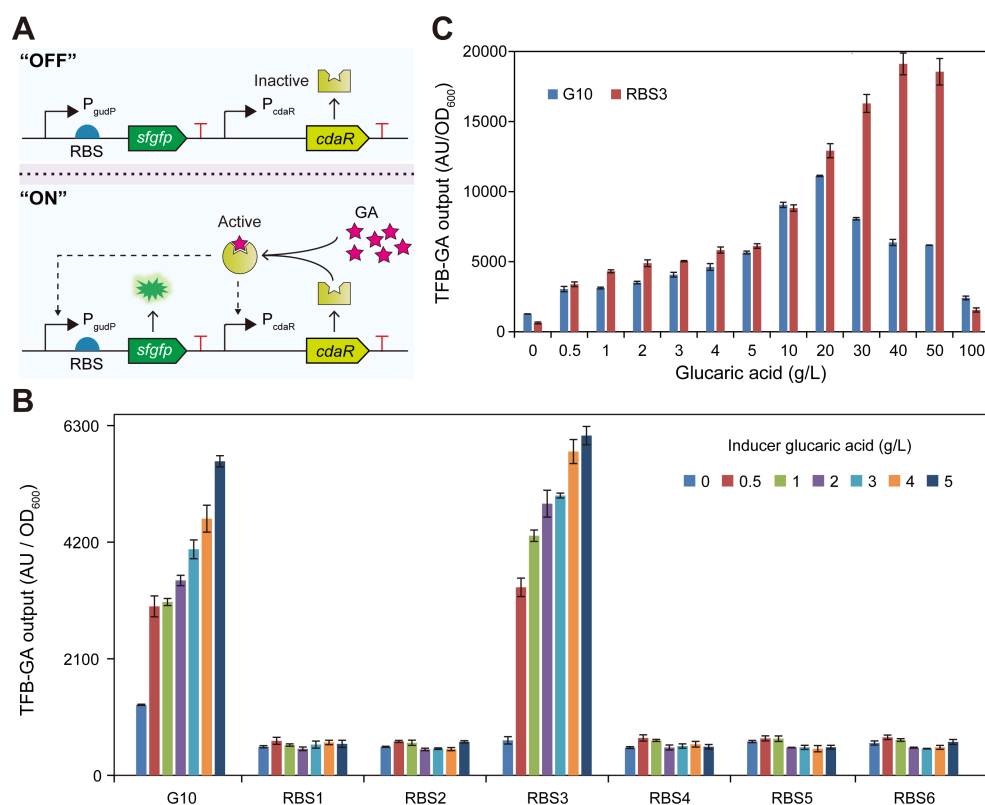


FIG 1 Effects of RBS on biosensor detection limit. (A) The working principle of TFB-GA. Inactive CdaR (“OFF” state) is activated by GA while increasing its own and P_{gudP} -controlled gene expression (“ON” state). Dashed arrows represent feedback activation. (B) The effect of gradient-strength RBSs on TFB-GA output induced by various concentrations of GA within 5 g/L. (C) Comparison of the detection limit for biosensors TFB-GA-G10 and TFB-GA-RBS3 based on TFB-GA output induced by different concentrations of GA within 100 g/L. Data represent the mean and standard deviation for three replicates. AU, arbitrary unit; OD₆₀₀, optical density at 600 nm.

leakage expression than G10 by analyzing the TFB-GA output induced by 0-g/L GA (see “Fluorescence assays” in Materials and Methods and Fig. 1B). This implied that TFB-GA controlled by RBS3 might have a higher upper limit than G10.

To further explore the upper limit of TFB-GA-G10 and TFB-GA-RBS3, we expanded the GA concentration to analyze the TFB-GA output (see “Optimization of TFB-GA detection limit” in Materials and Methods). It showed that the TFB-GA output of TFB-GA-RBS3 peaked at 40-g/L GA at 12 h, whereas maximum TFB-GA output with TFB-GA-G10 occurred at 20-g/L GA at 12 h (Fig. 1C). This indicated that the above assumption was reliable, and RBS3 was conducive to optimizing the TFB-GA upper limit. Thus, the TFB-GA-RBS3 was superior for detecting the strain that produced more GA in *E. coli*. In addition, we found that when the GA concentration exceeded the detection upper limit of TFB-GA-G10 (20 g/L) and TFB-GA-R3 (40 g/L), respectively, the TFB-GA output showed a downward trend. The reason may be that the higher concentrations of GA led to slower cell growth (Fig. S1A) or cell death.

To verify whether excessive GA concentration would lead to cell death, we selected 0- (control), 20-, 50-, and 100-g/L GA at 12 h to perform the percentage of analysis of cell death using the propidium iodide (PI) Red Dead nucleic acid staining kit by flow cytometry (see “Percentage analysis of cell death” in Materials and Methods; Fig. S1B through I). The results showed that at 20-, 50-, and 100-g/L GA, the percentages of TFB-GA-G10 cell death were 17.8%, 11.6%, and 3.38%, respectively (Fig. S1C through E), and the percentages of TFB-GA-R3 cell death were 20.2%, 13.5%, and 4.7%, respectively (Fig. S1G through I). It indicated that the percentage of cell death decreased with the increase in GA concentration. This implied that higher concentrations of GA might lead to slower cell growth (Fig. S1A), thereby prolonging the apoptosis period and ultimately reducing the percentage of cell death. Thus, the possible reason for the decrease in TFB-GA output at high concentration of GA was slow cell growth rather than cell death.

Random mutagenesis of *miox* to increase glucaric acid production

During GA production, the intermediate *myo*-inositol is oxidized to glucuronic acid by MIOX. However, MIOX was extremely unstable (29, 30). Although the enzyme stability could be increased by supplementing with *myo*-inositol in the culture medium (11), obtaining MIOX with high stability and activity remains a huge challenge. Therefore, based on TFB-GA output, we screened MIOX superior mutant strains with the efficient production of GA. In many cases, the lack of structural information required random methods such as error-prone polymerase chain reaction (PCR) and whole-cell mutagenesis to generate sequence/structural diversity (31–33). Thus, we performed error-prone PCR amplification of the *miox* coding sequence in the pRSF-*miox-ino1-udh* (pRSF-MIU) plasmid and identified 84 *miox*-mutated transformants (M1–M84) (see method 4.6).

To evaluate the ability of these variants to produce GA, we transformed the TFB-GA-RBS3 and the control plasmid pRSF-MIU or mutant plasmid pRSF-M-*miox-ino1-udh* (pRSF-M-MIU) into *E. coli* BL21 (DE3) cells. The mutant strains (M1–M84) were screened in 96-well plates using TFB-GA output (see “Screening of GA high-titer strains based on TFB-GA and *miox* mutation” in Materials and Methods). Here, to eliminate the detection deviation, we defined that the mutant strain was meaningless to improve GA production when the TFB-GA output ratio of the mutant strain and control strain was less than 3 (Fig. 2A). The results showed that the TFB-GA output ratios of the M6 and M33 mutants were 4.6 and 5.2, respectively (Fig. 2A). It indicated that the M6 and M33 mutants could produce more GA. Subsequently, the M6, M33, and control strains were fermented to detect GA production. The results showed that the GA titers were 0.33, 0.42, and 0.325 g/L in M6, M33, and control strains, respectively (Fig. 2B and C). The GA titer of M33 was 1.3 times higher than that of the control strain at 73 h (Fig. 2C), and the *miox* of M33 was mutated from “A” to “G” at position 616. Additionally, although the TFB-GA output of the M6 mutant was much higher than that of the control strain, the GA titer was not significantly altered. It indicated that the cell systems were complex. Therefore, real-time

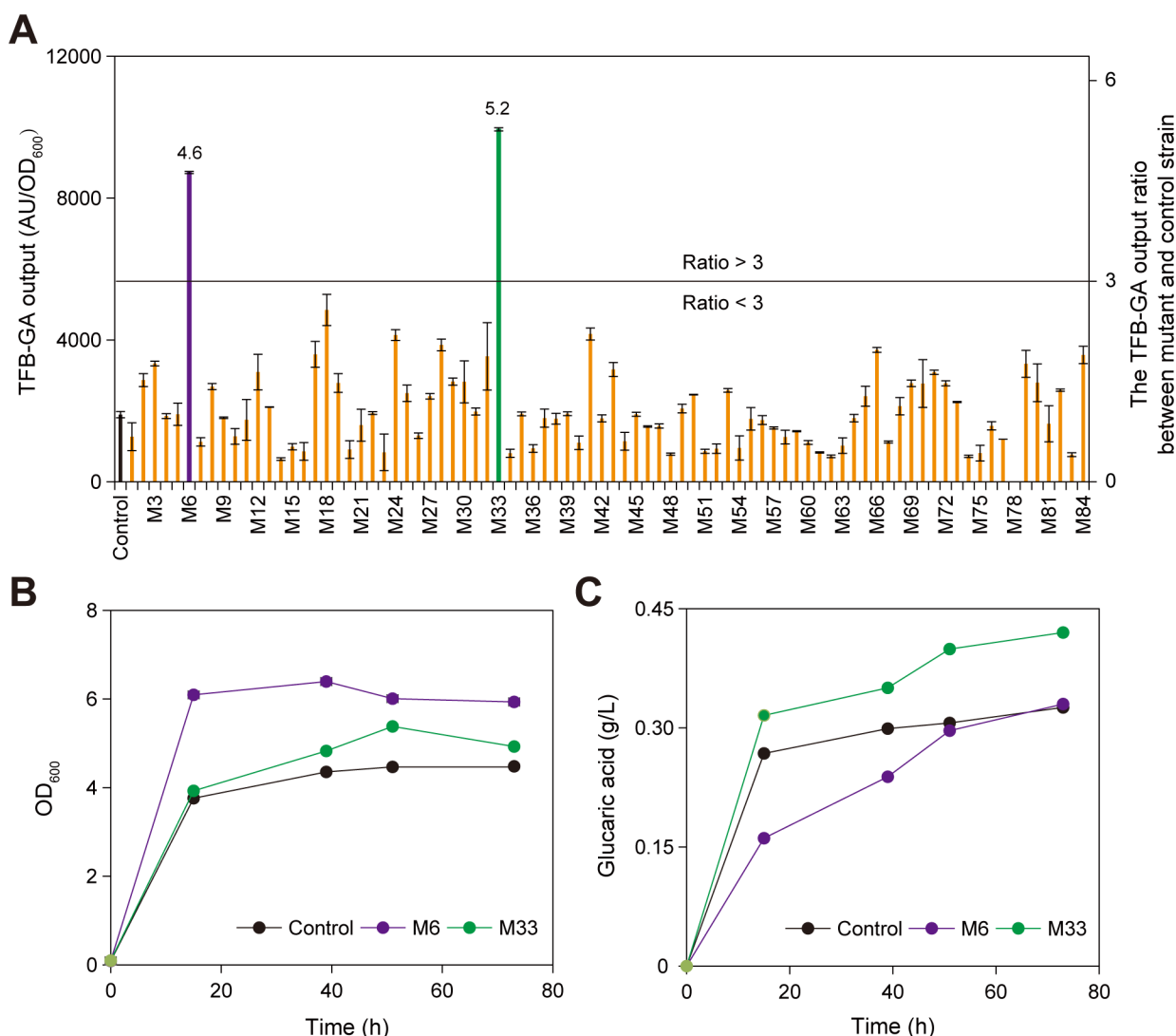


FIG 2 Improvement of the GA titer based on *mioX* mutation. (A) Analysis of the difference of GA production caused by *mioX* mutation based on TFB-GA output. The control represents *E. coli* BL21 (DE3) with GA production plasmid pRSF-MIU. The M6 and M33 represent *E. coli* BL21 (DE3) with GA production mutant plasmid pRSF-M-MIU. The TFB-GA output of M6 and M33 was higher than that of the control strain, and they were selected for further fermentation to determine the GA titer. (B) The optical density at 600 nm of control, M6, and M33 strains. (C) The GA titer of control, M6, and M33 strains. Data represent the mean and standard deviation for two replicates.

and accurate evaluation of intracellular target metabolite concentrations remains a huge challenge.

Establishment of glucaric acid bioselector and SUMO-MIOX to further improve glucaric acid production

Although we identified a suitable MIOX mutant, GA production remained low in a shake flask fermentation because of low MIOX stability and activity. At present, many studies have shown that SUMO can be used as a molecular chaperone to increase protein expression, promote correct protein folding, enhance tolerance to heat and protein degradation, and help maintain protein stability (34, 35). Therefore, we used SUMO fusion to express *mioX* for optimizing MIOX stability to improve GA production. In addition, the cells with a poor ability decreased GA production. Thus, to prevent low-performing cells from wasting nutrients and to improve GA production, we constructed the GA bioselector (pJKR-H-*cdaR*-RBS3-*tetA*) based on the PopQC approach

(27, 36), including the TetA and a flexible linker sequence encoding (Gly-Gly-Gly-Ser)₄ between TetA and sfGFP to facilitate the proper folding of each protein (37). For GA-producing cells, CdaR was activated and increased the expression of P_{gudP}-controlled genes, turning on the expression of the TetA-sfGFP fusion protein. Then, the TetA pumped the Tc out of the cell, thereby ensuring cell viability, while cells that did not produce GA would die due to Tc buildup (Fig. 3A).

To evaluate whether there were cell-to-cell variations in GA-producing strains, the strain CTM-R3 (QC_{GA+}) and the control strain constitutively expressing sfGFP were used for fermentation. Strain QC_{GA+} was added to Tc at a final concentration of 20 mg/L. The results showed that the strain QC_{GA+} had a wider sfGFP fluorescence distribution than the control strain based on the flow cytometry at 36, 48, and 60 h (Fig. 3B through D). In addition, QC_{GA+} with Tc had higher sfGFP fluorescence intensity than QC_{GA+} without Tc at 36, 48, and 60 h (Fig. 3B through D). It also indicated that the variations of the GA production by QC_{GA+} with and without Tc were 1.77-, 1.94-, and 2.15-fold at 36, 48, and 60 h, respectively (Fig. 3E through G; Fig. S2). Correspondingly, the difference in sfGFP fluorescence was also greater at 36, 48, and 60 h (Fig. 3B through D). These results suggested that there were variations between cells in the production of GA. In the absence of Tc, cells that do not produce GA will waste resources (carbon sources and energy), resulting in inefficient synthesis of GA. In the presence of Tc, the cells producing GA will be enriched, and the variations between cells will be eliminated, which is expected to achieve efficient synthesis of GA.

Then, to evaluate the effect of the GA bioselector and the SUMO-MIOX fusion on the GA production under selection pressure, fermentation of the CM-R3, CM-SUMO-R3, CTM-R3, and CTM-SUMO-R3 strains was carried out in shake flasks with *myo*-inositol as substrate (see “Assay conditions for glucaric acid production” and “Fermentation conditions in shake flask” in Materials and Methods). Strains CTM-R3 and CTM-SUMO-R3 were added to Tc at a final concentration of 20 mg/L. The results showed that the GA titer was 0.72 g/L for CM-R3 at 60 h, 3.10 g/L for CM-SUMO-R3 at 48 h, 1.29 g/L for CTM-R3 at 48 h, and 3.33 g/L for CTM-SUMO-R3 at 48 h supplemented with 11-g/L *myo*-inositol (Fig. 4A; Fig. S3A and B). However, the GA titer was relatively low and insufficient (0.75 g/L) for CTM-SUMO-R3 following without *myo*-inositol (Fig. 4A; Fig. S3A and B). It indicated that the SUMO-MIOX fusion and *myo*-inositol were key factors for improving GA production. In addition, we found that strains CTM-R3 and CTM-SUMO-R3 with GA bioselector had more GA titer than CM-R3 and CM-SUMO-R3 without GA bioselector, respectively. It demonstrated that strains with GA bioselector were superior to the efficient production of GA under Tc selection pressure. However, the Tc concentration that was most conducive to the efficient production of GA remained to be optimized.

Finally, to determine the optimal Tc concentration, strain CTM-SUMO-R3 was used to perform shake flask fermentation using yeast extract-peptone (YP) medium supplemented with 11-g/L *myo*-inositol in 0-, 15-, 20-, and 25-mg/L Tc, respectively. The results showed that the GA titers were 3.19, 4.36, 3.33, and 2.91 g/L, respectively, at 60 h (Fig. 4B; Fig. S3C and D). This revealed that the optimal Tc concentration for efficient GA production was 15 mg/L. Thus, strain CTM-SUMO-R3 was selected for fed-batch fermentation in a 5-L bioreactor using YP medium supplemented with 11-g/L *myo*-inositol and 15-mg/L Tc.

Production of glucaric acid in fed-batch fermentation

To further confirm the increased GA production by the GA bioselector, the strain CTM-SUMO-R3 and the control strain CTM-SUMO-G10 were selected to perform fed-batch fermentation using *myo*-inositol as the major substrate for supporting MIOX stability in a 5-L bioreactor (see “Fermentation conditions in a 5-L bioreactor” in Materials and Methods). Because oxygen was essential for MIOX, we maintained ventilation at 1.5 vvm, agitation at 400 rpm, and dissolved oxygen at >30%. The results showed that the GA titer of strain CTM-SUMO-R3 reached a maximum of 5.52 g/L, and the concentration of *myo*-inositol was decreased by about 16% from the initial concentration of 11.0 to

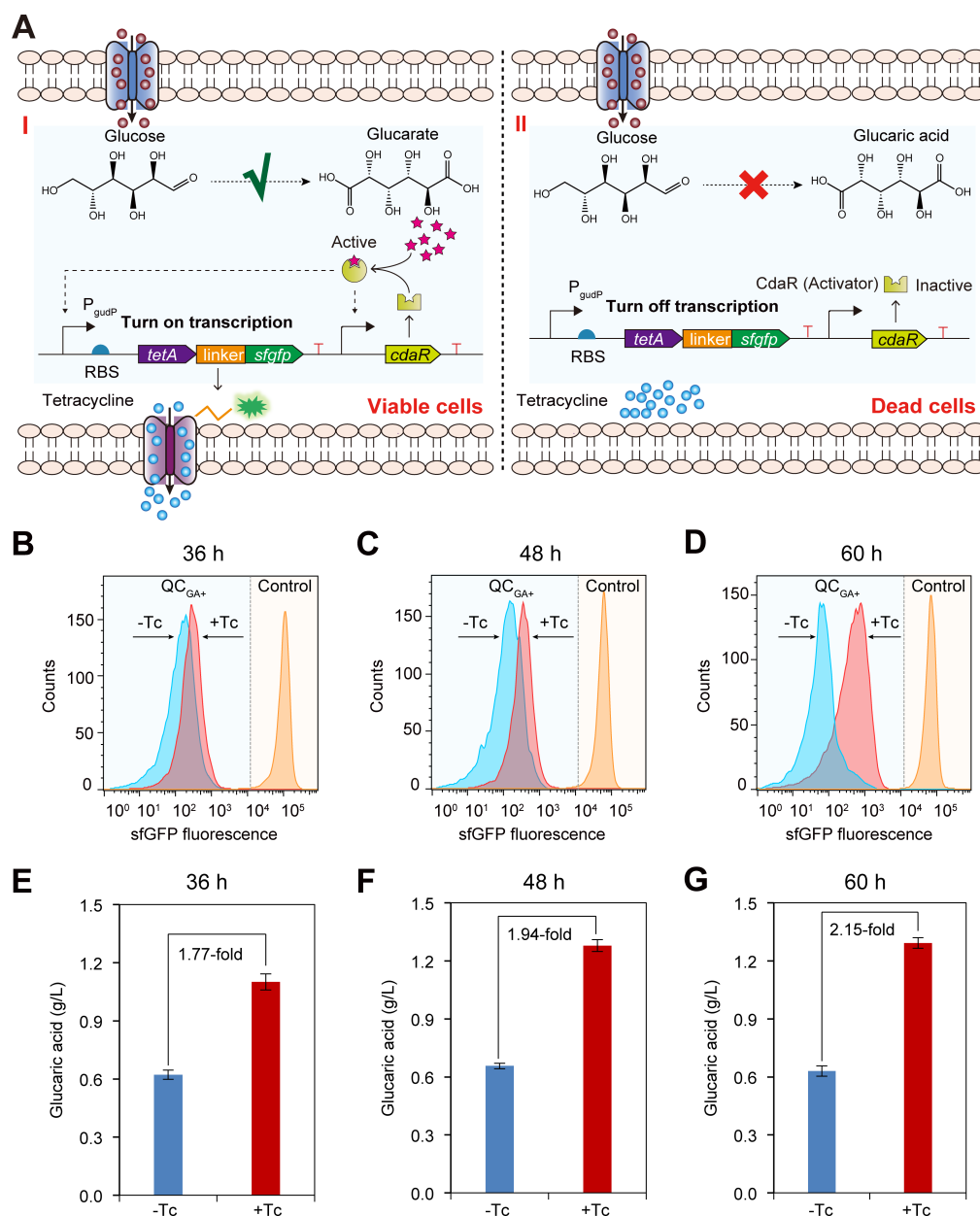


FIG 3 Construction of the GA bioselector and flow cytometry suggests cell-to-cell variation in GA production. (A) The working principle of the GA bioselector: (I) when cells produce GA, the CdaR is activated by binding to the GA, turning on the expression of the TetA-sfGFP fusion protein, and the TetA pumps Tc out of the cell to ensure cell viability; (II) when cells do not produce GA, the CdaR is inactive, turning off the expression of the TetA-sfGFP, and the cells will die due to Tc pressure. The sfGFP fluorescence intensity of strain QC_{GA+} (expressing both a GA biosynthetic pathway and a GA-responsive biosensor, sfGFP as output) cultivated with (red) or without (blue) PopQC pressure Tc and the control strain (expressing a constitutive sfGFP reporter) was analyzed by the flow cytometry at 36 h (B), 48 h (C), and 60 h (D). A wider distribution of sfGFP fluorescence was apparent in strain QC_{GA+} relative to the control strain, suggesting variation in GA production. A shift to higher sfGFP fluorescence was noted with the addition of selection pressure, suggesting an increase in both GA titer and the number of high-performing variants at 36 h (E), 48 h (F), and 60 h (G). Data represent the mean and standard deviation for two replicates.

9.2 g/L at 65 h, with a maximal optical density at 600 nm (OD₆₀₀) value of 3.0 achieved at 24 h (Fig. 5). However, the GA titer of control strain CTM-SUMO-G10 reached only 3.12 g/L, and the *myo*-inositol concentration decreased by about 9.3% at 60 h, reaching

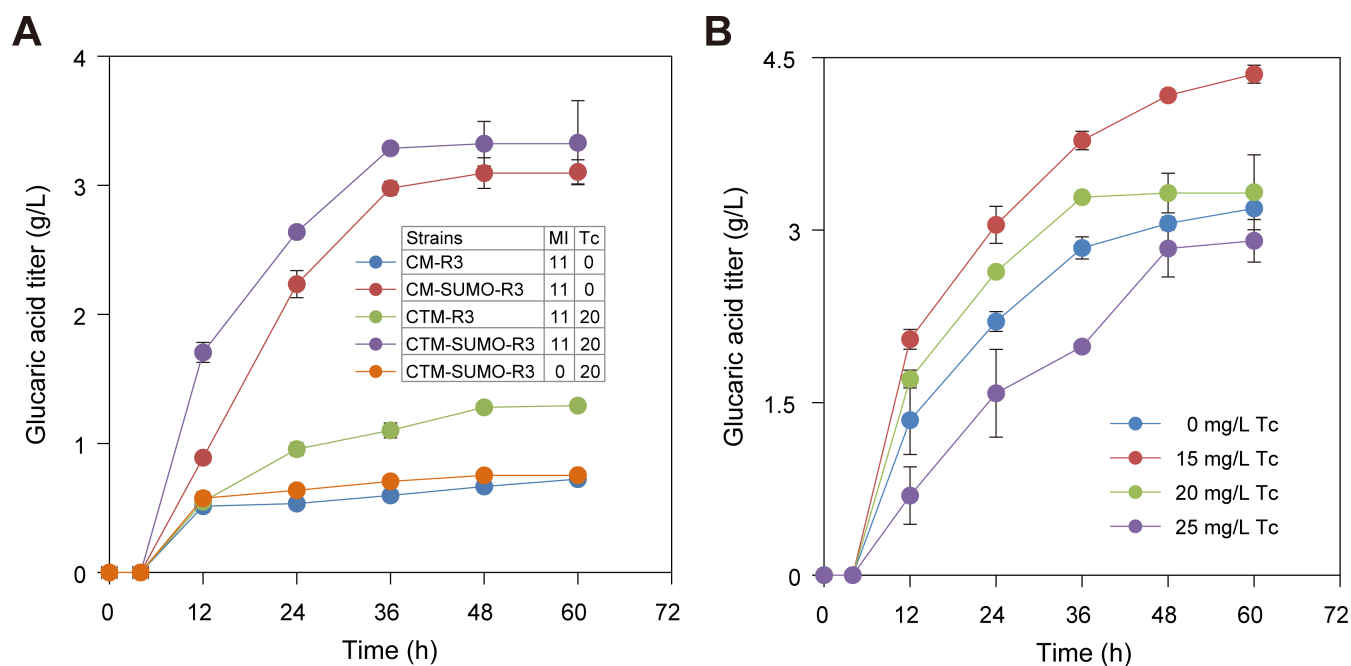


FIG 4 Improvement of GA production based on the GA bioselector. (A) The effects of SUMO, *myo*-inositol, and GA bioselector on GA production. The concentration of *myo*-inositol and Tc was 11 g/L and 20 mg/L, respectively. (B) The effect of various Tc concentrations on GA production by strain CTM-SUMO-R3. Data represent the mean and standard deviation for two replicates. CM-R3, strain *E. coli* BL21 (DE3) containing GA biosensor pJKR-H-*cdaR*-RBS3 and GA production plasmid pRSF-MIU; CM-SUMO-R3, strain *E. coli* BL21 (DE3) containing GA biosensor pJKR-H-*cdaR*-RBS3 and GA production plasmid pRSF-sumo-MIU; CTM-R3, strain *E. coli* BL21 (DE3) containing GA bioselector pJKR-H-*cdaR*-tetA-RBS3 and GA production plasmid pRSF-MIU; CTM-SUMO-R3, strain *E. coli* BL21 (DE3) containing GA bioselector pJKR-H-*cdaR*-tetA-RBS3 and GA production plasmid pRSF-sumo-MIU; MI, *myo*-inositol; Tc, tetracycline.

a maximum OD₆₀₀ value of 2.1 at 24 h (Fig. S4). This implied that RBS could fine-tune the performance of the GA bioselector to control the GA production. Specifically, RBS could regulate the TetA expression, which affected the amount of Tc pumped out of cells, and ultimately controlled the GA titer by adjusting the elimination degree of cell-to-cell variation (Fig. 3A). Therefore, the RBS modification in the GA bioselector was conducive to the efficient biosynthesis of GA.

DISCUSSION

TFBs responding to small-molecule inducers are receiving increasing attention (19, 38). Based on TFB output, real-time quantitative detection of GA can be achieved *in vivo*. This eliminates the need for high-performance liquid chromatography (HPLC) and other time-consuming methods with complex sample processing. However, the previously reported upper limit of TFB-GA was 3.15 g/L (22). This can only detect the GA production within 36 h in this study, which may not be sufficient for large-scale production of GA. Increasing the upper limit can be achieved by improving the TFB-GA output at high concentrations of GA. Our previous studies had shown that RBS could improve TFB-GA output by controlling protein expression and folding (23). Therefore, we designed the RBS library to improve the upper limit of TFB-GA, suggesting the TFB-GA-RBS3 had a higher upper limit (40 g/L) than TFB-GA-G10 (20 g/L). The possible reason was that the expression level and misfolding rate of sfGFP regulated by G10 were lower and higher than those of sfGFP controlled by RBS3, respectively. The misfolded sfGFP could not produce fluorescence. Thus, the TFB-GA-G10 output was lower than that of TFB-GA-RBS3 at a high concentration of GA, which ultimately led to the lower upper limit of TFB-GA-G10 than that of TFB-GA-RBS3. It indicated that the TFB-GA-RBS3 was conducive to real-time monitoring of large-scale GA production and efficient screening of GA high-performance production strains.

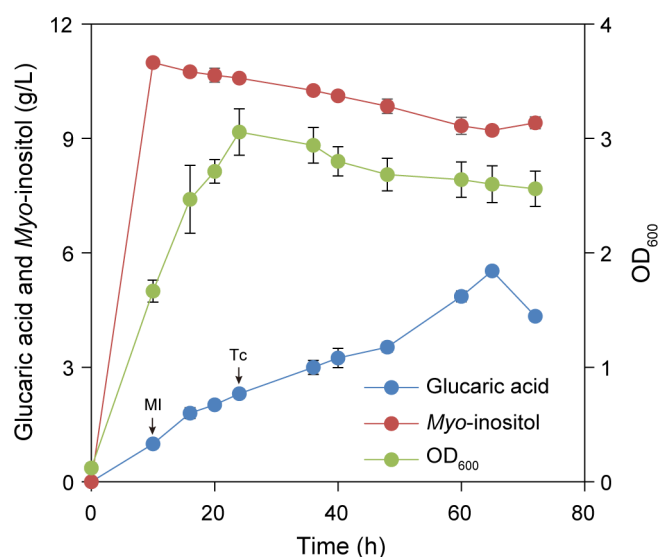


FIG 5 Fed-batch fermentation of strain CTM-SUMO-R3. The highest titer was achieved in the 5-L bioreactor with an aeration rate of 1.5 vvm and 400-rpm agitation in YP medium. Data represent the mean and standard deviation for two replicates. MI, myo-inositol; Tc, tetracycline.

The GA titer was still low due to the low stability and activity of MIOX in *E. coli*. To improve the activity of MIOX, we performed a *miox* random mutation to obtain a large mutant library. However, screening the GA-efficient production strains was time-consuming and costly by HPLC. Therefore, we used the superior TFB-GA for screening the mutant strains of GA-efficient production based on fluorescence output. We found that the TFB-GA output of M6 and M33 mutant strains was higher than that of the control strain, suggesting that the GA titer of M6 and M33 was theoretically higher than that of the control strain. However, the best M33 mutant strain only showed a 1.3-fold increase in GA titer. The possible reason was that the complex cell environment and the existing potential energy of GA concentration led to the significant difference of TFB-GA output at the same GA concentration *in vitro* and *in vivo*, indicating that the determination of GA production *in vivo* was unreliable through the TFB-GA output. This is a common problem for TFB accurately detecting target products *in vivo*. To solve this challenge, a novel method for real-time and precise detection of GA *in vivo* using TFB-GA output will be established based on deep learning in the future (39, 40), and the internal mechanism of TFB-GA output and GA production will be intelligently analyzed by embedding an algorithm into the neural network (41).

To increase GA production, we designed a GA bioselector for screening the GA-efficient biosynthesis cells based on TFB-GA and Tc efflux pump protein TetA. We found that the addition of Tc inhibited cell growth of the CTM-R3 strain compared to the CTM-SUMO-R3 strain (Fig. 4A; Fig. S3A). One of the main reasons was that the GA production of CTM-R3 was lower than that of CTM-SUMO-R3, resulting in less TetA expression in the CTM-R3 strain. Thus, Tc could not all be pumped out of the cells, leading to cell death, thereby reducing the absorbance at 600 nm. Through this approach, only GA-producing cells could survive and avoid the waste of nutrients and resources of GA-free cells.

The constructed GA bioselector had great potential to improve the GA titer. However, the tolerance of *E. coli* cells to GA remained still unclear. Here, we determined the tolerance based on cell growth. We added various neutralized GA concentrations to the culture medium. The effect of many GA concentrations on cell growth was then determined by measuring the absorbance at 600 nm. The results showed that the *E. coli* BL21 cells could tolerate 50 g/L of neutralized GA (Fig. S5), suggesting that *E. coli* had high GA tolerance and was suitable for large-scale GA production. To expand GA production, we performed fed-batch fermentation using strain CTM-SUMO-R3 in a

5-L bioreactor. The results showed that the titer of GA was increased to 5.52 g/L, the highest titer reported so far in *E. coli*. Additionally, the production speed of GA reached 0.085 g/L/h, which was higher than the previous report (0.074 g/L/h) (15). Therefore, it had great potential to realize the industrial production of GA using strain CTM-SUMO-R3 combined with various metabolic engineering strategies.

In conclusion, we optimized the upper limit of TFB-GA based on the RBS library, reaching 40-g/L GA. It showed that RBS was a key factor in regulating the detection limit of TFBs. The GA production was then increased to 0.42 g/L based on MIOX mutation. Subsequently, to further improve the GA titer, we constructed a GA biosensor based on TFB-GA and TetA and fused SUMO to MIOX for enhancing its stability and activity. In addition, the optimal screening concentration of Tc was 15 mg/L. These strategies led to a GA titer of 4.36 g/L in a shake flask fermentation for CTM-SUMO-R3 strain using 11-g/L *myo*-inositol as substrate. Finally, we fermented the CTM-SUMO-R3 strain in a 5-L bioreactor and achieved a GA titer of 5.52 g/L.

MATERIALS AND METHODS

Strains and culture conditions

All strains used in this study are listed in Table S3. *E. coli* JM109 and *E. coli* BL21 (DE3) cells were used for plasmid cloning and protein expression, respectively. Luria-Bertani (LB) medium, consisting of NaCl (10 g/L), peptone (10 g/L), and yeast extract (5 g/L), was used for seed cultures at 37°C and plasmid amplification. YP medium, consisting of yeast extract (10 g/L) and peptone (20 g/L), supplemented with 11-g/L *myo*-inositol during the logarithmic growth period was used to provide a carbon source for GA production. M9 medium, consisting of Na₂HPO₄ (6.78 g/L), KH₂PO₄ (3.0 g/L), NaCl (0.5 g/L), MgSO₄·7H₂O (0.5 g/L), CaCl₂ (0.011 g/L), and NH₄Cl (1.0 g/L), supplemented with 8-g/L glucose was used to wash the bacteria for fluorescence intensity assessment to reduce the error caused by the color of the YP medium. The final concentration of ampicillin, kanamycin, and isopropyl β-D-1-thiogalactopyranoside (IPTG) employed in this study was 100 µg/mL, 50 µg/mL, and 1 mM, respectively.

Plasmid construction

All plasmids and primers used in this study are listed in Tables S3 and S4, respectively. To construct the biosynthetic pathway of GA, the *miox*, *ino1*, and *udh* genes were synthesized and ligated into plasmid pRSFDuet-1 by Genewiz (Suzhou, China). The final plasmid was named pRSF-MIU. To further increase the GA production, the plasmid pRSF-*sumo-miox-ino1-udh* was constructed through *Nco* I/*Bam* H I digestion and Gibson assembly methods using primers F/R-*sumo* and F/R-*miox*-2, and the ligation products were introduced into *E. coli* JM109 for screening by colony PCR and Sanger sequencing. Moreover, there was no termination codon between the *sumo* and *miox* genes. All the sequences of GA biosynthetic pathway genes and plasmids are provided in Table S5.

To analyze the effect of mutant *miox* (M-*miox*) on GA titer, the Mn²⁺ was added into the conventional PCR system using the pRSF-MIU plasmid as a template to increase the mutation frequency in the PCR amplification process, and random mutagenesis of the *miox* gene was performed at the optimum frequency to obtain *miox* mutant libraries. The *miox* gene was amplified with primers F/R-*miox*-1. The pRSF-MIU was digested with *Nco* I and *Bam* H I, and the pRSF-*ino1-udh* DNA fragment was recovered. Then, the M-*miox* gene was ligated into pRSF-*ino1-udh* using T4 DNA ligase. The ligation product was introduced into *E. coli* JM109 and cultured overnight at 37°C. The colonies grown on the plate were placed separately in 50-mL LB medium supplemented with 50-µg/mL kanamycin, cultured at 37°C with shaking at 200 rpm for 8 h, and the plasmid pRSF-M-MIU was extracted.

The pJKR-H-*cdaR* plasmid for the TFB-GA was purchased from Addgene (#62557). In addition to G10, the RBS calculator (28, 42)-designed six RBSs were cloned into

TFB-GA vectors. The primer pairs F/R-RBS1-6 were designed based on the different RBS sequences, and the pJKR-H-*cdaR* plasmid was used as the template for whole-plasmid PCR. Plasmids pJKR-H-*cdaR*-RBSs ($s = 1-6$) were constructed through *Dpn* I digestion at 37°C for 30 min, and the digestion products were transformed into *E. coli* JM109 cells for screening by colony PCR and Sanger sequencing. To establish an efficient high-through screening method of GA, we constructed the GA bioselector based on TFB-GA-G10/RBS3, TetA, and linker sequences synthesized by Genewiz using the Gibson assembly method. The final plasmids were named pJKR-H-*cdaR*-*tetA*-G10 and pJKR-H-*cdaR*-*tetA*-RBS3.

Fluorescence assays

E. coli BL21 (DE3) cell growth was monitored by measuring OD₆₀₀. Before fluorescence measurement, cultures were diluted to ensure the OD₆₀₀ value was ~0.5. Fluorescence intensity of sfGFP (TFB-GA output) was measured by a Biotek HT plate reader (Winooski, VT, USA) using the same settings, with excitation at 485/20, emission at 528/20, a temperature of 37°C, a gain of 60, and fast shaking. TFB-GA output was measured in arbitrary units (AU), and the OD was determined by absorbance. For a given measurement, normalized TFB-GA output was determined by dividing the observed fluorescence by the OD₆₀₀. The ratio of TFB-GA output to OD₆₀₀ was used to compensate for the changes in cell density over time and between experiments (AU/OD).

Optimization of TFB-GA detection limit

To improve the TFB-GA upper limit, plasmids pJKR-H-*cdaR*-G10/RBS1-6 were transformed into *E. coli* BL21 (DE3). The correct transformants were cultured in LB medium overnight and then inoculated at 1% vol into a 96-well plate containing M9 medium supplemented with 8-g/L glucose. After 4 h, expression of sfGFP was induced by adding various GA concentrations *in vitro*. To assess the upper limit of TFB-GA-G10 and TFB-GA-R3, we expanded the GA concentration and made a GA stock solution at a concentration of 200 g/L. A detailed description is shown in Table S6. TFB-GA-G10 and TFB-GA-R3 were cultured in LB medium overnight and then inoculated at 1% vol into a 15-mL Eppendorf (EP) tube containing 3 mL of mother liquor. After 4 h, various GA concentrations were added to the EP tube. The normalized fluorescence intensity was then measured as described above.

Percentage analysis of cell death

The CTM-SUMO-R3 and CTM-SUMO-G10 cells induced by 0-, 20-, 50-, and 100-g/L GA at 12 h were collected by centrifugation (4°C, 5 min, 5,000 rpm) and then washed twice with cold PBS and resuspended to an OD₆₀₀ of 0.1. Then, 5-μL 2-mg/mL PI Red Dead nucleic acid stain was added to the 95-μL sample. After incubating the sample at room temperature, avoiding light for 30 min, the sample droplets were added to the slide, and the coverslip was covered. Next, the cell fluorescence was measured by flow cytometry within an hour. The phycoerythrin PI channel of a BD FACS Aria III instrument was used for determination. The voltage was set to 520 V according to the TFB-GA output induced by 0-g/L GA at 12 h. Each sample was recorded 20,000 counts at a flow rate of 0.01 mL/s. All data were exported in FCS format and processed using FlowJo software (FlowJo, LLC).

Screening of GA high-titer strains based on TFB-GA and *miox* mutation

To achieve the high-throughput screening of GA high-titer strains, the TFB-GA and plasmid pRSF-MIU or pRSF-M-MIU were transformed into *E. coli* BL21 (DE3) cells for obtaining the control strain and the mutation strains (Ms) ($s = 1-84$). After overnight incubation, the control strain and the 84 mutant strains were inoculated into 15-mL centrifuge tubes containing 2-mL LB medium supplemented with 100-μg/mL ampicillin and 50-μg/mL kanamycin, and cultured for 10 h. Then, a 200-μL aliquot was then transferred into a 15-mL centrifuge tube containing 2-mL M9 medium supplemented with 3-g/L glucose, 1-g/L *myo*-inositol, 100-μg/mL ampicillin, and 50-μg/mL kanamycin.

After 4 h, cultures were induced with 0.1-M IPTG and grown overnight at 30°C. The broth was diluted to an OD₆₀₀ value of ~0.5 and transferred to a 96-well plate. Finally, the normalized fluorescence intensity was measured as described above. The remaining bacteria were added to the same volume of 30% glycerol and stored at –80°C.

Assay conditions for glucaric acid production

Cell culture samples (1 mL) were collected by centrifugation at 12,000 × *g* for 10 min, and supernatants were used for detection following passage through a 0.22-μm filter membrane. Metabolites such as *myo*-inositol and GA were measured by HPLC (Hitachi, Japan) equipped with an HPX-87H column (Bio-Rad, Hercules, CA, USA). The mobile phase was 5-mM H₂SO₄; the flow rate was 0.6 mL/min; and the column temperature was maintained at 30°C. *Myo*-inositol and GA were measured by a refractive index detector. The reported *myo*-inositol and GA concentrations were the average of two samples, and the error bars represent the means ± standard deviations.

Fermentation conditions in shake flask

For fermentation in shake flasks, JM109 strains containing GA biosensor and production pathway plasmids stored at –80°C in 30% glycerol were first activated on LB agarose plates supplemented with appropriate antibiotics. After cultivating overnight, plasmids were extracted separately and transformed into *E. coli* BL21 (DE3) cells. Single colonies were cultivated overnight in LB medium at 37°C and diluted 1:100 into 250-mL shake flasks containing 50-mL YP medium. When the OD₆₀₀ value reached 0.8 following incubation at 37°C, 1-mM IPTG was added, and culturing continued at 30°C. For TFB-GA output measurements, cultures were diluted 1:100 into M9 medium after growth overnight in LB medium and incubated at 37°C. After 6 h, the 150-μL sample of logarithmic growth cells was transferred to the 96-well plate, and the normalized TFB-GA output was measured as described above. Strains BL21-(pJKR-H-*cdaR*-RBS3)-(pRSF-MIU) (CM-R3), BL21-(pJKR-H-*cdaR*-RBS3)-(pRSF-sumo-MIU) (CM-SUMO-R3), BL21-(pJKR-H-*cdaR*-*tetA*-RBS3)-(pRSF-MIU) (CTM-R3), and BL21-(pJKR-H-*cdaR*-*tetA*-RBS3)-(pRSF-sumo-MIU) (CTM-SUMO-R3) were fermented using the above methods.

Fermentation conditions in a 5-L bioreactor

GA production was conducted for culturing cells at 37°C and for inducing gene expression at 30°C with 1.5-vvm aeration, 400-rpm agitation, and 1-mM IPTG in a 5-L bioreactor (Baoxing, Shanghai, China). The fermentation strategy involved initial culturing at 37°C. During the first stage, 2% of the working volume seed culture was inoculated into the bioreactor and appropriate antibiotics were added. During the second stage, IPTG and 11-g/L *myo*-inositol were added into the YP medium once the OD₆₀₀ value reached ~1, and the culturing temperature was decreased to 30°C. During the third stage, tetracycline at a final concentration of 15 mg/L was added to the bioreactor. The pH was maintained at 7 by online measurement using a pH sensor and the addition of 2-M NaOH. The aeration and agitation rates varied according to the different conditions.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (32301218 and 31600070), the Zhejiang Provincial Natural Science Foundation of China (LZ22C200001), the Scientific Research Development Foundation of Zhejiang A&F University (2023LFR020), and the Open Project Program of State Key Laboratory of Food Science and Resources, Jiangnan University (SKLF-KF-202309).

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FUNDING

Funder	Grant(s)	Author(s)
MOST National Natural Science Foundation of China (NSFC)	32301218	Nana Ding
MOST National Natural Science Foundation of China (NSFC)	31600070	Lianghong Yin
Zhejiang Provincial Natural Science Foundation of China	LZ22C200001	Lianghong Yin
Scientific Research Development Foundation of Zhejiang A&F University	2023LFR020	Nana Ding
Open Project Program of State Key Laboratory of Food Science and Resources, Jiangnan University	SKLF-KF-202309	Nana Ding

DATA AVAILABILITY

Flow cytometry data for the effect of glucaric acid concentrations on the percentage of cell death and the cell-to-cell variation in GA production were deposited at Flow Repository and are directly accessible at <http://flowrepository.org/id/FR-FCM-Z7CD> and <http://flowrepository.org/id/FR-FCM-Z7CE>, respectively.

ADDITIONAL FILES

The following material is available [online](#).

Supplemental Material

Supplemental material (AEM00149-24-s0001.pdf). Figures S1 to S5; Tables S1 to S6.

REFERENCES

- Petroll K, Care A, Bergquist PL, Sunna A. 2020. A novel framework for the cell-free enzymatic production of glucaric acid. *Metab Eng* 57:162–173. <https://doi.org/10.1016/j.ymben.2019.11.003>
- Werpy TA, Petersen GE. 2004. Top value added chemicals from biomass, volume 1: results of screening for potential candidates from sugars and synthesis gas. Washington, DC US Department of Energy
- Chen LZ, Huang SL, Hou J, Guo XP, Wang FS, Sheng JZ. 2020. Cell-based and cell-free biocatalysis for the production of D-glucaric acid. *Biotechnol Biofuels* 13:203. <https://doi.org/10.1186/s13068-020-01847-0>
- Walaszek Z. 1990. Potential use of D-glucaric acid derivatives in cancer prevention. *Cancer Lett* 54:1–8. [https://doi.org/10.1016/0304-3835\(90\)90083-a](https://doi.org/10.1016/0304-3835(90)90083-a)
- Iglesias J, Martínez-Salazar I, Maireles-Torres P, Martín Alonso D, Mariscal R, López Granados M. 2020. Advances in catalytic routes for the production of carboxylic acids from biomass: a step forward for sustainable polymers. *Chem Soc Rev* 49:5704–5771. <https://doi.org/10.1039/d0cs00177e>
- Choi H, Soland NE, Buss BL, Honeycutt NC, Tomashek EG, Haugen SJ, Ramirez KJ, Miscall J, Tan ECD, Smith TN, Saboe PO, Karp EM. 2022. Separation of bio-based glucaric acid via antisolvent crystallization and azeotropic drying. *Green Chem* 24:1350–1361. <https://doi.org/10.1039/D1GC03984A>
- Zhang QZ, Wan ZH, Yu IKM, Tsang DCW. 2021. Sustainable production of high-value gluconic acid and glucaric acid through oxidation of biomass-derived glucose: a critical review. *J Clean Prod* 312:127745. <https://doi.org/10.1016/j.jclepro.2021.127745>
- Teržan J, Sedminek A, Lavrič Ž, Grilc M, Huš M, Likozar B. 2023. Selective oxidation of biomass-derived carbohydrate monomers. *Green Chem* 25:2220–2240. <https://doi.org/10.1039/D2GC04623G>
- Pellis A, Cantone S, Ebert C, Gardossi L. 2018. Evolving biocatalysis to meet bioeconomy challenges and opportunities. *New Biotechnol* 40:154–169. <https://doi.org/10.1016/j.nbt.2017.07.005>
- Ölçücü G, Klaus O, Jaeger K-E, Drepper T, Krauss U. 2021. Emerging solutions for *in vivo* biocatalyst immobilization: tailor-made catalysts for

- industrial biocatalysis. *ACS Sustainable Chem Eng* 9:8919–8945. <https://doi.org/10.1021/acssuschemeng.1c02045>
11. Shiue E, Prather KLJ. 2014. Improving D-glucaric acid production from MYO-Inositol in *E. coli* by increasing MIOX stability and myo-Inositol transport. *Metab Eng* 22:22–31. <https://doi.org/10.1016/j.ymben.2013.12.002>
 12. Moon TS, Yoon S-H, Lanza AM, Roy-Mayhew JD, Prather KLJ. 2009. Production of glucaric acid from a synthetic pathway in recombinant *Escherichia coli*. *Appl Environ Microbiol* 75:589–595. <https://doi.org/10.1128/AEM.00973-08>
 13. Zheng S, Hou J, Zhou Y, Fang H, Wang TT, Liu F, Wang FS, Sheng JZ. 2018. One-pot two-strain system based on glucaric acid biosensor for rapid screening of myo-inositol oxygenase mutations and glucaric acid production in recombinant cells. *Metab Eng* 49:212–219. <https://doi.org/10.1016/j.ymben.2018.08.005>
 14. Qu YN, Yan HJ, Guo Q, Li JL, Ruan YC, Yue XZ, Zheng WX, Tan TW, Fan LH. 2018. Biosynthesis of D-glucaric acid from sucrose with routed carbon distribution in metabolically engineered *Escherichia coli*. *Metab Eng* 47:393–400. <https://doi.org/10.1016/j.ymben.2018.04.020>
 15. Su HH, Peng F, Ou XY, Zeng YJ, Zong MH, Lou WY. 2020. Combinatorial synthetic pathway fine-tuning and cofactor regeneration for metabolic engineering of *Escherichia coli* significantly improve production of D-glucaric acid. *New Biotechnol* 59:51–58. <https://doi.org/10.1016/j.nbt.2020.03.004>
 16. Reizman IMB, Stenger AR, Reisch CR, Gupta A, Connors NC, Prather KLJ. 2015. Improvement of glucaric acid production in *E. coli* via dynamic control of metabolic fluxes. *Metab Eng Commun* 2:109–116. <https://doi.org/10.1016/j.meten.2015.09.002>
 17. Doong SJ, Gupta A, Prather KLJ. 2018. Layered dynamic regulation for improving metabolic pathway productivity in *Escherichia coli*. *Proc Natl Acad Sci U S A* 115:2964–2969. <https://doi.org/10.1073/pnas.1716920115>
 18. Yu WW, Xu XH, Jin K, Liu YF, Li JH, Du GC, Lv XQ, Liu L. 2023. Genetically encoded biosensors for microbial synthetic biology: from conceptual frameworks to practical applications. *Biotechnol Adv* 62:108077. <https://doi.org/10.1016/j.biotechadv.2022.108077>
 19. Pham C, Stogios PJ, Savchenko A, Mahadevan R. 2022. Advances in engineering and optimization of transcription factor-based biosensors for plug-and-play small molecule detection. *Curr Opin Biotechnol* 76:102753. <https://doi.org/10.1016/j.copbio.2022.102753>
 20. Ding N, Zhou S, Deng Y. 2021. Transcription-factor-based biosensor engineering for applications in synthetic biology. *ACS Synth Biol* 10:911–922. <https://doi.org/10.1021/acssynbio.0c00252>
 21. Rogers JK, Guzman CD, Taylor ND, Raman S, Anderson K, Church GM. 2015. Synthetic biosensors for precise gene control and real-time monitoring of metabolites. *Nucleic Acids Res* 43:7648–7660. <https://doi.org/10.1093/nar/gkv616>
 22. Rogers JK, Church GM. 2016. Genetically encoded sensors enable real-time observation of metabolite production. *Proc Natl Acad Sci U S A* 113:2388–2393. <https://doi.org/10.1073/pnas.1600375113>
 23. Ding NN, Yuan ZQ, Zhang XJ, Chen J, Zhou SH, Deng Y. 2020. Programmable cross-ribosome-binding sites to fine-tune the dynamic range of transcription factor-based biosensor. *Nucleic Acids Res* 48:10602–10613. <https://doi.org/10.1093/nar/gkaa786>
 24. Liu X, Silverman AD, Alam KK, Iverson E, Lucks JB, Jewett MC, Raman S. 2020. Design of a transcriptional biosensor for the portable, on-demand detection of cyanuric acid. *ACS Synth Biol* 9:84–94. <https://doi.org/10.1021/acssynbio.9b00348>
 25. Zhou GJ, Zhang F. 2023. Applications and tuning strategies for transcription factor-based metabolite biosensors. *Biosensors (Basel)* 13:428. <https://doi.org/10.3390/bios13040428>
 26. Rugbjerg P, Sommer MOA. 2019. Overcoming genetic heterogeneity in industrial fermentations. *Nat Biotechnol* 37:869–876. <https://doi.org/10.1038/s41587-019-0171-6>
 27. Xiao Y, Bowen CH, Liu D, Zhang FZ. 2016. Exploiting nongenetic cell-to-cell variation for enhanced biosynthesis. *Nat Chem Biol* 12:339–344. <https://doi.org/10.1038/nchembio.2046>
 28. Salis HM, Mirsky EA, Voigt CA. 2009. Automated design of synthetic ribosome binding sites to control protein expression. *Nat Biotechnol* 27:946–950. <https://doi.org/10.1038/nbt.1568>
 29. Zhao YY, Zuo FY, Shu QX, Yang XY, Deng Y. 2023. Efficient production of glucaric acid by engineered *Saccharomyces cerevisiae*. *Appl Environ Microbiol* 89:e0053523. <https://doi.org/10.1128/aem.00535-23>
 30. Zhao YY, Li J, Su RF, Liu YL, Wang J, Deng Y. 2021. Effect of magnesium ions on glucaric acid production in the engineered *Saccharomyces cerevisiae*. *J Biotechnol* 332:61–71. <https://doi.org/10.1016/j.jbiotec.2021.03.020>
 31. Choudhury A, Fenster JA, Fankhauser RG, Kaar JL, Tenaillon O, Gill RT. 2020. CRISPR/Cas9 recombineering-mediated deep mutational scanning of essential genes in *Escherichia coli*. *Mol Syst Biol* 16:e9265. <https://doi.org/10.15252/msb.20199265>
 32. Wang YJ, Xue P, Cao MF, Yu TH, Lane ST, Zhao HM. 2021. Directed evolution: methodologies and applications. *Chem Rev* 121:12384–12444. <https://doi.org/10.1021/acs.chemrev.1c00260>
 33. Yang XW, Huo XJ, Tang YQ, Zhao MY, Tao Y, Huang JZ, Ke CR. 2023. Integrating enzyme evolution and metabolic engineering to improve the productivity of gamma-aminobutyric acid by whole-cell biosynthesis in *Escherichia coli*. *J Agric Food Chem* 71:4656–4664. <https://doi.org/10.1021/acs.jafc.2c07613>
 34. Hendriks IA, Vertegaal ACO. 2016. A comprehensive compilation of SUMO proteomics. *Nat Rev Mol Cell Biol* 17:581–595. <https://doi.org/10.1038/nrm.2016.81>
 35. Molfetta R, Petillo S, Cippitelli M, Paolini R. 2023. SUMOylation and related post-translational modifications in natural killer cell anti-cancer responses. *Front Cell Dev Biol* 11:1213114. <https://doi.org/10.3389/fcell.2023.1213114>
 36. Zhou SH, Alper HS, Zhou JW, Deng Y. 2023. Intracellular biosensor-based dynamic regulation to manipulate gene expression at the spatiotemporal level. *Crit Rev Biotechnol* 43:646–663. <https://doi.org/10.1080/07388551.2022.2040415>
 37. Edwards DT, LeBlanc M-A, Perkins TT. 2021. Modulation of a protein-folding landscape revealed by AFM-based force spectroscopy notwithstanding instrumental limitations. *Proc Natl Acad Sci U S A* 118:e2015728118. <https://doi.org/10.1073/pnas.2015728118>
 38. Ding NN, Zhang GK, Zhang LP, Shen ZY, Yin LH, Zhou SH, Deng Y. 2023. Engineering an AI-based forward-reverse platform for the design of cross-ribosome binding sites of a transcription factor biosensor. *Comput Struct Biotechnol J* 21:2929–2939. <https://doi.org/10.1016/j.csbj.2023.04.026>
 39. Cheng Y, Bi XY, Xu YM, Liu YF, Li JH, Du GC, Lv XQ, Liu L. 2023. Artificial intelligence technologies in bioprocess: opportunities and challenges. *Bioresour Technol* 369:128451. <https://doi.org/10.1016/j.biortech.2022.128451>
 40. Zhang XE, Liu CL, Dai JB, Yuan YJ, Gao CX, Feng Y, Wu B, Wei P, You C, Wang XW, Si T. 2023. Enabling technology and core theory of synthetic biology. *Sci China Life Sci* 66:1742–1785. <https://doi.org/10.1007/s11427-022-2214-2>
 41. Wei Z, Hua K, Wei L, Ma S, Jiang R, Zhang X, Li Y, Wong WH, Wang X. 2023. NeuronMotif: deciphering cis-regulatory codes by layer-wise demixing of deep neural networks. *Proc Natl Acad Sci U S A* 120:e2216698120. <https://doi.org/10.1073/pnas.2216698120>
 42. Chen RN, Liu YL, Zhong W, Hao XM, Mu TZ, Yang MH, Xing JM. 2022. Ribosome-binding sequences (RBS) engineering of key genes in *Escherichia coli* for high production of fatty alcohols. *Biotechnol Bioproc* E 27:615–623. <https://doi.org/10.1007/s12257-021-0354-0>