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# Engineering the Reductive TCA Pathway to Dynamically Regulate the Biosynthesis of Adipic Acid in *Escherichia coli*

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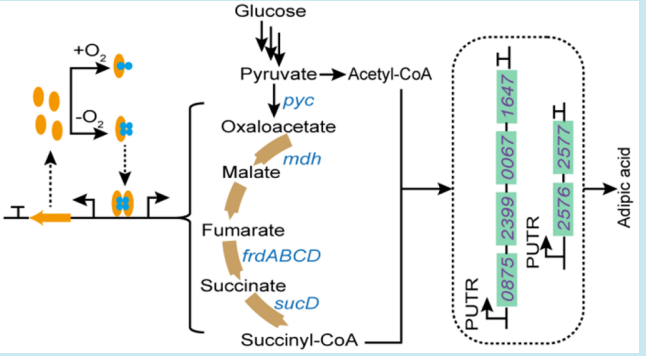
**ABSTRACT:** Adipic acid is a versatile aliphatic dicarboxylic acid. It is applied mainly in the polymerization of nylon-6,6, which accounts for 50.8% of the global consumption market of adipic acid. The microbial production of adipic acid avoids the usage of petroleum resources and the emission of harmful nitrogen oxides that are generated by traditional chemical synthetic approaches. However, in the fermentation process, the low theoretical yield and the usage of expensive inducers hinders the large-scale industrial production of adipic acid. To overcome these challenges, we established an oxygen-dependent dynamic regulation (ODDR) system to control the expression of key genes (*sucD*, *pyc*, *mdh*, and *frdABCD*) that could be induced to enhance the metabolic flux of the reductive TCA pathway under anaerobic conditions. Coupling of the constitutively expressed adipic acid synthetic pathway not only avoids the use of inducers but also increases the theoretical yield by nearly 50%. After the gene combination and operon structure were optimized, the reaction catalyzed by *frdABCD* was found to be the rate-limiting step. Further optimizing the relative expression levels of *sucD*, *pyc*, and *frdABCD* improved the titer of adipic acid 41.62-fold compared to the control strain Mad1415, demonstrating the superior performance of our ODDR system.




**KEYWORDS:** *adipic acid, biosensor, dynamic regulation, oxygen, organic acid*



## INTRODUCTION

Adipic acid is one of the most important dicarboxylic acids among the various commercial chemicals and is used as the monomers of nylon-6,6,[1] food additives,[2] and hot melt adhesives.[3] In the past ten years, the global market value of adipic acid has experienced significant growth. This uptrend is expected to continue at a global annual rate of 4%−5% over the next 10 years.[4] In industry, almost all of the adipic acid is produced on the basis of the oxidation of aromatics (e.g., ketone alcohol oil, KA oil), which was established by DuPont in the early 1930s.[5] However, such methods use cyclohexanone/cyclohexanol, which is derived from fossil fuels, and lead to a considerable amount of greenhouse gas ($NO_x$) emissions.[5,6] With increasing concerns about fossil fuels and environmental pollution, more attention is being focused on the sustainable green biosynthesis of adipic acid *via* renewable sources.[5,7]

A combined biological and chemical synthesis route was established in 1994,[8] during which renewable sources were bioconverted into adipic acid precursors, such as *cis*,*cis*-muconic acid[9] and glucaric acid.[10] These precursors were then further catalyzed into adipic acid in a hydrogenation process using the precious metal Pt as a catalyst.[9,11,12] However, the use of Pt greatly increases production costs. Recently, muconic acid reductase (MAR) has been proven to reduce *cis*,*cis*-muconic acid to adipic acid and has been applied for the full biological production of adipic acid.[13,14] However, the low titer and high price of *cis*,*cis*-muconic acid still hinder the large-scale industrial production of adipic acid. In addition, various biosynthetic routes that are independent of the chemical synthesis processes have been established in recent years, such as the reversal of the $\beta$-oxidation pathway[15,16] and the $\omega$-oxidation of the fatty acids pathway[1,17] and the $\alpha$-keto acid pathway.[18,19] However, these approaches are limited by the low adipic acid titer and the expensive precursors. For example, the reversal of the $\beta$-oxidation pathway resulted in only 36 mg/L adipic acid in *Escherichia coli* BL21(DE3).[16] Furthermore, using nondecarboxylative Claisen condensation, only 2.5 g/L adipic acid was obtained in a controlled bioreactor.[20] Even though it was reported in a recent study that it could reach 66 g/L adipic acid in *E. coli* through a one-pot biocatalytic route (similar to $\omega$-oxidation of fatty acids),

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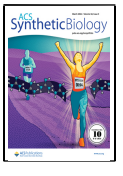




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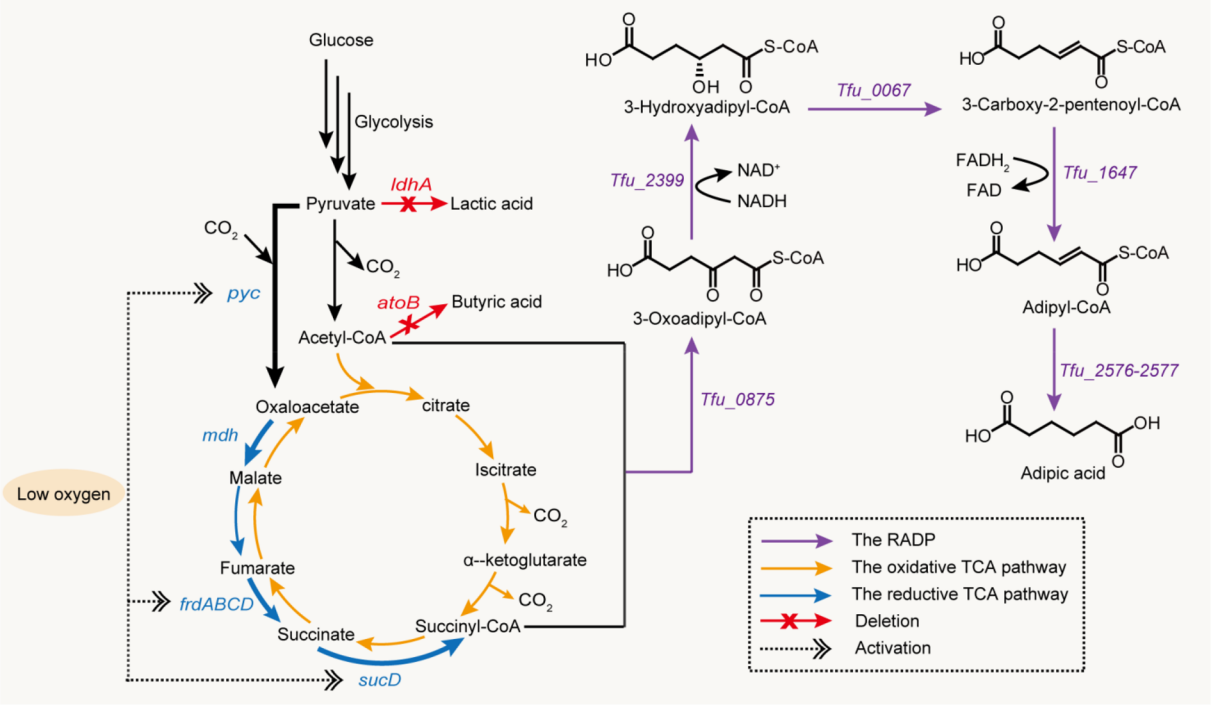


**Figure 1.** Regulation mechanisms of the ODDR system for the synthesis of adipic acid. Dashed double arrows represent the activation of the oxygen-dependent dynamic regulation (ODDR) system. Multiple arrows indicate multiple catalyzation steps. The blue arrows indicate the reductive TCA pathway controlled by the oxygen-dependent biosensor. The orange arrows indicate the oxidative TCA pathway. The purple arrows indicate the reversal of the adipic acid degradation pathway (RADP). The red arrows indicate knocked-out genes. The bold arrows indicate the genes expected to be overexpressed. *pyc*, pyruvate carboxylase; *mdh*, malate dehydrogenase; *frdABCD*, fumarate reductase; *sucD*, succinyl-CoA synthetase $\alpha$ subunit; *ldhA*, L-lactate dehydrogenase; *atoB*, acetyl-CoA C-acetyltransferase; *Tfu-0875*, $\beta$-ketothiolase; *Tfu-2399*, 3-hydroxyacyl-CoA dehydrogenase; *Tfu-0067*, 3-hydroxyadipyl-CoA dehydrogenase; *Tfu-1647*, 5-carboxy-2-pentenoyl-CoA reductase; *Tfu-2576−2577*, adipyl-CoA synthetase.

the high price of the biobased precursor $\varepsilon$-caprolactone still hinders industrialization.[21]

As one of the most efficient adipic acid synthetic pathways, the reversal of the adipic acid degradation pathway (RADP)[22,23] from *Thermobifida fusca* achieved the highest titer of 68.0 g/L adipic acid in *E. coli* by fed-batch fermentation.[22] However, the use of an inducer isopropyl $\beta$-D-1-thiogalactopyranoside (IPTG) significantly increases the fermentation cost. To alleviate the high cost caused by the addition of IPTG, Zhou et al. constitutively overexpressed the RADP in *E. coli* and obtained 57.6 g/L adipic acid in a 5 L bioreactor.[24] In reality, acetyl-CoA and succinyl-CoA are the precursors of the RADP for the biosynthesis of adipic acid. The generation of succinyl-CoA from glucose through the oxidative TCA pathway would release half of the carbon atoms in the form of carbon dioxide due to the two decarboxylation steps (Figure 1), which resulted in a theoretical yield of adipic acid of only 54.07%.[22] Hence, the existing low theoretical yield was the biggest limiting factor for increasing productivity.

The generation of succinyl-CoA through the reductive TCA pathway involves the conversion of only four-carbon compounds and without decarboxylation (Figure 1). This achieved a high theoretical yield for the biosynthesis of malic acid,[25] fumaric acid,[26] and succinic acid[27] through the reductive TCA pathway, which was superior to the oxidative TCA pathway, glyoxylate pathway, and noncyclic glyoxylate.[28] Furthermore, this study aimed to enhance the reductive TCA pathway and the $CO_2$ fixation process coupled with the RADP to avoid the emission of $CO_2$ and increase the theoretical yield of adipic acid (Figure 1). Compared with the previous pathway,[24] the theoretical yield of the re-engineered pathway increased by 50% (81.11% vs 54.07%). In the pathway engineering process, we introduced an oxygen-dependent dynamic regulation (ODDR) system and optimized the gene combination and operon structure. In addition, we also coordinated gene expression levels using promoter engineering. The dynamic control strategy in this study not only achieved a breakthrough in improving the theoretical yield of adipic acid by reductive TCA pathway but also could provide a paradigm for the production of a wide range of other compounds.

## RESULTS AND DISCUSSION

**Designing and Verifying the Feasibility of the Reductive TCA Pathway for the Production of Adipic Acid.** Normally, the oxidative TCA pathway is the preferred metabolic pathway for microorganisms under aerobic conditions. However, it leads to a decrease in the utilization rate of the carbon source due to decarboxylation reactions (Figure 1). Hence, using the intermediate metabolites (acetyl-CoA and succinyl-CoA) of the oxidative TCA pathway as precursors for the biosynthesis of adipic acid would significantly reduce the theoretical yield from glucose. Theoretically, the oxidative TCA pathway would be reversed under anaerobic conditions and usually would be called the reductive TCA pathway (Figure 1).[28] The reductive TCA pathway has a great advantage for the biosynthesis of succinyl-CoA since no carbon atom is wasted. Hence, by combining a $CO_2$ fixation reaction, the reductive TCA pathway, and the RADP, zero


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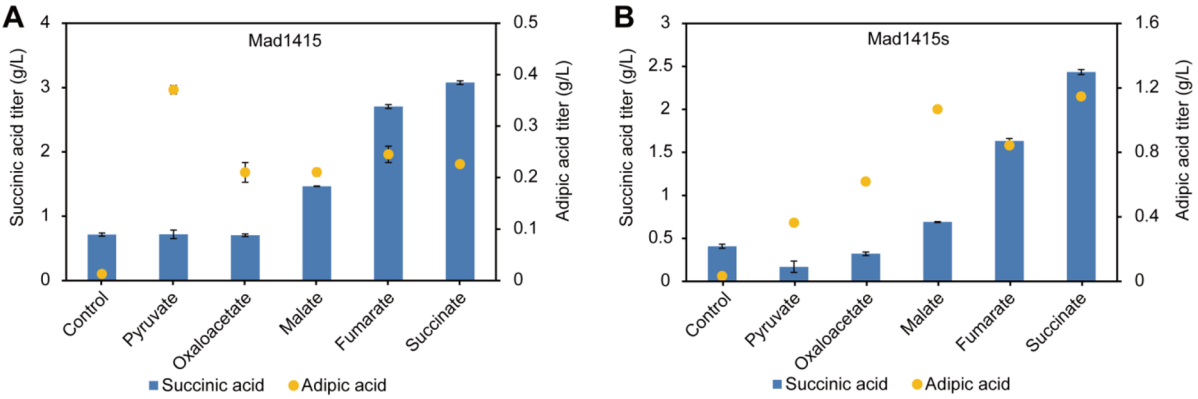


**Figure 2.** Feasibility analysis of the metabolic pathway. Mad1415: constitutively overexpressed the RADP in MG1655 (Δ*sucD*Δ*ldhA*Δ*atoB*). Mad1415s: restored *sucD* expression based on Mad1415.

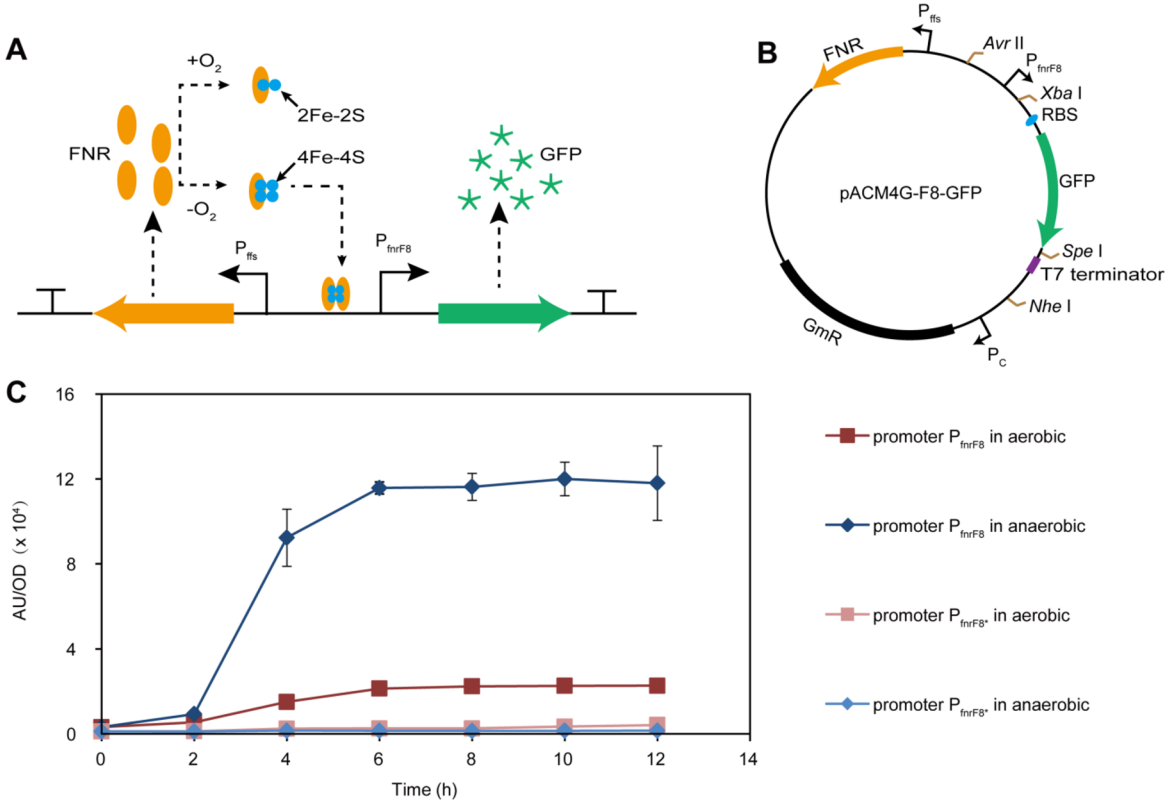


**Figure 3.** Design and dynamic range of anaerobic inducible biosensor. (A) Working principle of the anaerobic inducible biosensor. (B) Schematic of the construction of the plasmid pACM4G-F8-GFP. (C) Dynamic range of the anaerobic inducible biosensor in aerobic and anaerobic conditions. The promoter $P_{fnrF8}$ was used with the FNR binding sites, and $P_{fnrF8*}$ was used without the FNR binding sites.

carbon emission could probably be achieved (Figure 1). The stoichiometry calculation associated with producing adipic acid from glucose is shown in Table S4. This reveals that 1 mol of adipic acid is generated on the basis of 1 mol of glucose, with a resulting theoretical yield of 0.81 g of adipic acid per gram of glucose, which is increased by 50% compared to the oxidative TCA pathway.[22]

In previous studies, the adipic acid producing strain Mad1415 was obtained by knocking out *sucD*, *ldhA*, and *atoB* and constitutively expressing the RADP.[24] Due to the *sucD* gene defect in the Mad1415 strain, we additionally restored its expression to reduce succinate to succinyl-CoA and finally obtained the Mad1415s strain. As a proof-of-concept, we took the Mad1415 and Mad1415s strains as the research objects and explored the feasibility of producing adipic acid by the reductive TCA pathway by adding different intermediate organic acid precursors under anaerobic conditions. We found that Mad1415 had weak adipic acid production activity and that the addition of pyruvate had no influence on the accumulation of succinic acid while the accumulation of adipic acid increased by 88% (Figure 2A). It was speculated that the addition of exogenous pyruvate resulted in the production of a large amount of acetyl-CoA, which flowed into the oxidative TCA pathway even under anaerobic conditions and generated succinyl-CoA for the production of adipic acid. The lack of pyruvate carboxylase (*pyc*) to drive pyruvate into the reductive TCA pathway probably was the reason for the low succinic acid accumulation. With the addition of oxaloacetate, malate,


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and fumarate for Mad1415, there was no significant change in the accumulation of adipic acid (Figure 2A). However, it is worth noting that the addition of malate and fumarate led to a significant increase in succinic acid (Figure 2A), indicating that the reductive TCA pathway was functional under anaerobic conditions. However, when adding oxaloacetate, the accumulation of succinic acid was basically unchanged (Figure 2A). It was possibly because the pathway from oxaloacetate to malate was blocked by the limited activity of malate dehydrogenase (*mdh*).

Compared with Mad1415, when adding oxaloacetate, malate, fumarate, and succinate for Mad1415s, the accumulation of succinic acid generally decreased and caused an overall increase in the biosynthesis of adipic acid (Figure 2B). Furthermore, the addition of succinate resulted in the highest adipic acid titer, which was 1.36-fold the titer when adding fumarate, indicating that fumarate reductase (*frdABCD*) was probably another rate-limiting enzyme. Taken together, we concluded that the corresponding enzymes pyruvate carboxylase (*pyc*), malate dehydrogenase (*mdh*), and fumarate reductase (*frdABCD*), catalyzing the metabolic transition to oxaloacetate, malate, and succinate, could contribute significantly to the synthesis of adipic acid (Figure 2B).

**Constructing an Oxygen-Dependent Dynamic Regulated Reductive TCA Pathway.** Generally, the reductive TCA pathway in microorganisms could be activated under anaerobic conditions.[28] With this background, using an oxygen-dependent biosensor to regulate the expression of the reductive TCA pathway could achieve the dynamic regulatory purpose and avoid the use of an expensive inducer. The fumarate and nitrate reductase (FNR) transcription activator-dependent biosensor has been the most studied dissolved oxygen response biosensor. FNR activity was regulated by the transition between 4Fe-4S and 2Fe-2S clusters (Figure 3A).[29,30] The FNR dimer was activated under the anaerobic conditions and acted as an activator of the anaerobic metabolism genes. In order to obtain an oxygen-dependent biosensor, we used the anaerobic inducible synthetic promoter $P_{fnrF8}$ to control the expression of a green fluorescence protein (GFP) (Figure 3B).[31] In addition, we chose the promoter $P_{fnrF8*}$ without FNR binding sites as a control.[31] Compared to aerobic conditions, the promoter $P_{fnrF8}$ produced a 6.14-fold induction under anaerobic conditions (Figure 3C). These results indicate that the constructed biosensor would be a good regulation center for the expression of the reductive TCA pathway when shifting from aerobic to anaerobic conditions.[31]

We selected *pyc*, *mdh*, and *frdABCD* to overexpress in the Mad1415s strain to establish the ODDR system. These genes were combinational overexpressed with a monocistronic structure under the control of the promoter $P_{fnrF8}$. The overexpression of *pyc*, *mdh*, or *frdABCD* in Mad1415s enhanced the titer of adipic acid at least 2.71-fold (Figure 4). The expression of *frdABCD* significantly enhanced the titer to 0.27 g/L, 17.50-fold of Mad1415s, demonstrating that the step catalyzed by *frdABCD* was the rate-limiting step in our ODDR system. Then, we combined the overexpression of *pyc* and *mdh* in Mad1415-F8NAsf. The further expression of *mdh* in Mad1415-F8NAsf improved cell growth but had no benefit for adipic acid production. The overexpression of *pyc* further improved the titer of adipic acid to 0.30 g/L, 1.13-fold of Mad1415-F8NAsf. Although the difference is not significant, the expression of *pyc* is necessary because its key role in catalyzing carbon fixation. Finally, compared with the control

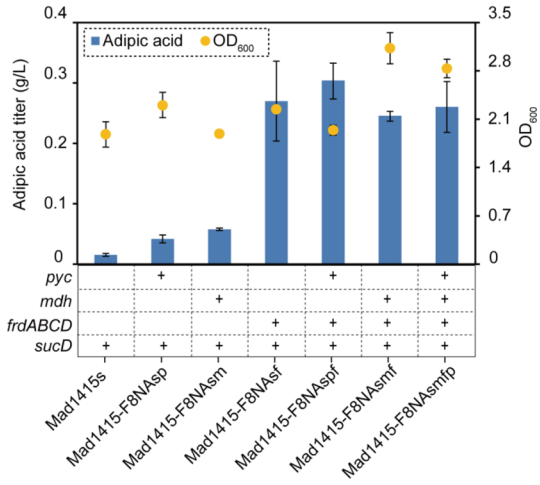


**Figure 4.** Optimization of the combination of key genes related to the reductive TCA pathway for adipic acid production. The symbol "+" means that the gene is overexpressed.

strain Mad1415s, there was a 19.70-fold improvement in the titer of adipic acid in Mad1415-F8NAspf. Overall, the coexpression of *sucD*, *pyc*, and *frdABCD* in Mad1415 was the best gene combination for adipic acid production.

**Optimizing the Operon Structure of the Reductive TCA Pathway.** The prokaryote expression system often contains a complicated operon structure that would influence the expression level of genes.[32] Hence, optimizing the structure of the reductive TCA pathway probably could further enhance the production of adipic acid. Three types of structural units were superimposed for gene expression. The first one was the classical operon structure, in which all genes were controlled by only one promoter and one terminator. The second was the pseudo-operon structure in which all genes were controlled by their own promoter and a common terminator. The last one was the monocistronic structure in which each gene was under the control of an independent promoter and terminator.[32] We constructed another two strains, Mad1415-F8SXspf and Mad1415-F8SAspf, which held the classical operon structure and the pseudo-operon structure, respectively (Figure 5). After 72 h of anaerobic fermentation, we found that the monocistronic structure strain Mad1415-F8NAspf was still the best. However, the Mad1415-F8SAspf strain exhibited a better growth profile than the others, demonstrating that the arranged genetic structure could influence gene expression.

**Coordinating Gene Expression by Different Strength Anaerobic Promoters.** The activated transcription factor FNR could target specific domains of the DNA sequence TTGA(T/C)NNNN(A/G)TCAA that was located in the upstream region of the promoter $P_{fnrF8}$ (Figure 6A).[33] We speculated that changing the distance between the FNR binding site and the −35 region might affect the performance of the anaerobic activation, leading to different levels of gene expression.[34] As a proof-of-concept, we tried to increase and decrease the distance between the FNR binding site and the −35 region. Increasing this distance reduced the sensitivity of the activation signal and instead caused the promoter to be inhibited under anaerobic conditions (Figure 6B). However, deleting 3, 4, or 7 bp between the FNR binding site and the −35 region obtained gradient strength variants, $P_{fnrD3F8}$, $P_{fnrD4F8}$, and $P_{fnrD7F8}$, which were 71.55%, 55.24%, and 10.71% of the promoter $P_{fnrF8}$ (Figure 6B and Table S3).


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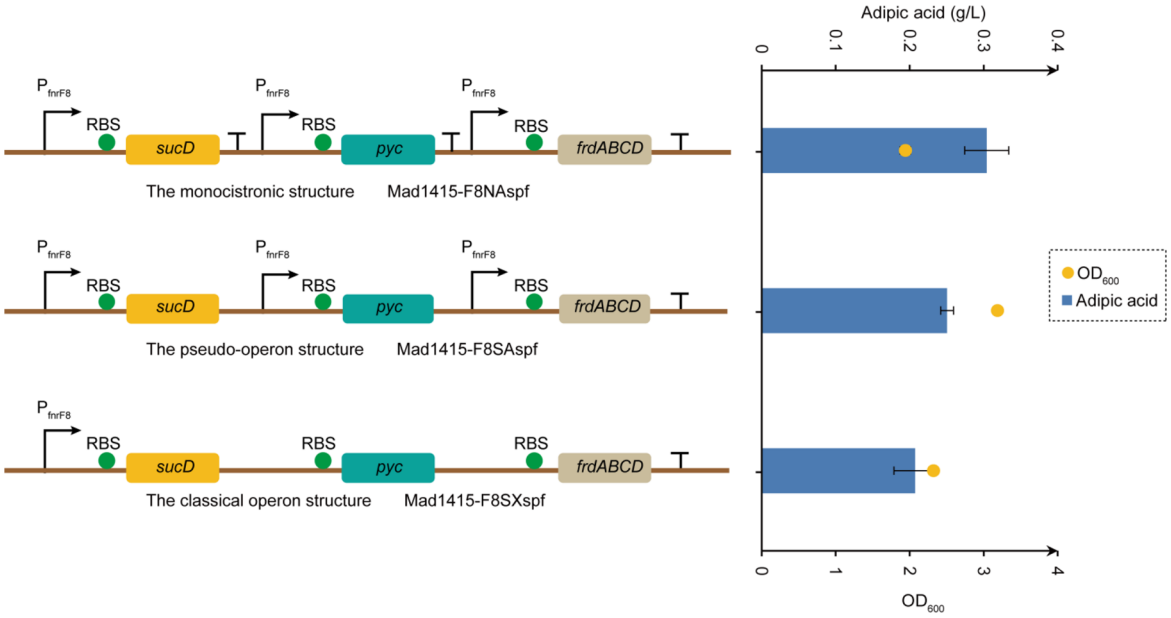


**Figure 5.** Optimization about the operon structure of the *sucD-pyc-frdABCD* gene string for adipic acid production.

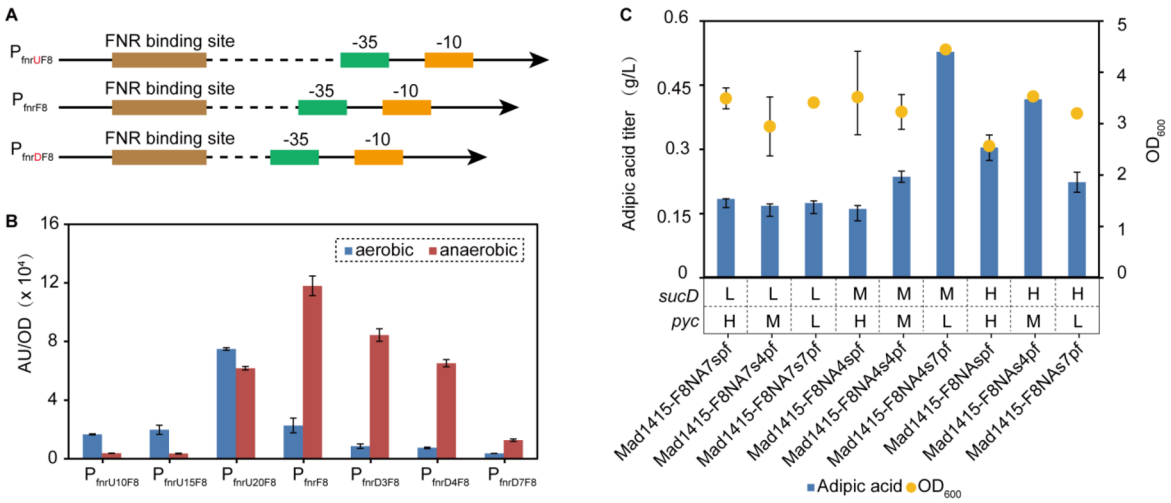


**Figure 6.** Promoter screening and coordinated gene expression for adipic acid production. Note: $P_{fnrU10F8}$, $P_{fnrU15F8}$, and $P_{fnrU20F8}$ increased the distance by 10, 15, and 20 bp between the FNR binding site and the −35 region of the promoter. $P_{fnrD3F8}$, $P_{fnrD4F8}$, and $P_{fnrD7F8}$ reduced the distance by 3, 4, and 7 bp between the FNR binding site and the −35 region of the promoter. H, controlled by a high strength promoter $P_{fnrF8}$; M, controlled by a medium strength promoter $P_{fnrD4F8}$; L, controlled by a low strength promoter $P_{fnrD7F8}$.

Then, we chose high ($P_{fnrF8}$), medium ($P_{fnrD4F8}$), and low ($P_{fnrD7F8}$) strength promoters to regulate the expression level of the reductive TCA pathway.

The gene *frdABCD* was a limiting step for the production of adipic acid, and thus, we used a strong promoter $P_{fnrF8}$ to maintain its high expression level. Then, we optimized the expression level of *sucD* and *pyc* to balance the metabolic flux of the reductive TCA pathway (Figure 6C). We found that the low expression level of *sucD* would significantly negatively affect the production of adipic acid, no matter the expression level of *pyc*. Using the medium strength promoter to control the expression of *sucD*, the lower expression level of *pyc* generated a higher titer of adipic acid and optimum cell growth (Figure 6C). The high expression level of *sucD* was beneficial for the production of adipic acid but impaired cell growth. Finally, a medium expression for *sucD* and a low expression for *pyc* were identified as the best combination. The adipic acid titer of the optimized strain Mad1415-F8NA4s7pf achieved was 0.53 g/L, which was 41.62-fold that of the control strain Mad1415.

## DISCUSSION

Currently, the RADP is the most efficient adipic acid synthetic pathway in *E. coli*. However, in the biosynthesis of the adipic acid precursors acetyl-CoA and succinyl-CoA through the oxidative TCA pathway, half of the carbon atoms are lost through the formation of $CO_2$.[22,24] Hence, the theoretical yield of adipic acid is radically restricted. To overcome this challenge, we used the constitutive strain Mad1415 as a chassis and established an ODDR system by coupling the reductive TCA pathway and the RADP. This avoided the loss of carbon atoms and resulted in a 50% increase in the theoretical yield (0.81 g of adipic acid per gram of glucose). Using an anaerobic biosensor as a control center, we


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systemically optimized the ODDR system and obtained 0.53 g/L adipic acid in a shake flask under anaerobic conditions, 41.62-fold that of the control strain Mad1415. In summary, the reductive TCA pathway and the ODDR system were first applied in the bioproduction of adipic acid and significantly improved the titer under anaerobic conditions.

The reductive TCA pathway has been successfully applied to the biosynthesis of various organic acids by virtue of its high theoretical yield advantages.[25−27] For example, even if there was no decarboxylation step in the glyoxylic acid pathway, the theoretical yield would be only 1 mol of succinic acid per mol of glucose, but the theoretical yield using the reductive TCA pathway would be 2 mol of succinic acid per mol of glucose.[28] The reason for this is that there is a carbon fixation reaction in the reductive TCA pathway. Therefore, increasing the utilization of $CO_2$ to enhance the carbon fixation reaction is beneficial for producing the target product. Except for overexpressing genes related to carbon fixation, such as phosphoenolpyruvate carboxylase (PPC),[35] phosphoenolpyruvate carboxykinase (PCK),[35] and pyruvate carboxylase (*pyc*),[36] it could exogenously supply $CO_2$ in the form of bicarbonate salts at the same time.[37] In our study, we overexpressed pyruvate carboxylase (*pyc*) to enhance the carbon fixation and expected to add calcium carbonate in the optimized medium to maximize the carbon sequestration node.

Traditional metabolic engineering approaches are static and depend on artificial intervention.[38] However, in this study, fermentation was always accompanied by changing conditions, such as oxygen changes. The aerobic fermentation conditions from the beginning to the end would put the reductive TCA pathway at a disadvantage, and the anaerobic conditions throughout the process would lead to a low cell density and insufficient growth energy.[31] To balance the trade-offs between growth and productivity, the application of an oxygen-dependent biosensor could monitor environmental signals from aerobic to anaerobic conditions in real-time and transform the genetic expression signals, dynamically regulate target gene expression, and make an optimal transition from cell growth to organic acid production.[31,39] Wherein, the performance and sensitivity of a sensor response to signals closely affects the metabolic flow.[40,41] To obtain desired biosensors, we modified the distance between the transcription factor binding site and the −35 region of the promoter to coordinate and control target gene expression in our study. Moreover, other biosensor engineering strategies, such as modeling-based optimum ribosome binding sites (RBS) and promoter prediction strategies would also help to more precisely design biosensors with desired performances.[34,42] Therefore, the systemic optimization of the promoter $P_{ffs}$ and the RBSs in an oxygen-dependent biosensor can obtain a superior ODDR system in the near future.

Overall, our established ODDR system breaks through the inherent limitation of current adipic acid production strategies and provides a new strategy with dynamic regulation to enhance the theoretical yield of adipic acid. Additionally, the anaerobic fermentation stage does not require the participation of oxygen in this strategy, which greatly simplifies the fermentation equipment and process operations. Nevertheless, there is still a long way to large-scale industrial production of adipic acid. The main challenge is to explore the two-stage fermentation methods and balance the cell growth, byproducts accumulation, and adipic acid production under anaerobic conditions. On the contrary, due to the limited reduction equivalent under anaerobic conditions, cofactor engineering strategies might be applied to balance the NADH/$NAD^+$ in the near future.[43,44] Taken together, assisted with various advanced synthetic biology methods, our established ODDR system would provide a paradigm for the bioproduction of other chemical compounds.

## METHODS

**Strains, Medium, and Cultivation.** *E. coli* JM109 or *E. coli* DH5$\alpha$ was used as the host for plasmid cloning. *E. coli* Mad1415 was used as the host for the constitutive expression of the target genes.[24] The strains used in this study are listed in Table S1. Lysogeny broth (LB) (Supporting Information) was used for plasmid cloning and seed cultivation. Super optimal broth (SOB) (Supporting Information) was used for adipic acid production in the serum bottles. In the fermentation process, rubber stoppers were added when needed to achieve anaerobic conditions.[45,46] Overnight cultured seed inoculum (4 mL) was added to 250 mL serum bottles containing 200 mL of SOB medium (supplemented with 4 g/L glucose) for culturing at 37 °C and 250 rpm. After cultivation in the aerobic phase for 12 h, the glucose was depleted. Then, the anaerobic culture was started with the supplementation of another 4 g/L glucose. Equimolar organic acid salts were added to the medium for specific fermentation. Ampicillin (100 $\mu$g/mL), gentamicin (50 $\mu$g/mL), and kanamycin (50 $\mu$g/mL) were added to the media as required.

**Plasmid Construction.** All plasmids and primers used in this study are listed in Tables S1 and S2, respectively. All DNA polymerases and DNA restriction enzymes were purchased from Takara (Dalian, China). The pACM4 plasmid was an ePathBrick vector with four compatible restriction enzyme sites, which were *Spe* I, *Avr* II, *Xba* I, and *Nhe* I.[32] The pACM4G plasmid was obtained by replacing the original chloramphenicol resistance gene of the pACM4 plasmid with a gentamicin resistance gene. The FNR transcription activator from *E. coli* K12 MG1655 was cloned and expressed under the control of the promoter $P_{ffs}$ on pACM4G.[47] The T7 promoter of pACM4G was replaced with the anaerobic inducible promoter $P_{fnrF8}$ and promoter $P_{fnrF8*}$, whose FNR binding site was removed,[31] generating plasmids of pACM4G-F8 and pACM4G-F8*, respectively. Then, a green fluorescence protein (GFP) was cloned into the site of *Nde* I/*Xho* I of pACM4G-F8 and pACM4G-F8*, generating an anaerobic biosensor pACM4G-F8-GFP and its negative control pACM4G-F8*-GFP (Figure S1). To obtain anaerobic inducible promoters with gradient strength, $P_{fnrF8}$ of pACM4G-F8-GFP was replaced by the truncated and extended $P_{fnrF8}$ variants (Table S3).

The genes *sucD*, *frdABCD*, and *mdh* were amplified from the genomic DNA of *E. coli* K12 MG1655 by the primer pairs of sucD-F/sucD-R, frdABCD-F/frdABCD-R, and mdh-F/mdh-R, respectively. The gene *pyc* was amplified from *Corynebacterium crenatum* genomic DNA by the primer pairs pyc-F/pyc-R. Their PCR products were cloned into the *Nde* I/*Xho* I site of the pACM4G-F8 plasmid, generating the pACM4G-F8s, pACM4G-F8f, pACM4G-F8m, and pACM4G-F8p plasmids, respectively (Figure S1). According to the principle of ePathBrick vector, we constructed different plasmids with different operon structures for stacking gene expression. All plasmid schematic diagrams and their construction processes are illustrated in Figures S1−S5.


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**Metabolite Quantification.** The optical density was measured at 600 nm ($OD_{600}$) with a spectrophotometer (UV-1800, AOE instruments, Shanghai, China). The fermentation supernatants were obtained by centrifugation at 16 000*g* for 5 min and then diluted for metabolite quantification. The concentrations of glucose, adipic acid, acetic acid, lactic acid, and succinic acid were measured by high-performance liquid chromatography (HPLC; Agilent 1260 II, Santa Clara, CA) equipped with an HPX-87H ion-exclusion column (Bio-Rad, Hercules, CA). The mobile phase flowed with 5 mM $H_2SO_4$ at a rate of 0.6 mL/min, and the column temperature was maintained at 30 °C. The organic acids were measured by an UV−vis detector at 210 nm, and glucose was measured by a refractive index (RI) detector.

**Fluorescence Measurement.** The overnight seed solution was transferred into fresh SOB medium with 2% inoculum and cultured at 37 °C and 250 rpm. For aerobic culturing, strains were cultured in 250 mL Erlenmeyer flasks containing 50 mL of SOB media. For anaerobic culturing, strains were cultured in 1.5 mL centrifuge tubes containing 1.5 mL of SOB media. Samples taken at different times were diluted with 10 mM phosphate buffered saline (PBS, 7.0) to ensure that the $OD_{600}$ was in the range 0.2−0.8. The fluorescence was measured using a BioTek HT plate reader (Winooski, VT) under an excitation wavelength of 485 ± 20 nm and an emission wavelength of 528/20 nm at room temperature. Finally, the fluorescence was normalized by dividing the $OD_{600}$ and fluorescence (AU).[42]

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.0c00648.

Tables of strains and plasmids used, primers used, synthesized oligonucleotides, and stoichiometry of producing adipic acid from glucose, figures of plasmid schematic diagrams and construction processes, and discussions of synthetic methods for medium (PDF)

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### Author Contributions

Y.D., S.Z., and T.H. designed the study and wrote the manuscript. Y.D., S.Z., and G.L. critically revised the manuscript. T.H. performed the experiments and analyzed the results. All authors reviewed, approved, and contributed to the final version of the manuscript.

### Notes

The authors declare no competing financial interest.

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