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Research review paper

# Metabolic engineering in the biotechnological production of organic acids in the tricarboxylic acid cycle of microorganisms: Advances and prospects

Xian Yin<sup>a,b,c</sup>, Jianghua Li<sup>a,c</sup>, Hyun-dong Shin<sup>d</sup>, Guocheng Du<sup>a,c</sup>, Long Liu<sup>a,b,c,\*</sup>, Jian Chen<sup>a,e,\*\*</sup><sup>a</sup> Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, China<sup>b</sup> State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, China<sup>c</sup> Synergetic Innovation Center of Food Safety and Nutrition, Wuxi 214122, China<sup>d</sup> School of Chemical and Biomolecular Engineering, GA Institute of Technology, Atlanta 30332, USA<sup>e</sup> National Engineering of Laboratory for Cereal Fermentation Technology, Jiangnan University, Wuxi 214122, China

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## ABSTRACT

Organic acids, which are chemically synthesized, are also natural intermediates in the metabolic pathways of microorganisms, among which the tricarboxylic acid (TCA) cycle is the most crucial route existing in almost all living organisms. Organic acids in the TCA cycle include citric acid,  $\alpha$ -ketoglutaric acid, succinic acid, fumaric acid, L-malic acid, and oxaloacetate, which are building-block chemicals with wide applications and huge markets. In this review, we summarize the synthesis pathways of these organic acids and review recent advances in metabolic engineering strategies that enhance organic acid production. We also propose further improvements for the production of organic acids with systems and synthetic biology-guided metabolic engineering strategies.

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\* Correspondence to: L. Liu, Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, China. Tel.: +86 510 85918312; fax: +86 510 85918309.

\*\* Correspondence to: J. Chen, Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, China. Tel.: +86 510 85913661; fax: +86 510 85910799.

E-mail addresses: [longliu@jiangnan.edu.cn](mailto:longliu@jiangnan.edu.cn) (L. Liu), [jchen@jiangnan.edu.cn](mailto:jchen@jiangnan.edu.cn) (J. Chen).

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## 1. Introduction

Organic acids are low-molecular-weight organic compounds with one or more acidic groups such as carboxyl, sulfonic, alcohol, and thiol groups. These functional groups make organic acids key building-block chemicals, which have huge markets and are commonly refined from petroleum. Because petroleum is an unsustainable resource and its use generates greenhouse gas emissions, petroleum-based chemical production must be shifted to biotechnological processes (Sauer et al., 2008). Most organic acids are intermediates in the metabolic pathways that occur naturally in microorganisms; among these pathways the tricarboxylic acid (TCA) cycle is the most crucial. The TCA cycle exists in almost all living organisms. Chemical compounds in the TCA cycle include citric acid (CA),  $\alpha$ -ketoglutaric acid (KGA), succinic acid (SA), fumaric acid (FA), L-malic acid (MA), and oxaloacetate (OAA). The chemical structures of these compounds are listed in Fig. 1. These acids have key positions in the organic acid industry owing to their broad applications.

CA is a bulk product used mainly in food, pharmaceutical and other industrial applications. It is also used as a biocompatible polymeric material for nanomedicine (Naeini et al., 2010). KGA is used mainly as a dietary supplement but also has uses in diagnostic assays (Morgunov et al., 2013). SA, FA, MA, and OAA, which constitute the C<sub>4</sub>-dicarboxylic acid family, have similar molecular structures and can be easily interconverted (Cao et al., 2011). The U.S. Department of Energy has proposed these acids as building-block chemicals that can be produced from biomass.

The potential market for these acids can be expanded if fermentation costs can be reduced. Strain improvement is crucial for fermentation and has occurred through random mutagenesis and screening processes, rational metabolic engineering, and currently, systems metabolic engineering. No review has summarized developments in the metabolic

engineering of strains for the production of these organic acids. This review compares the existing synthesis pathways of these organic acids and summarizes the metabolic engineering strategies for bacteria, yeasts, and filamentous fungi implemented to improve performance. We also suggest strategies for combining systems biology, synthetic biology, and transporter engineering as efficient tools for improving the production and productivity of these organic acids in the future.

## 2. Citric acid

CA (2-hydroxy-propane-1,2,3-tricarboxylic acid) is used extensively in the food and pharmaceutical industries owing to its safety, pleasant acidic taste, high water solubility, and chelating and buffering properties. CA is also widely used in detergents, cosmetics, and a variety of other industrial applications (Soccol et al., 2006).

The work of Currie formed the basis of industrial CA fermentation in 1917 (Papagianni, 2007). Currently, 99% of the world's CA output is obtained through fermentation, and approximately 80% is obtained through submerged fermentation using *Aspergillus niger* (Dhillon et al., 2011). CA fermentation requires several critical nutrient conditions, including excessive concentrations of carbon source, low pH, sufficient dissolved oxygen, and suboptimal concentrations of Mn<sup>2+</sup> and phosphate (Karaffa and Kubicek, 2003; Papagianni, 2007). The best strains produce CA at yields up to 0.95 g per gram of sugar. However, potential remains for a fivefold increase in CA productivity according to the model of Alvarez-Vasquez et al. (2000).

CA is formed mainly via the cytosolic glycolytic pathway and subsequent mitochondrial condensation of a C<sub>4</sub> and a C<sub>2</sub> moiety (Karaffa and Kubicek, 2003). Glycolysis catabolizes glucose to 2 mol pyruvate. One mole is transformed into the mitochondria and converted to acetyl-coenzyme A (acetyl-CoA) via the release of 1 mol CO<sub>2</sub>, and the other is converted to OAA via the fixing of 1 mol CO<sub>2</sub>. OAA is subsequently

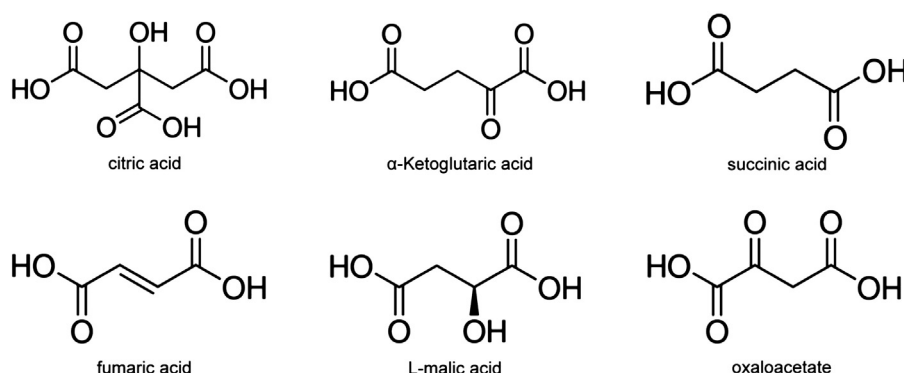
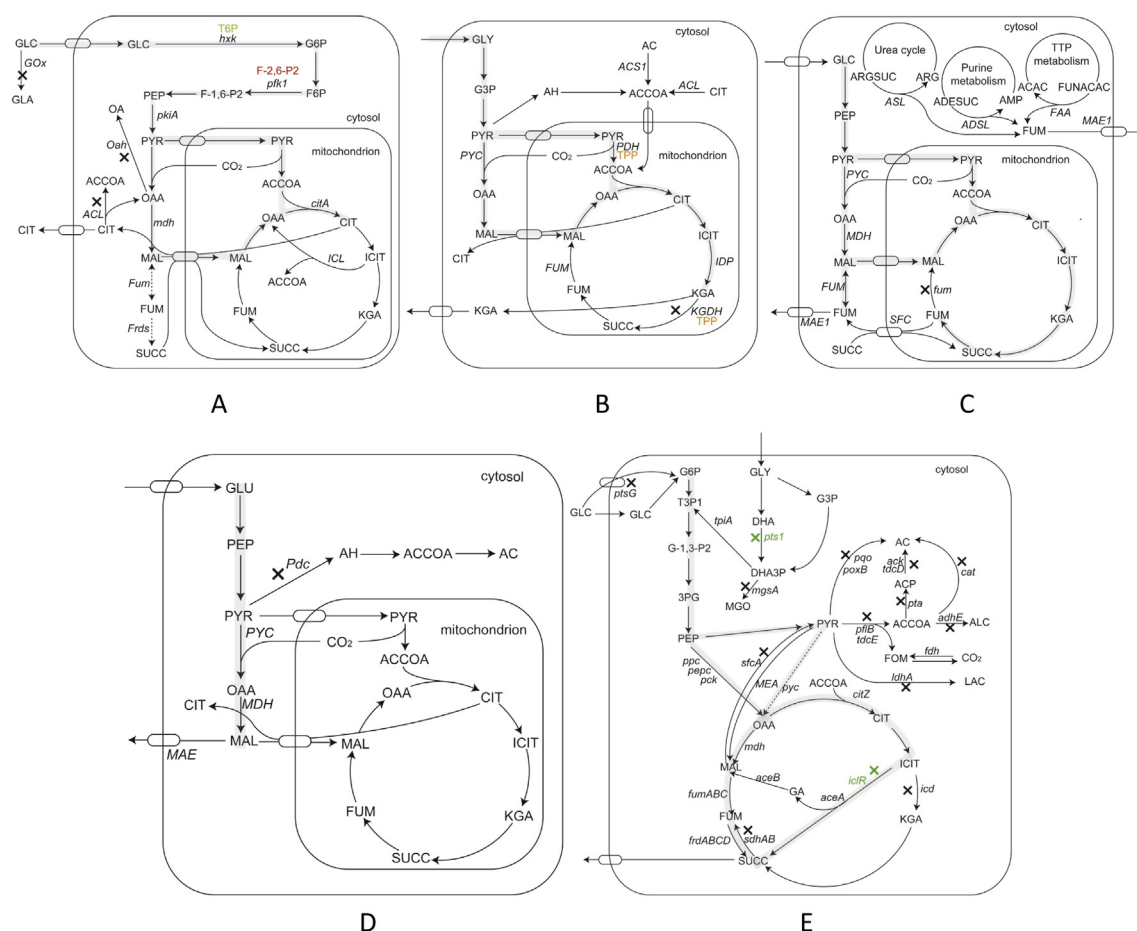


Fig. 1. Chemical structures of organic acids.



**Fig. 2.** Engineering strategies for enhancing organic acid production. Metabolic flux of (A) citric acid in *A. niger* and *Y. lipolytica*, (B) KGA in *Y. lipolytica*, (C) fumaric acid in *S. cerevisiae* and *T. glabrata*, (D) malic acid in *S. cerevisiae*, (E) succinic acid in *E. coli*. Gray line shows the main pathway of acid synthesis. The dotted line indicates exogenous pathway that does not exist in natural strain. Letters in green indicates inhibitor of the enzyme, while in red enhancer and in orange co-factor. The cross mark indicates the gene is knocked out. Abbreviations: 3PG, glycerate-3-phosphate; AC, acetate; ACAC, acetoacetate; ACCOA, Acetyl-CoA; ACP, acetyl phosphate; ADESUC, adenylosuccinate; AH, aldehyde; ALC, alcohol; AMP, adenosine monophosphate; ARG, arginine; ARGUSC, argininosuccinate; CIT, citrate; DHA, dihydroxyacetone; DHA3P, dihydroxyacetone 3-phosphate; G6P, glucose-6-phosphate; F-1,6-P2, fructose-1,6-bisphosphate; F-2,6-P2, fructose 2,6-bisphosphate; F6P, fructose-6-phosphate; FMA, formate; FUM, fumarate; FUMACAC, fumarylacetoacetate; G-1,3-P2, glyceralate-1,3-bisphosphate; G3P, glycerol-3-phosphate; GA, glyoxylate; GLA, gluconic acid; GLC, glucose; GLY, glycerol; G6P, glucose-6-phosphate; KGA,  $\alpha$ -ketoglutarate; ICIT, isocitrate; LAC, lactic acid; MAL, malate; MGO, methylglyoxal; OA, oxalic acid; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PYR, pyruvate; SUCC, succinate; T3P1, glyceraldehyde-3-phosphate; T6P, trehalose-6-phosphate; TPP, thiamine pyrophosphate; *aceA*, isocitrate lyase; *aceB*, malate synthase; *ackA*, acetate kinase; *ACL*, ATP-citrate lyase; *ADSL*, adenylosuccinate lyase; *ASL*, argininosuccinate lyase; *cat*, acetyl-CoA:CoA transferase; *cit*, citrate synthase; *eqo*, pyruvate:menaquinone oxidoreductase; *FAA*, fumarylacetoacetate; *fdh*,  $NAD^+$ -coupled formate dehydrogenase; *fum*, fumarase; *frd*, fumarate reductase; *gapA*, glyceraldehyde 3-phosphate dehydrogenase; *Gox*, glucose oxidase; *hxx*, hexokinase; *icd*, isocitrate dehydrogenase; *ICL*, isocitrate lyase; *ICLR*, Isocitrate lyase repressor; *IDP1*, isocitrate dehydrogenase; *KGDH*,  $\alpha$ -ketoglutarate dehydrogenase; *ldhA*, lactate dehydrogenase; *MAE*,  $C_4$ -dicarboxylic acids transporter; *mdh*, malate dehydrogenase; *MEA*, malate dehydrogenase; *mgsA*, methylglyoxal synthase; *oah*, oxaloacetate acetylhydrolase; *pck*, phosphoenolpyruvate carboxykinase; *Pdc*, pyruvate decarboxylase; *PDHC*, pyruvate dehydrogenase complex; *pepc*, phosphoenolpyruvate carboxykinase; *pfk1*, phosphofructokinase; *pfl*, pyruvate:formate lyase; *pkfA*, phosphofructokinase; *poxB*, pyruvate oxidase; *ppc*, phosphoenolpyruvate carboxykinase; *pta*, phosphotransacetylase; *pyc*, pyruvate carboxylase; *sdhAB*, succinate dehydrogenase; *SFC*, succinate-fumarate transporter; *sfcA*,  $NAD^+$ -linked malic enzyme; *tcdD*, threonine decarboxylase; *tcdE*, 2-ketobutyrate formate-lyase. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

reduced to MA and enters the mitochondria via a malate-citrate antiporter. Mitochondrial MA participates in the TCA cycle and forms CA (Fig. 2A). As a result, the maximum theoretical CA yield is 1 mol per mole glucose. The conversion of glucose to CA generates 1 mol ATP and 3 mol of nicotinamide adenine dinucleotide (NADH), which is redundant and must be turned over through an alternative respiratory pathway.

## 2.1. Engineering glycolysis and the TCA pathway for enhanced CA production

Metabolic engineering through the overexpression of rate-limiting enzymes involving glycolysis and the TCA cycle to increase CA productivity has had little success because of the stringent regulation of central carbon metabolism. For example, overexpression of pyruvate kinase and phosphofructokinase did not increase CA production; neither the intermediary metabolite levels nor the activities of other enzymes in

the pathway changed. Although phosphofructokinase was overexpressed, enzyme activity decreased owing to a reduction of the level of fructose 2,6-bisphosphate, which was a positive allosteric effector. The constructed strain produced CA at a titer of 55 g/L, which was identical to the yield of 0.64 mol/mol in the wild-type strain (Ruijter et al., 1997). Similarly, increasing the expression level of citrate synthase (CS) did not improve CA production; the mutant strain produced approximately 46 g/L CA (Ruijter et al., 2000). When *ACO1*, which encodes aconitase, was expressed in *Yarrowia lipolytica*, the product proportion of isocitrate increased, but the total amount of acids produced was not influenced (Holz et al., 2009).

## 2.2. Engineering to eliminate inhibitor effect and by-product formation

Deletion of the gene encoding trehalose-6-phosphate synthase A decreased the level of trehalose-6-phosphate, an inhibitor of hexokinase. As a result, CA accumulation was initiated earlier (Arisan-Atac et al.,

1996). The gene *Brsa-25* is responsive to  $Mn^{2+}$ , the concentration of which influences the morphology of *A. niger* and CA production. Antisense expression of *Brsa-25* facilitates pelleted growth and increases CA production in the presence of  $Mn^{2+}$  (Dai et al., 2004).

Oxalic acid is produced by *A. niger* when the culture pH is above 3, and oxaloacetate acetylhydrolase drives oxalate synthesis. A recombinant strain, which lacked both glucose oxidase and oxaloacetate acetylhydrolase, tolerated a pH of 5 and the presence of  $Mn^{2+}$  to produce CA (Ruijter et al., 1999).

### 2.3. Engineering to enhance the supplement pathway

The influence of the cytosol reductive TCA (rTCA) cycle on CA production has been evaluated through individual or combined overexpression of genes including fumarases (*Fum1s* and *FumRs*), fumarate reductase (*Frds1*), and malate dehydrogenase (*mdh2*). Compared with the wild-type strain, all the engineered strains produced CA at higher yields and productivity. The finding that the *Mdh2*-overexpressing strain increased the CA production rate earlier in the initial phase supports the theory that the accumulation of cytosol MA triggers CA production. The overexpression of fumarase, which converts FA to MA, provided more substrate to the mitochondrial malate-citrate antiporter and increased CA secretion, but a large amount of by-product oxalate was generated. Higher CA productivity was obtained with the *Frds1*-overexpressing strain. *Frds1* catalyzed SA formation from FA. The relationship of cytosol SA synthesis and CA production suggests that SA is a potential substrate for the mitochondrial CA antiporter. The best result came from the strain co-expressing *fumRs* and *Frds1*, which created a cytosolic rTCA pathway from MA toward SA, which was then used to exchange mitochondrial CA (de Jongh and Nielsen, 2008).

### 2.4. Engineering to enhance diverse carbon absorption in *Y. lipolytica*

A CA-producing *Y. lipolytica* strain was constructed to express inulinase *INU1* from *Kluyveromyces marxianus*. The strain utilized inulin to synthesize CA at a titer of 68.9 g/L, with 4.1 g/L isocitric acid as a by-product (Liu et al., 2010). Deleting *ACL1*-encoding ATP-citrate lyase and expressing isocitrate lyase (*ICL1*) produced a final strain that yielded CA from 10% inulin at a titer of 84 g/L (Liu et al., 2013).

CA production was enhanced through the expression of invertase *SUC2* from *Saccharomyces cerevisiae* for the utilization of sucrose and *ICL1* in *Y. lipolytica*. The reconstructed strain H222-S4 (p67ICL1) T5 produced 140 g/L CA from sucrose, with a high productivity at 0.73 g/L per hour in a fed-batch culture (Forster et al., 2007).

## 3. $\alpha$ -Ketoglutaric acid

KGA (2-oxopentanedioic acid; 2-ketoglutaric acid) is used in dietary supplements as a direct precursor for glutamine and glutamate. It is also a building-block chemical for the synthesis of heterocycles, which are used as antitumor agents (Otto et al., 2011; Stottmeister et al., 2005). KGA is used as a substrate for 2-oxoglutarate dehydrogenase (EC 1.2.4.2), glutamate dehydrogenase (EC 1.4.1.2), aspartate transaminase (EC 2.6.1.1), alanine transaminase (EC 2.6.1.2), cysteine transaminase (EC 2.6.1.3), and others in diagnostic assays for a wide range of diseases (Morgunov et al., 2013).

KGA is crucial in the coordination of carbon and nitrogen utilization due to its role as a key intermediate in the Krebs cycle and amino acid metabolism (Doucette et al., 2011). It is mainly synthesized through the TCA cycle (Fig. 2B).

Using *Y. lipolytica* as a KGA producer yields 195 g/L KGA when n-paraffins are used as carbon sources, but the high price of paraffin inhibits the industrial application of this method. In a recent study, a *Y. lipolytica* strain utilized glycerol, ethanol, and vegetable oils as carbon sources (Yu et al., 2012b). The thiamine-auxotrophic *Y. lipolytica* is a robust KGA producer. A deficiency of thiamine, which is a cofactor of

pyruvate dehydrogenase (PDH) and  $\alpha$ -ketoglutarate dehydrogenase, inhibited KGA oxidation and caused the accumulation of KGA in broth (Kamzolova et al., 2012).

### 3.1. Cofactor engineering for enhanced KGA production

The effect of components of the pyruvate dehydrogenase complex on KGA production has been discussed by Guo et al. (2014). When the alpha or beta subunits of the PDH complex E1 were overexpressed, which is involved in the thiamine pyrophosphate binding, the recombinant strains accumulated pyruvate at low levels and were insensitive to exogenous thiamine concentration. This outcome indicated that the competition for the cofactor could be switched to ketoglutarate dehydrogenase. Among these mutants, *Y. lipolytica* T1, which expressed the PDH complex E1 component  $\alpha$  subunit, yielded the highest production at 43.3 g/L (Guo et al., 2014).

In another study, cofactor metabolism was modulated by the overexpression of the acetyl-CoA synthetase gene *ACS1* from *S. cerevisiae* and the ATP citrate lyase gene *ACL* from *Mus musculus* to induce carbon flux flow from pyruvate to KGA. Both mutants increased acetyl-CoA formation and enhanced KGA production when either of these genes was expressed. The strain *Y. lipolytica*-ACL performed best, with a KGA titer of 56.5 g/L (Zhou et al., 2012).

### 3.2. By-product elimination and TCA cycle enhancement

A screened *Y. lipolytica* strain produced KGA at a titer of 39.2 g/L, with pyruvate as a by-product (Zhou et al., 2010). The pyruvate carboxylation pathway was enhanced by overexpressing pyruvate carboxylase genes with the aim of decreasing pyruvate production and replenishing the TCA cycle to increase KGA formation. The constructed strain increased KGA yield by 35.3% at a final titer of 62.5 g/L, with pyruvate yield decreased by 69.8% (Yin et al., 2012). The influence of cytosol pyruvate carboxylase (PYC) and mitochondrial fumarase (FUM) on KGA production was studied by overexpressing enzyme genes individually or simultaneously. These strains showed different product selectivities with identical KGA production of approximately 130 g/L (Otto et al., 2012).

The combination of TCA cycle enhancement and the elimination of pyruvate as a by-product improved KGA production. Dose-dependent gene overexpression was used to construct strains harboring multiple copies of the nicotinamide adenine dinucleotide phosphate ( $NADP^+$ )-dependent isocitrate dehydrogenase gene (*IDP1*) and PYC gene (*PYC1*). Reconstructed *Y. lipolytica* strains containing either of these genes increased KGA secretion. Overexpression of both genes further enhanced KGA production to 186 g/L (Yovkova et al., 2014).

## 4. Succinic acid

SA (1,2-ethanedicarboxylic acid) has broad applications in the food industry as an additive and flavoring agent and in pharmaceuticals as a supplement. It is also used as a surfactant, detergent extender, ion chelator, foaming agent, and aviation de-icer.

*Anaerobiospirillum succiniciproducens* and *Actinobacillus succinogenes* are natural SA producers, yielding SA as the dominant product at 50 g/L and 90 g/L, respectively, during anaerobic fermentation. However, these bacteria are unstable, tend to degenerate, and require strict anaerobic conditions for culture. These disadvantages disqualify them from commercial fermentation.

Three routes are used for SA formation (Cheng et al., 2013). The first route is the reductive TCA cycle under anaerobic conditions. OAA is synthesized from phosphoenolpyruvate (PEP) via  $CO_2$  fixation and converted to MA, which subsequently forms FA and finally SA. The theoretical yield is 2 mol SA per mole glucose. Two moles of NADH are required for 1 mol SA formation from phosphoenolpyruvate. However, 1 mol glucose generates 2 mol NADH during the formation of 2 mol PEP, leading

to NADH limitation in SA production. The second pathway is a glyoxylate route under aerobic conditions. Two moles of acetyl-CoA are converted to 1 mol SA, with 1 mol NADH formation, thereby forming extra NADH. The third pathway is the oxidative TCA cycle. Two moles of pyruvate are converted to 1 mol SA, generating 3 mol NADH. The theoretical yield is 1 mol SA per mole glucose (Fig. 2E).

*Escherichia coli* has been used as a host to produce SA, and several engineered strains have shown high productivity and yield.

#### 4.1. Engineering to block by-product formation

*E. coli* strains naturally synthesize large amount of acetate, lactate, and ethanol from pyruvate. As a result, *E. coli* engineering must first block by-product synthesis pathways. Genes involved in these pathway are the phosphotransacetylase (*pta*) plus acetate kinase (*ackA*), pyruvate:menaquinone oxidoreductase (*pqo*), acetyl-CoA:CoA transferase (*cat*), and L-lactate dehydrogenase (*ldhA*) genes.

Gene deletions and metabolic evolution resulted in strain KJ060, which lacked *ldhA*, *adhE*, *ackA*, *focA*, and *pflB*. This strain showed increased ATP production and produced SA at a titer of 86.6 g/L, with a yield of 1.41 mol/mol. Another strain, KJ071, in which *mgsA* was also deleted, produced MA instead of SA at a titer of 516 mM (Jantama et al., 2008a).

The additional deletion of *tdcD* encoding threonine decarboxylase and *tdcE* encoding 2-ketobutyrate formate-lyase in *E. coli* strain KJ091 ( $\Delta$ *ldhA*,  $\Delta$ *adhE*,  $\Delta$ *ackA*,  $\Delta$ *focA-pflB*,  $\Delta$ *mgsA*,  $\Delta$ *poxB*) inhibited acetate formation and increased SA yield by 10%. Individual deletion of *aspC* encoding aspartate amino-transferase and *sfcA* encoding nicotinamide adenine dinucleotide (NAD<sup>+</sup>)-linked malic enzyme did not affect SA yield, but deleting them simultaneously increased SA yield to 1.5 mol/mol (near theoretical yields) at a titer of 700 mM (Jantama et al., 2008b).

A set of mutants was constructed to evaluate the influence of genes in the by-product pathway on SA production. The strains ( $\Delta$ *adhE*,  $\Delta$ *pta*,  $\Delta$ *poxB*,  $\Delta$ *ldhA* [pZS-*pyc*] and  $\Delta$ *pflB*,  $\Delta$ *ldhA*,  $\Delta$ *poxB*,  $\Delta$ *adhE*,  $\Delta$ *ackA*,  $\Delta$ *pta* [pZS-*pyc*]) performed best, with yields of 60% and 54%, respectively (Blankschien et al., 2010).

The genes *ptsI*, which is involved in the phosphotransferase system, and *pflB*, which encodes pyruvate formate-lyase, were disrupted with the aim of eliminating formate by-product synthesis and using glycerol as a carbon source. The expression of *pck* was enhanced by mutating the promoter. The recombinant *E. coli* strain XZ721 produced SA at a yield of 0.8 mol/mol glycerol (Zhang et al., 2010).

#### 4.2. Engineering to enhance flux for OAA generation

OAA is a core chemical in SA production as a precursor in both the TCA and rTCA cycles. Enzymes linked to OAA formation are PYC and phosphoenolpyruvate carboxylase (PEPC). Overexpression of the *pyc* gene in *E. coli* resulted in a low SA production of 1.77 g/L. Overexpressing the PEPC gene improved SA production to 10.7 g/L (Millard et al., 1996). *E. coli* strain AFP111 was derived from NZN111 through spontaneous chromosome mutation. The mutation site encoded phosphotransferase, which transported PEP as a co-substrate for glucose transport. The strain generated acetic acid and ethanol as well as SA, for which the yield was 0.7 g/g (Donnelly et al., 1998). When expressing the pyruvate carboxylase gene *pyc* from *Rhizobium etli*, an engineered strain AFP111/pTrc99A-*pyc* produced SA at a yield of 0.96 g/g (Vemuri et al., 2002a). When dual-phase fermentation was used in this strain, production increased to 99.2 g/L at a yield of 1.1 mol/mol glucose and a productivity of 1.3 g/L per hour (Vemuri et al., 2002b). Strain SBS110MG, an *adhE* and *ldhA* double mutant, was constructed to express *pyc* and achieved an SA production of 15.6 g/L at a yield of 1.3 mol/mol (Sanchez et al., 2005a). YBS132, an *ackA-pta* and *ldhA* mutant, was constructed to co-express *pepc* from *Sorghum vulgare* and *pyc*

from *Lactococcus lactis*, improving SA production to 3.4 g/L (Lin et al., 2005e).

A recombinant *Corynebacterium glutamicum* strain was constructed to express *pyc*. The *ldhA* gene, encoding lactate dehydrogenase, was also knocked out in the strain. SA production by this strain reached 146 g/L within 46 h at a yield of 1.4 mol/mol glucose (Okino et al., 2008).

#### 4.3. Engineering to enhance the TCA cycle, rTCA cycle, and glyoxylate flux

The strain *C. glutamicum* ZX1 ( $\Delta$ *sdhCAB*,  $\Delta$ *ldhA*,  $\Delta$ *pqo*,  $\Delta$ *cat*,  $\Delta$ *pta*) was constructed to express *acsA* encoding acetyl-CoA synthase and *gltA* encoding CS to enhance the TCA cycle and increase the expression levels of *ppc* and *pyc* via promoter exchange. The strain produced 28.5 g/L SA at a yield of 0.63 mol/mol glucose (Zhu et al., 2013).

The deletion of the pyruvate:formate lyase gene *pfl* and lactate dehydrogenase gene *ldh*, resulted in a recombinant *E. coli* strain that synthesized abundant pyruvate. The introduction of an engineered malate dehydrogenase (MDH) gene, *MEA*, into the mutant to enhance the rTCA cycle yielded a final strain that produced SA at 7.07 g/L (Stols et al., 1997).

The glyoxylate pathway was reconstructed to produce SA. Five genes, including *pta-ackA*, *pqo*, *cat*, and *ldhA*, were first deleted. A recombinant *C. glutamicum* strain expressing the PYC gene *pyc*, PEPC gene *ppc*, ICL gene *aceA*, malate synthase gene *aceB*, CS gene *gltA*, and succinate exporter gene *SucE* produced 0.93 M SA, with a productivity of 9.4 mM/h and yield of 1.32 mol/mol glucose (Zhu et al., 2014).

#### 4.4. Manipulation of reducing power balance and cofactor engineering

The NAD<sup>+</sup>-coupled formate dehydrogenase gene (*fdh*) catalyzes NADH formation. The *fdh* and pyruvate carboxylase (*pyc*<sup>P458S</sup>) genes were integrated into the chromosome of *C. glutamicum* BOL-1 ( $\Delta$ *pta-ackA*,  $\Delta$ *pqo*,  $\Delta$ *cat*,  $\Delta$ *ldhA*), and the glyceraldehyde 3-phosphate dehydrogenase gene (*gapA*) was overexpressed from plasmid. The final strain produced SA at 133 g/L in 53 h with a yield of 1.67 mol/mol glucose (Litsanov et al., 2012). *E. coli* BA16 was derived from BA002, which lacked the *ldhA* and *pflB* genes and overexpressed *pncB*, which encodes a rate-limiting enzyme for NAD(H) synthesis and *pyc* from *L. lactis*. The strain produced 25 g/L SA under anaerobic fermentation in a 3-L bioreactor (Ma et al., 2013).

Pantothenate kinase was expressed in combination with PEPC or PYC in two strains to increase cytosol acetyl-CoA and CoA levels. Both strains showed improved SA production with a titer of approximately 20 mM (Lin et al., 2004).

#### 4.5. Strain improvement with in silico modeling

The construction of *E. coli* strains must always use several engineering strategies to coordinate the carbon flux for SA production, by-product elimination with energy and reducing power balance to achieve the highest SA yield. A mutant strain, SBS550MG, lacking *adhE*, *ldhA*, *ack-pta*, and *iclR* was constructed to express *pyc*. The strain produced SA at a yield of 1.59 mol/mol. When the *adhE*, *ldhA*, and *ack-pta* gene mutant strain SBS990MG expressed *citZ*, SA fermentation yield reached 1.58 mol/mol (Sanchez et al., 2005b). Further expression of *fdh1*, encoding NAD<sup>+</sup>-dependent formate dehydrogenase, and *pycA* from *L. lactis* improved SA productivity to 2 g/L per hour when formate was present in the fermentation culture owing to NADH and CO<sub>2</sub> supplementation through formate degradation (Balzer et al., 2013).

Three pathways were redesigned in *E. coli* GJT001 for SA production. The TCA cycle was blocked by deleting *sdhAB* and *icd*, the glyoxylate cycle was enhanced by disrupting the ICL depressor gene *iclR*, and the acetate pathway was inhibited via deletion of *poxB* and *ackA-pta*. The final strain, HL27615k, produced SA under aerobic conditions at a yield of 0.7 mol/mol glucose (Lin et al., 2005a). Strain HL27659k ( $\Delta$ *sdhAB*,  $\Delta$ *ackA-pta*,  $\Delta$ *poxB*,  $\Delta$ *iclR*,  $\Delta$ *ptsG*), expressing *pepc* from

*S. vulgare*, increased the SA yield to 0.94 mol/mol (Lin et al., 2005c). When the *pepc*<sup>SD</sup> mutant was expressed instead of natural *pepc*, the strain achieved an SA yield of 1.09 mol/mol (Lin et al., 2005b). Further study showed that strain HL27659K had higher CS, ICL, MDH, and malate synthase activities (Lin et al., 2005d).

According to the CAASOP (computational approach for strain optimization aiming at high productivity) analysis of *E. coli* ZJG13 ( $\Delta$ *sdhA*,  $\Delta$ *ackA-pta*,  $\Delta$ *poxB*,  $\Delta$ *mgsA*,  $\Delta$ *iclR*), the strain was transformed with plasmid pT184pyc to overexpress pyruvate carboxylase. The SA production of the constructed strain was 36.1 g/L (Yang et al., 2014).

*Mannheimia succiniciproducens* MBEL55E, a capnophilic bacterium, natively accumulates SA as a major product at a yield of 0.68 g/g glucose (Lee et al., 2002). The genome sequence of the strain and in silico genome-scale metabolic network were reported (Hong et al., 2004). Based on this work, *ldhA*, *pflB*, *pta*, and *ackA* were disrupted to reduce by-product formation, and the final strain, *M. succiniciproducens* LPK7, produced SA at 52.4 g/L, with a productivity of 1.8 g/L per hour (Lee et al., 2006).

#### 4.6. Engineering *S. cerevisiae* for SA production

Acid-tolerant *S. cerevisiae* was selected as an SA production host (Raab et al., 2010). Through deletion of the succinate dehydrogenase subunits *SDH1* and *SDH2*, isocitrate dehydrogenase subunit *IDH1*, and mitochondrial isocitrate dehydrogenase isoenzyme *IDP1*, the engineered strain enhanced the glyoxylate pathway and achieved an SA titer of 3.62 g/L at a yield of 0.11 mol/mol glucose.

With the help of a genome-scale metabolic model, two pathways involving three targets, including *Sdh3p* encoding succinate dehydrogenase complex and *Ser3p/Ser33p* encoding 3-phosphoglycerate dehydrogenase, were predicted to consume SA and needed to be deleted. The three-gene disrupted mutant was selected through directed evolution for faster growth. Finally, a plasmid-harbored isocitrate lyase gene, *icl1p*, was transformed into the strain. The constructed strain, 8D Evolved (pICL1), produced SA at a titer of 0.9 g/L (Otero et al., 2013).

### 5. Fumaric acid

FA (2-butenedioic acid trans; 1,2-ethylenedicarboxylic acid) is a valuable platform chemical used mainly in the food and beverage industries (Xu et al., 2012a,b,c). It is also used as a starting material for the synthesis of polymers and resins because it has a carbon–carbon double bond and two carboxylic acid groups (Roa Engel et al., 2008). FA is used to treat the skin condition psoriasis and is commonly added to cattle feed as an antibacterial factor and physiologically active agent (McGinn et al., 2004).

Two metabolic pathways can be used to produce FA (Xu et al., 2013). The first is the rTCA cycle, which provides a maximum theoretical yield of 2 mol FA per mole glucose through CO<sub>2</sub> fixation. However, the energy and reducing power are unbalanced, which causes cell maintenance and acid transport problems. The second pathway is the oxidative TCA cycle, which limits the maximum theoretical yield to 1 mol FA per mole glucose but provides a stable recombinant strain (Xu et al., 2012a,b,c). *Rhizopus* species such as *Rhizopus arrhizus*, *Rhizopus delemar*, and *Rhizopus oryzae*, can produce high levels of FA (Das and Brar, 2014; Wen et al., 2013; Zhou et al., 2011; Zhou et al., 2014).

#### 5.1. Engineering to enhance metabolic flux toward the rTCA and TCA cycles

Enhancing FA production by yeast involves the modification of two pathways (Fig. 2C). One is the rTCA cycle, in which the maximum theoretical FA yield is 2 mol per mole glucose, and the other is the oxidative TCA cycle.

The rTCA cycle was introduced in *S. cerevisiae* by overexpressing genes encoding MDH and FUM from *R. oryzae* and endogenous PYC. Compared with the control, the recombinant strain expressing PYC and MDH produced a higher titer of FA. However, the production was low at 3.18 g/L due to energy imbalance (Xu et al., 2012a).

Simultaneous introduction of the oxidative and reductive routes to *S. cerevisiae* strain FMME003, a pyruvate-producing strain, yielded FA at a titer of 5.64 g/L. In this study, native *fum1* involved in the oxidative route was knocked out, and *RoMDH* and *RoFUM1* from *R. oryzae*, which are involved in the reductive route, were overexpressed; the expression of *RoPYC* showed extreme importance in the balancing of intermediate metabolites for final FA production (Xu et al., 2013).

#### 5.2. Systems metabolic engineering

The oxidative TCA cycle has been enhanced to manage the problem of energy deficiency and improve FA production. Flux balance analysis with a genome-scale metabolic model predicted that mitochondrial FUM1 deletion, pyruvate carboxylase expression, and succinate-fumarate transporter SFC1 introduction in *S. cerevisiae* would enhance FA synthesis. The metabolic engineered strain improved FA production step by step when deletion/insertion was conducted in turn, achieving a final titer of 1.67 g/L (Xu et al., 2012b).

A pyruvate-producing strain of *Torulopsis glabrata* was engineered to produce FA. According to the genome-scale metabolic model, four cytosolic enzymes—argininosuccinate lyase in the urea cycle; adenylosuccinate lyase involved in purine metabolism; fumarylacetoacetase involved in tyrosine, tryptophan, and phenylalanine metabolism, and FUM1 in the TCA cycle—were overexpressed. The recombinant strain with high-level expression of argininosuccinate lyase and low-level expression of adenylosuccinate lyase showed high FA production of 5.62 g/L. Further expression of the *SpMAE1* gene, which encoded a C<sub>4</sub>-dicarboxylic acid transporter, increased the FA titer to 8.83 g/L (Chen et al., 2015).

Gu et al. (2014) proved that the hypothetical protein AOX mediates the alternative respiration pathway of *R. oryzae*, which is required for FA production. The alternative respiration pathway was essential in replenishing redundant redox power to produce CA in *A. niger* (Papagianni, 2007). The role of the pathway for FA synthesis, which lacked reducing power for high production, was interesting. Systems biology will determine the mechanism in future studies.

#### 5.3. Enhancement of the rTCA cycle in *R. oryzae*

A strategy of increasing precursor OAA has been used with the aim of enhancing FA production. Overexpressing exogenous PEPC in *R. oryzae* successfully increased FA yield by 26% at 1.21 mol per mole glucose, with a titer of 24 g/L. However, the endogenous pyruvate carboxylase-expressing mutants produced low levels of FA with poor growth due to ATP shortage and pyruvate flux imbalance (Zhang et al., 2012a). Endogenous fumarase was overexpressed in *R. oryzae* to eliminate by-product MA, but adversely, the mutant strain increased MA production at a yield of 0.38 g/g, which is twice that of the wild type (Zhang and Yang, 2012b).

#### 5.4. Combined enhancement of the noncyclic glyoxylate route with the rTCA cycle in *E. coli*

The noncyclic glyoxylate route has been used to produce FA in *E. coli*. The isocitrate lyase repressor gene *iclR* was deleted to enhance the carbon flow into a glyoxylate shunt. *fumA*, *fumB*, and *fumC* were knocked out for FA accumulation. Endogenous *ppc* encoding PEPC was overexpressed to increase rTCA cycle flux. Additionally, *arcA*, *aspA*,

*lacL*, and *ptsG* were deleted. The FA production of the final strain was 28.2 g/L, with a productivity of 0.448 g/L per hour (Song et al., 2013).

## 6. Malic acid

MA (2-hydroxybutanedioic acid; 2-hydroxysuccinic acid) has wide applications in the food and beverage industries as an acidulant and flavor enhancer (Goldberg et al., 2006). Compared with CA, MA provides a softer taste. MA also has nonfood applications in metal cleaning and finishing, textile finishing, cosmetics, pharmaceuticals, hospital infusions, and paints.

Microbial fermentation of MA as a by-product of yeast fermentation dates back to 1924. As a key constituent of the TCA cycle, MA is generated by variety of microorganisms including bacteria, yeast, and fungi, among which *Aspergillus flavus* is a well-known producer, achieving 63% of the highest theoretical yield of MA on glucose (Battat et al., 1991). Nevertheless, *A. flavus* is disqualified for industrial MA production due to aflatoxin production. Other *Aspergillus* species and *Schizophyllum commune* are also candidate MA producers, but their yields and productivity are low (Kawagoe et al., 1997). Through random mutagenesis and screening processes, a mutant malate-producing strain that lacks alcohol dehydrogenase activity and belongs to *R. delemar* has been isolated. The final mutant, HF-121, produced 120 g/L MA, with a productivity of 2.03 g/L/h—the highest productivity reported to date (Li et al., 2014).

Four possible metabolic pathways exist for MA production (Goldberg et al., 2006; Zelle et al., 2008). The first is the rTCA pathway, in which pyruvate is converted to OAA by pyruvate carboxylase and subsequently transformed to MA by MDH. The pathway is ATP neutral and involves CO<sub>2</sub> fixation, leading to a maximum theoretical yield of 2 mol MA per mole glucose (Fig. 2D). The second pathway involves the classic TCA cycle. Because two CO<sub>2</sub> molecules are released via the oxidation of CA to MA, the theoretical yield is limited to 1 mol per mole glucose. The third route involves the glyoxylate cycle in which OAA is replenished by MA and the theoretical yield is 1 mol per mole glucose due to CO<sub>2</sub> release during the conversion of pyruvate to acetyl-CoA. The fourth route is a noncyclic glyoxylate shunt, in which OAA is supplied via pyruvate carboxylation leading to a theoretical yield of 1.33 mol/mol glucose. When yield and simplicity are considered, the cytosolic rTCA pathway is optimal and has been used for the metabolic design of MA production in *E. coli*, *S. cerevisiae*, and *Aspergillus oryzae*.

### 6.1. Combined enhancement of the rTCA cycle and transporter engineering

Isolated by directed evolution, a pyruvate decarboxylase-negative (*Pdc*<sup>−</sup>) *S. cerevisiae* strain, which was alcohol-eliminated and produced high levels of pyruvate, was selected as a platform to introduce *PYC2* encoding the cytoplasmic pyruvate carboxylase gene *MDH3* encoding the MDH gene, in which the peroxisomal targeting sequence was modified for cytosol location, and *SpMAE1* encoding the malate transporter gene from *Schizosaccharomyces pombe*. When these modifications were introduced simultaneously, the engineered strain improved MA production significantly to a titer of 59 g/L at a yield of 0.42 mol/mol glucose, both of which were the highest reported for *S. cerevisiae*. However, the strain generated large amounts of by-products including glycerol, pyruvate, SA, and FA (Zelle et al., 2008).

*A. oryzae*, which has generally recognized as safe (GRAS) status, is a basic MA producer. Despite large transforming and screening challenges, this fungus was used to overexpress a native C<sub>4</sub>-dicarboxylate transporter, pyruvate carboxylase, and MDH. Microarray experiments that revealed the expression profiles of several candidate genes during the fermentation were coupled with protein localization prediction by WoLF PSORT software to identify the primary genes involved in the cytosolic rTCA pathway and MA transportation. Overexpression of the C<sub>4</sub>-dicarboxylate transporter (C4T318) greatly improved MA production to

122 g/L. Additional expression of the native cytosolic pyruvate carboxylase and MDH yielded an additional 27% increase in the MA production rate. The final recombinant strain achieved the highest-ever MA titer of 154 g/L, with a high yield of 1.38 mol/mol on glucose (Brown et al., 2013). Transcription levels of the three genes—pyruvate carboxylase, MDH, and the MA transporter—were evaluated with quantitative polymerase chain reaction. The results indicated that the genes were integrated once, three times, and twice, respectively, into the genome. Thus, further expression of *pyc* might lead to an additional improvement in MA production. Because small amounts of SA and CA are produced as by-products, future strategies to improve MA production may be based on the formation mechanisms of SA and CA. The cytosol route of the rTCA branch from MA via FA to SA must be verified. One means of reducing CA formation may be to knock out the tricarboxylate transporter, which exchanges cytosolic MA for mitochondrial CA; alternatively, the export capacity of MA can be increased (Knuf et al., 2014).

### 6.2. Engineering of *E. coli* for MA production

When two additional genes, *poxB* and *mgsA*, were knocked out in *E. coli* KJ060 to generate strain KJ071, the mutant produced MA at a titer of 62.9 g/L, with a yield of 1.42 mol/mol. However, SA also accumulated in large amounts as a by-product (Jantama et al., 2008a). A new strategy for enhancing MA production in *E. coli* was reported by Moon et al. (2008). The results of metabolic flux analysis suggested that increased PEP carboxylation flux facilitates MA production. When energy neutrality was considered, PEP carboxykinase was chosen instead of PEP carboxylase to engineer the *E. coli* strain. The *pta* mutant strain WGS-10, which limited the conversion of acetyl-CoA to acetate, was constructed by transforming the PEP carboxykinase gene *pckA* from *M. succiniciproducens*. Although the final titer was low at 9.25 g/L, yield and productivity were considerable at 0.74 mol/mol and 0.75 g/L per hour, respectively.

Zhang et al. (2011) also developed a succinate-producing *E. coli* KJ060 strain for MA production. OAA was generated through PEP carboxylation by *pck* and subsequently reduced to MA, which was converted to FA and then SA. Deletion of fumarase isoenzymes *fumB* and *fumAC* and especially fumarate reductase (*frdBC*) redirected the carbon flow toward MA. By-products, including lactate, acetate, and ethanol, were inhibited by the deletion of eight additional genes. The final engineered strain, XZ658, produced MA in a high yield of 1.42 mol/mol glucose in a two-stage fermentation.

### 7. Oxaloacetate

OAA (2-oxosuccinic acid) is a four-carbon dicarboxylic acid-like material that is a starting material in the TCA cycle and a precursor of amino acid synthesis (Kwon et al., 2007). Attempts to improve OAA production have been achieved by engineering *E. coli* to overexpress codon-optimized PEPC genes from *Dunaliella salina* (Park et al., 2013) and *Photobacterium profundum* SS9 (Park et al., 2014). The strain produced OAA at a yield of approximately 5% mol/mol glucose and MA at a yield of 0.9% mol/mol (Park et al., 2013).

## 8. Conclusions and perspective

Bacteria, yeasts, and filamentous fungi are candidates for organic acid production through metabolic engineering (see Table 1). Bacteria such as *E. coli* are well-established industrial production hosts (Yu et al., 2011). They grow fast, their genetic backgrounds are clear, and many feasible genetic tools have been developed (Kirchner and Tauch, 2003). However, these prokaryotic cells require neutral pH growth conditions and consequently secrete the salt forms of organic acids, which require cost-intensive acidification and precipitation for conversion to the desired products. Yeasts—for instance, *S. cerevisiae*—are also good candidate biocatalysts for organic acid production. They are generally

**Table 1**  
Production of organic acids in terms of performance and engineering strategies.

| Strain  | Engineering strategy  | Culture method                   | Titer (g/L) | Yield (mol/mol)   | Productivity (g/L/h) | By-product                         | References                  |
|---|---|----------------------------------|-------------|-------------------|----------------------|------------------------------------|-----------------------------|
| <b>Citric acid</b>  |   |                                  |             |                   |                      |                                    |                             |
| <i>A. niger</i> Δ1-3  | Δ <i>ggsA</i>   | Aerobic, batch                   | 115         | ns                | ns                   | ns                                 | Arisan-Atac et al. (1996)   |
| <i>A. niger</i> NW185   | Δ <i>goxC17</i> , Δ <i>prtF28</i>   | Aerobic, batch                   | 90          | 1.15 <sup>a</sup> | 0.375                | No oxalate                         | Ruijter et al. (1999)       |
| <i>A. niger</i> 50-2-12   | ↑ <i>pfl</i> , ↑ <i>pki</i>   | Aerobic, batch                   | 55          | 0.64              | 0.33                 | ns                                 | Ruijter et al. (1997)       |
| <i>A. niger</i> 55-13   | ↑ <i>citA</i>   | Aerobic, batch                   | 46          | 0.54              | 0.27                 | ns                                 | Ruijter et al. (2000)       |
| <i>A. niger</i> Frds(V)-FumRs   | ↑ <i>Frds1</i> , ↑ <i>FumRs</i>   | Aerobic, batch                   | 20          | 0.43              | 0.10                 | Oxalate                            | de Jongh and Nielsen (2008) |
| <i>Y. lipolytica</i> H222-S4 (p67ICL1) T5   | ↑ <i>SUC2</i> , ↑ <i>ICL1</i>   | Aerobic, batch                   | 140         | 1.46 <sup>a</sup> | 0.73                 | ns                                 | Forster et al. (2007)       |
| <i>Y. lipolytica</i> 30   | Δ <i>ACL1</i> , ↑ <i>ICL1</i> , ↑ <i>INU1</i>   | Aerobic, batch                   | 84          | 0.93 <sup>b</sup> | 0.39                 | Iso-citrate                        | Liu et al. (2013)           |
| <i>Y. lipolytica</i> 87   | ↑ <i>INU1</i>   | Aerobic, batch                   | 68.9        | ns                | 0.22                 | Iso-citrate                        | Liu et al. (2010)           |
| <b>α-Ketoglutaric acid</b>  |   |                                  |             |                   |                      |                                    |                             |
| <i>Y. lipolytica</i> H355A (PYC1-IDP1) T5   | ↑ <i>PYC1</i> , ↑ <i>IDP1</i>   | Aerobic, pH control, fed-batch   | 186         | 0.23 <sup>c</sup> | 1.59                 | Pyruvate                           | Yovkova et al. (2014)       |
| <i>Y. lipolytica</i> H355A (FUM1-PYC1) T4   | ↑ <i>FUM1</i> , ↑ <i>PYC1</i>   | Aerobic, pH control, fed-batch   | 138         | ns                | 1.21                 | Malate, pyruvate                   | Otto et al. (2012)          |
| <i>Y. lipolytica</i> -RoPYC2  | Thiamine <sup>+</sup> , ↑ <i>PYC2</i>   | Two-stage pH control, fed-batch  | 62.5        | ns                | 0.434                | Pyruvate                           | Yin et al. (2012)           |
| <i>Y. lipolytica</i> -ACL   | ↑ <i>ACL</i>  | Two-stage pH control, batch      | 56.5        | 0.36 <sup>c</sup> | 0.39                 | Pyruvate                           | Zhou et al. (2012)          |
| <i>Y. lipolytica</i> T1   | ↑ <i>PDA1</i>   | Aerobic, pH control, batch       | 42.5        | ns                | 0.29                 | Pyruvate                           | Guo et al. (2014)           |
| <b>Succinic acid</b>  |   |                                  |             |                   |                      |                                    |                             |
| <i>M. succiniciproducens</i> LPK7   | Δ <i>ldhA</i> , Δ <i>pflB</i> , Δ <i>pta</i> , Δ <i>ackA</i>  | Anaerobic, fed-batch             | 52.4        | 1.16              | 1.8                  | Pyruvate, malate                   | Lee et al. (2006)           |
| <i>C. glutamicum</i> (Δ <i>ldhA</i> -pCRA717)   | Δ <i>ldhA</i> , ↑ <i>pyc</i>  | Micro-aerobic, fed-batch         | 146         | 1.4               | 3.17                 | Acetate                            | Okino et al. (2008)         |
| <i>C. glutamicum</i> BOL-3  | Δ <i>cat</i> , Δ <i>pqo</i> , Δ <i>pta-ackA</i> , Δ <i>ldhA</i> , ↑ <i>pyc</i> , ↑ <i>fdh</i> , ↑ <i>gapA</i>   | Anaerobic, fed-batch             | 134         | 1.67              | 2.52                 | ns                                 | Litsanov et al. (2012)      |
| <i>C. glutamicum</i> SA5  | Δ <i>cat</i> , Δ <i>pqo</i> , Δ <i>pta-ackA</i> , Δ <i>ldhA</i> , ↑ <i>pyc</i> , ↑ <i>ppc</i> , ↑ <i>aceA</i> , ↑ <i>aceB</i> , ↑ <i>gltA</i> , ↑ <i>sucE</i> | Anaerobic, fed-batch             | 109         | 1.32              | 1.11                 | ns                                 | Zhu et al. (2014)           |
| <i>C. glutamicum</i> ZX1 (pEacsA <i>gltA</i> )  | Δ <i>sdhCAB</i> , Δ <i>ldhA</i> , Δ <i>pqo</i> , Δ <i>cat</i> , Δ <i>pta</i> , <i>acsA</i> , <i>gltA</i> , ↑ <i>ppc</i> , ↑ <i>pyc</i>                        | Aerobic, fed-batch               | 28.5        | 0.63              | 0.42                 | Pyruvate                           | Zhu et al. (2013)           |
| <i>E. coli</i> AFP111/pTrc99A- <i>pyc</i>   | Δ <i>ptsG</i> , Δ <i>pfl</i> , Δ <i>ldh</i> , ↑ <i>pyc</i>  | Anaerobic, fed-batch, dual-phase | 99.2        | 1.1               | 1.3                  | Ethanol                            | Vemuri et al. (2002b)       |
| <i>E. coli</i> KJ060  | Δ <i>ldhA</i> , Δ <i>adhE</i> , Δ <i>ackA</i> , Δ <i>focA</i> , Δ <i>pflB</i>   | Anaerobic, fed-batch             | 86.6        | 1.41              | 0.72                 | Acetate, malate                    | Jantama et al. (2008a)      |
| <i>E. coli</i> KJ122  | Δ <i>ldhA</i> , Δ <i>adhE</i> , Δ <i>focA-pflB</i> , Δ <i>mgsA</i> , Δ <i>poxB</i> , Δ <i>tdcDE</i> , Δ <i>citF</i> , Δ <i>aspC</i> , Δ <i>sfcA</i>           | Anaerobic, fed-batch             | 82.7        | 1.5               | 0.9                  | ns                                 | Jantama et al. (2008b)      |
| <i>E. coli</i> HL27659k (pKK313)  | Δ <i>sdhAB</i> , Δ <i>ackA-pta</i> , Δ <i>poxB</i> , Δ <i>icIR</i> , Δ <i>ptsG</i> , ↑ <i>pepc</i>  | Aerobic, fed-batch               | 58.3        | 0.85              | 1.08                 | Pyruvate, acetate                  | Lin et al. (2005c)          |
| <i>E. coli</i> ZJG13 (pT184 <i>pyc</i> )  | Δ <i>sdhA</i> , Δ <i>ackA-pta</i> , Δ <i>poxB</i> , Δ <i>mgsA</i> , Δ <i>icIR</i> , ↑ <i>pyc</i>  | Aerobic, fed-batch               | 36.1        | 0.72              | 0.694                | Acetate, lactate, KGA              | Yang et al. (2014)          |
| <i>E. coli</i> BA016  | Δ <i>ldhA</i> , Δ <i>pflB</i> , ↑ <i>pncB</i> , ↑ <i>pyc</i>  | Anaerobic, fed-batch             | 25          | 1.09              | 0.223                | Pyruvate                           | Ma et al. (2013)            |
| <i>E. coli</i> SBS550MG-Cms243 (pHL413KF1)  | Δ <i>adhE</i> , Δ <i>ldhA</i> , Δ <i>ack-pta</i> , Δ <i>icIR</i> , ↑ <i>fdh1</i> , ↑ <i>pycA</i>  | Anaerobic, shake flask           | 20.3        | 1.74              | ns                   | Formate, acetate                   | Balzer et al. (2013)        |
| <i>E. coli</i> SBS550MG (pHL413)  | Δ <i>adhE</i> , Δ <i>ldhA</i> , Δ <i>ack-pta</i> , Δ <i>icIR</i> , ↑ <i>pyc</i>   | Repeated feeding bioreactor      | 40          | 1.59              | 1.18                 | Formate, acetate                   | Sanchez et al. (2005b)      |
| <i>E. coli</i> SBS990MG (pHL413 + pHL531)   | Δ <i>adhE</i> , Δ <i>ldhA</i> , Δ <i>ack-pta</i> , ↑ <i>pyc</i> , ↑ <i>citZ</i>   | Anaerobic, batch                 | 18.2        | 1.58              | 0.76                 | Formate, acetate                   | Sanchez et al. (2005b)      |
| <i>E. coli</i> SBS110MG (pHL413)  | Δ <i>ldhA</i> , Δ <i>adhA</i> , ↑ <i>pyc</i>  | Anaerobic, batch                 | 15.6        | 1.27              | 0.65                 | Formate, acetate                   | Sanchez et al. (2005a)      |
| <i>E. coli</i> AFP111   | Δ <i>pfl</i> , Δ <i>ldh</i> , Δ <i>ptsG</i>   | Anaerobic, batch                 | 12.8        | 0.7               | ns                   | Acetate, ethanol                   | Donnelly et al. (1998)      |
| <i>E. coli</i> XZ721  | <i>pck*</i> Δ <i>pflB</i> , Δ <i>ptsI</i>   | Anaerobic, batch                 | 12          | 0.80 <sup>c</sup> | 0.083                | Acetate, lactate,                  | Zhang et al. (2010)         |
| <i>E. coli</i> JCL1208  | ↑ <i>PEPC</i>   | Anaerobic, batch                 | 10.7        | 0.44              | 0.59                 | Ethanol, acetate, lactate, formate | Millard et al. (1996)       |
| <i>E. coli</i> MG1655 Δ <i>adhE</i> , Δ <i>pta</i> , Δ <i>poxB</i> , Δ <i>ldhA</i> (pZS- <i>pyc</i> ) | Δ <i>adhE</i> , Δ <i>pta</i> , Δ <i>poxB</i> , Δ <i>ldhA</i> , ↑ <i>pyc</i>   | Microaerobic, batch              | 8.8         | 0.47 <sup>c</sup> | 0.183                | Acetate, pyruvate                  | Blankschien et al. (2010)   |
| <i>E. coli</i> AFP111/pTrc99A- <i>pyc</i>   | Δ <i>ptsG</i> , Δ <i>pfl</i> , Δ <i>ldh</i> , ↑ <i>pyc</i>  | Anaerobic, batch                 | 8.0         | 1.46              | 0.286                | Acetate                            | Vemuri et al. (2002a)       |
| <i>E. coli</i> NZN111 (pMEA1)   | Δ <i>pfl</i> , Δ <i>ldh</i> , ↑ <i>MEA</i>  | Anaerobic, batch                 | 7.07        | 0.60              | 0.29                 | Ethanol, pyruvate                  | Stols et al.                |

Table 1 (continued)

| Strain  | Engineering strategy  | Culture method                                       | Titer (g/L) | Yield (mol/mol) | Productivity (g/L/h) | By-product                              | References                   |  |
|---|---|--|-------------|-----------------|----------------------|---|------------------------------|--|
| <i>E. coli</i> HL51276k (pKK313)  | $\Delta sdhAB, \Delta ackA-pta, \Delta poxB, \Delta iclR, \Delta ptsG, \uparrow pepc^{S8D}$   | Aerobic, batch                                       | 6.72        | 1.09            | 0.14                 | Pyruvate, acetate                       | (1997)<br>Lin et al. (2005b) |  |
| <i>E. coli</i> HL27615k   | $\Delta sdhAB, \Delta ackA-pta, \Delta poxB, \Delta icd, \Delta iclR$   | Aerobic batch  | 5.07        | 0.70            | 0.061                | Acetate, pyruvate                       | Lin et al. (2005a)           |  |
| <i>E. coli</i> YBS132 (pHL333, pHL413)  | $\Delta ack-pta, \uparrow PEPC, \uparrow PYC$   | Anaerobic, batch                                     | 3.4         | 0.3             | 0.14                 | Acetate, ethanol                        | Lin et al. (2005e)           |  |
| <i>S. cerevisiae</i>  | AH22ura3 $\Delta sdh1\Delta sdh2\Delta idh1\Delta idp1$   | $\Delta sdh1, \Delta sdh2, \Delta idh1, \Delta idp1$ | Shake flask | 3.62            | 0.11                 | 0.022                                   | Ethanol, glycerol, acetate   |  |
| Raab et al. (2010)<br><i>S. cerevisiae</i> 8D evolved (pICL1)                           | $\Delta sdh3, \Delta ser3, \Delta ser33, \uparrow icl$  | Aerobic, batch                                       | 0.9         | 0.076           | ns                   | ns                                      | Otero et al. (2013)          |  |
| Fumaric acid<br><i>R. oryzae</i> ppc  | $\uparrow pepc$   | Batch  | 24          | 1.21            | 0.25                 | Malate                                  | Zhang et al. (2012a)         |  |
| <i>R. oryzae</i> fumR2  | $\uparrow fumR2$  | Batch  | 21.5        | 1.00            | 0.22                 | Malate                                  | Zhang and Yang (2012b)       |  |
| <i>S. cerevisiae</i> FMME004-6  | $\Delta fum1, \uparrow RoPYC, \uparrow RoMDH, \uparrow RoFUM1$  | Shake flask  | 5.64        | 0.197           | ns                   | Ethanol, glycerol, pyruvate             | Xu et al. (2013)             |  |
| <i>S. cerevisiae</i> FMME-001 $\uparrow PYC2 + \uparrow RoMDH$                          | $\uparrow MDH, \uparrow PYC2$   | Aerobic, batch                                       | 3.18        | 0.10            | ns                   | Ethanol, glycerol, malate               | Xu et al. (2012a)            |  |
| <i>S. cerevisiae</i> FMME-002 $\Delta FUM1 + \uparrow RoPYC + \uparrow SFC1$            | $\Delta fum1, \uparrow PYC, \uparrow SFC1$  | Aerobic, batch                                       | 1.67        | 0.051           | 0.017                | ns                                      | Xu et al. (2012b)            |  |
| <i>E. coli</i> CWF812   | $\Delta iclR, \Delta fumC, \Delta fumA, \Delta fumB, \Delta arcA, \Delta ptsG, \Delta aspA, \Delta lacI, \uparrow ppc$                            | Fed-batch  | 28.2        | 0.59            | 0.448                | Acetic acid                             | Song et al. (2013)           |  |
| <i>T. glabrata</i><br>T.G-ASL <sub>(H)</sub> -ADSL <sub>(L)</sub> -SpMAE1               | $\uparrow ASL, \uparrow ADSL, \uparrow SpMAE1$  | Shake flask  | 8.83        | 0.23            | 0.12                 | Pyruvate                                | Chen et al. (2015)           |  |
| Malic acid<br><i>A. oryzae</i> SaMF2103a-68   | $\uparrow pyc, \uparrow mdh, \uparrow C4T318$   | Batch  | 154         | 1.38            | 0.94                 | Succinate                               | Brown et al. (2013)          |  |
| <i>S. cerevisiae</i> RWB525 $\uparrow PYC2 + \uparrow MDH3\Delta SKL + \uparrow SpMAE1$ | $\Delta Pdc, \uparrow PYC2, \uparrow MDH3, \uparrow SpMAE1$   | Shake flask  | 59          | 0.42            | 0.19                 | Glycerol, succinate, pyruvate, fumarate | Zelle et al. (2008)          |  |
| <i>E. coli</i> KJ071  | $\Delta ldhA, \Delta adhE, \Delta ackA, \Delta focA, \Delta pflB, \Delta mgsA$  | Anaerobic, fed-batch                                 | 69.2        | 1.4             | 0.72                 | Succinate, pyruvate, acetate            | Jantama et al. (2008a)       |  |
| <i>E. coli</i> XZ658  | $\Delta ldhA, \Delta ackA, \Delta adhE, \Delta pflB, \Delta mgsA, \Delta poxB, \Delta frdBC, \Delta sfcA, \Delta maeB, \Delta fumB, \Delta fumAC$ | Two-stage process                                    | 34          | 1.42            | 0.47                 | Lactate                                 | Zhang et al. (2011)          |  |
| <i>E. coli</i> WGS-10 (p104ManPck)  | $\Delta ldhA, \Delta pta, \Delta adh, \uparrow pckA$  | Batch  | 9.25        | 0.75            | 0.77                 | ns                                      | Moon et al. (2008)           |  |
| Oxaloacetate<br><i>E. coli</i> SGJS115  | $\uparrow DsPEPC$   | Shake flask  | ns          | 0.05            | ns                   | Malate, succinate                       | Park et al. (2013)           |  |

ns, not specified.

<sup>a</sup> mol/mol sucrose.<sup>b</sup> g/g inulin.<sup>c</sup> mol/mol glycerol.

regarded as safe, robust, and tolerant to acid, and they grow both aerobically and anaerobically on diverse carbon sources and chemically defined media. Moreover, genetic engineering tools, many omics tools, and synthetic biology approaches are available (Yadav et al., 2012), and complimentary methods for directed evolution and selection are well established (Otero et al., 2013). Filamentous fungi can spontaneously produce high levels of diverse organic acids. However, the tremendous differences in genetic backgrounds complicate the design of metabolic engineering strategies. In addition, the available genetic tools for manipulating metabolic pathways are limited compared to those for bacteria and yeasts, and targeting genes to the right genome locus is laborious. Nevertheless, advances and improvements in genetic tools (Delmas et al., 2014) and the application of omics analysis tools make engineered fungi candidates for environmentally responsible and economically feasible fermentations processes for the production of organic acids.

As shown in Fig. 3, the strategies for enhancing organic acid production can be summarized as follows: (1) overexpression of rate-limiting

enzymes or the introduction of new pathways to increase acid biosynthesis; (2) blocking of pathways for the synthesis of competing by-products; (3) overexpression of transporters to facilitate secretion; (4) coordination of sufficient energy, cofactor production, and supplement pathways for target product synthesis; and (5) elimination of inhibitor effects to enhance synthesis pathways. However, difficulties remain in the optimization of biological systems for designed tasks. First, optimizing the amount of carbon flux may require perfectly balanced enzymes expression levels, and the enzyme conversion direction in different environments and enzyme substrate specificity complicate the synthetic situation (Ma et al., 2014; Seo et al., 2013). Synthetic biology may provide approaches for optimizing a number of components, including genes from different sources and their mutants, devices, and novel pathways, in predictable and controllable manners to make biological production platforms more efficient (Seo et al., 2013).

Second, maintaining robustness and improving the product tolerance of microorganisms qualified for industrial production is critical. With various system-wide tools, including omics analyses (genome,

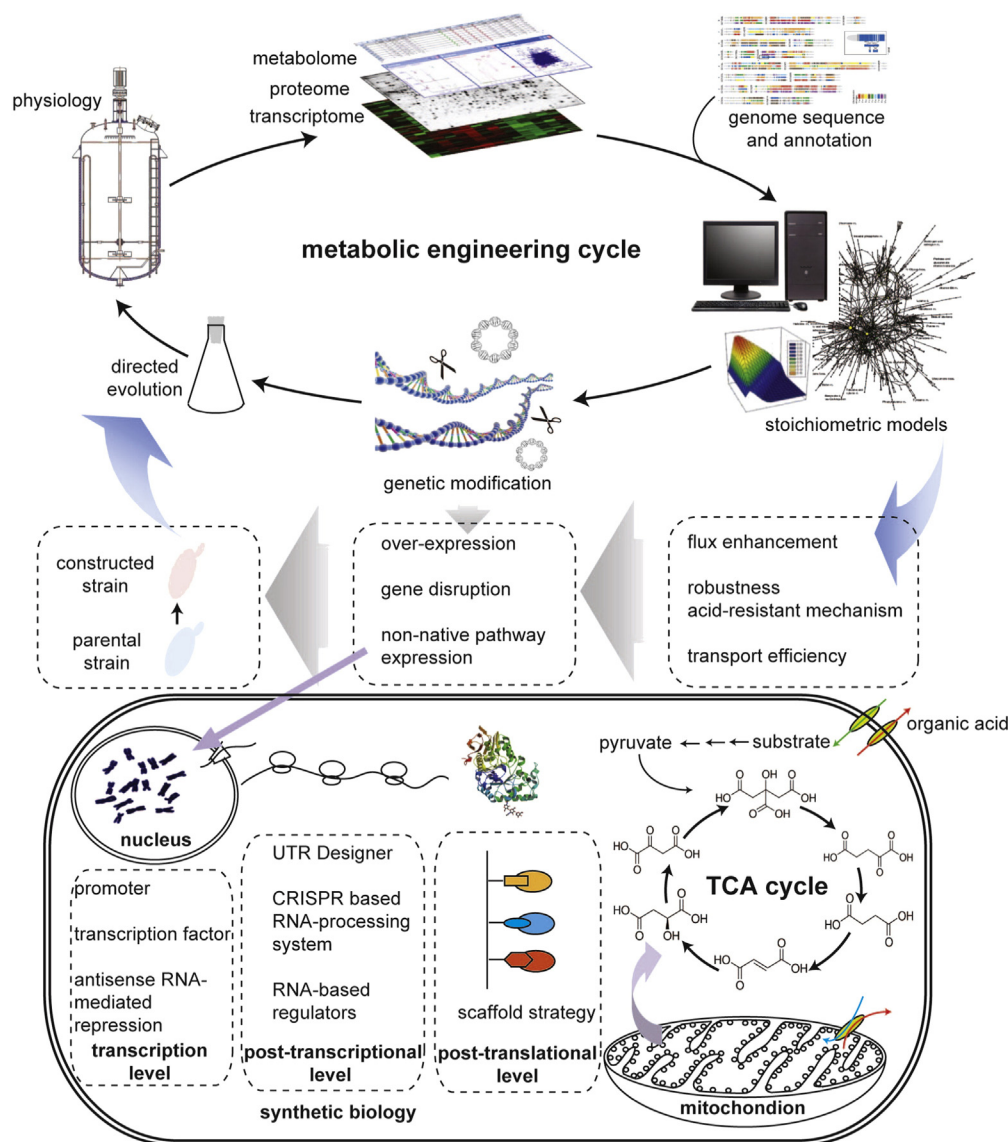


Fig. 3. Systems biology and synthetic biology guided metabolic engineering of microorganisms for organic acid production.

transcriptome, proteome, metabolome, and fluxome) and in silico modeling and simulation, metabolic engineering at the system level could help coordinate energy generation and cell growth with product synthesis (Chen et al., 2015; Cox et al., 2006; Kalinowski et al., 2003; Otero et al., 2013; Wang et al., 2013; Yu et al., 2012a). Systems biology may also detail stress responses to acids or salts, which can guide engineering to solve product resistance problems.

Third, the mechanisms of resource absorption, organic acid transportation from mitochondrion to cytosol, and secretion into the broth are rarely understood. With the help of omics technology, the putative pump candidates will be recognized and the mechanisms disclosed. Synthesis efficiency can be improved through transporter engineering that enhances substrate and product transportation.

Fourth, the compartmentalization of metabolic pathways in eukaryotes highlights the possibility of moving to the mitochondrion the syntheses of organic acids that usually take place in the cytosol. The benefits of compartmentalization are higher local concentrations of enzymes, substrates, and intermediates and weaker influence from competing pathways (Avalos et al., 2013).

Finally, it is important to pursue high yield and productivity as well as production to meet the economic needs of industry. Economic

production depends on an optimized combination of substrate absorption and assimilation, metabolic pathways, and transportation of intermediates and products.

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