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Metabolic evolution of two reducing equivalent-conserving pathways for high-yield succinate production in *Escherichia coli*

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ABSTRACT

Reducing equivalents are an important cofactor for efficient synthesis of target products. During metabolic evolution to improve succinate production in *Escherichia coli* strains, two reducing equivalent-conserving pathways were activated to increase succinate yield. The sensitivity of pyruvate dehydrogenase to NADH inhibition was eliminated by three nucleotide mutations in the *lpdA* gene. Pyruvate dehydrogenase activity increased under anaerobic conditions, which provided additional NADH. The pentose phosphate pathway and transhydrogenase were activated by increased activities of transketolase and soluble transhydrogenase SthA. These data suggest that more carbon flux went through the pentose phosphate pathway, thus leading to production of more reducing equivalent in the form of NADPH, which was then converted to NADH through soluble transhydrogenase for succinate production. Reverse metabolic engineering was further performed in a parent strain, which was not metabolically evolved, to verify the effects of activating these two reducing equivalent-conserving pathways for improving succinate yield. Activating pyruvate dehydrogenase increased succinate yield from 1.12 to 1.31 mol/mol, whereas activating the pentose phosphate pathway and transhydrogenase increased succinate yield from 1.12 to 1.33 mol/mol. Activating these two pathways in combination led to a succinate yield of 1.5 mol/mol (88% of theoretical maximum), suggesting that they exhibited a synergistic effect for improving succinate yield.

1. Introduction

Succinate is a bulk chemical that has been identified by the U.S. Department of Energy as one of the top 12 building block chemicals (Werpy and Petersen, 2004). Succinate and its derivatives have many applications in chemical, food, agricultural and plastics industries, with a potential market of $15 billion (McKinlay et al., 2007). Considerable interest in microbial production of succinate from sugars has emerged over the past two decades (Balzer et al., 2013; Jantama et al., 2008a; Jantama et al., 2008b; Lee et al., 2005; Lee et al., 2006; McKinlay et al., 2007; Samuelov et al., 1991; Sanchez et al., 2005; Scholten et al., 2009; Singh et al., 2011; Stols and Donnelly, 1997; Vemuri et al., 2002; Wang et al., 2011; Zhu et al., 2013).

In addition to inactivating competitive fermentation pathways, such as pyruvate formate-lyase (PflB), lactate dehydrogenase (LdhA) and aldehyde/alcohol dehydrogenase (AdhE) (Jantama et al., 2008a; Jantama et al., 2008b; Lee et al., 2005; Sanchez et al., 2005; Stols and Donnelly, 1997; Vemuri et al., 2002), supplies of energy, precursor and reducing equivalents are three key factors for efficient succinate production. First, net ATP formation is essential to maintain cell growth and metabolism. Several native microorganisms produce high amounts of succinate, utilizing phosphoenolpyruvate (PEP) carboxykinase (PCK) as the main enzyme for PEP carboxylation (Lee et al., 2006; Samuelov et al., 1991). This enzyme can conserve the high energy of PEP, thus leading to net ATP formation during succinate production (Zhang et al., 2009a). In contrast, *pck* gene expression is repressed by glucose in *Escherichia coli* but is activated in the reverse direction (gluconeogenesis) during the oxidative metabolism of organic acids (Kao et al., 2005; Oh et al., 2002). *E. coli* utilize PEP carboxylase (PPC) as the main enzyme for PEP carboxylation during glucose fermentation and waste the high energy of PEP, thus leading to zero ATP formation during succinate production (Zhang et al., 2009a). Recruiting PCK for PEP carboxylation in *E. coli* can solve the energy supply problem and improve succinate production significantly (Zhang et al., 2009a; Zhang et al., 2009b).

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Second, PEP is an essential precursor for succinate synthesis. However, the uptake and phosphorylation of glucose in *E. coli* are performed by PEP: carbohydrate phosphotransferase system (PTS), which consumes one mol of PEP for transport and phosphorylation of one mol of glucose (Flores et al., 1996; Postma et al., 1996). The evolution of *E. coli* strains having competitive fermentation pathways inactivated to improve succinate production resulted in mutation of either *ptsG* (encoding integral membrane permease $IICB^{Glc}$ of glucose-specific enzyme II^{Glc} complex of PTS) or *ptsI* (encoding non-sugar specific enzyme I of PTS) (Chatterjee et al., 2001; Zhang et al., 2009a). Both mutations lead to the inactivation of the PTS system, thus increasing PEP supply for succinate production.

Third, sufficient supply of reducing equivalents is essential to obtain the maximum yield of target fermentation products. The theoretical maximum yield for succinate is 1.71 mol per mol glucose if no exogenous electron is supplied (McKinlay et al., 2007; Vemuri et al., 2002). However, under anaerobic glucose fermentation conditions, only two mol of NADH is produced from one mol glucose, whereas the synthesis of one mol succinate requires two mol of NADH. Thus, only one mol succinate can be produced under this condition. Pyruvate dehydrogenase (PDH) activity in anaerobic *E. coli* cultures is either very low or undetectable (Snoep et al., 1993). If this enzyme is activated, additional NADH can be produced, leading to the production of 1.33 mol succinate (Vemuri et al., 2002). The oxidative TCA cycle and glyoxylate bypass are inactive in anaerobic *E. coli* cultures (Sanchez et al., 2005). If these enzymes are activated, the succinate yield can increase to 1.71 mol/mol (Sanchez et al., 2005; Vemuri et al., 2002).

In this study, *E. coli* was engineered for succinate production by inactivating competitive fermentation pathways and increasing energy and precursor supplies. Metabolic evolution was then performed to increase cell growth and succinate production. Through genome sequencing and transcriptome analysis, two reducing equivalent-conserving pathways were identified as responsible for increased succinate yield. PDH was activated by mutation of lipoamide dehydrogenase gene (*lpdA*). In addition, more carbon flux went through the pentose phosphate pathway (PPP), resulting in more NADPH production, which was then converted to NADH by transhydrogenase SthA for succinate production. Reverse metabolic engineering was further performed in the parent strain to verify the effectiveness of these two reducing equivalent-conserving pathways.

2. Materials and methods

2.1. Strains, media, and growth conditions

The strains constructed in this study are listed in Table 1. During strain construction, strains were cultured aerobically at 30 or 37 °C in Luria broth (10 g liter^{-1} Difco tryptone, 5 g liter^{-1} Difco yeast extract and 10 g liter^{-1} NaCl). Ampicillin (100 mg liter^{-1}), kanamycin (25 mg liter^{-1}), or chloramphenicol (17 mg liter^{-1}) were used where appropriate.

2.2. Genetic methods

Two-step recombination methods were used for markerless gene deletion and gene modulation (Jantama et al., 2008a; Shi et al., 2013). Red recombinase technology (Gene Bridges GmbH, Dresden, Germany) was used to facilitate chromosomal gene deletion and modulation (Datsenko and Wanner, 2000). The details of genetic methods were described in Text S1. All plasmids are listed in Table S1, and primers are listed in Table S2.

Table 1
E. coli strains used in this study.

Strains	Characteristics[a]
ATCC 8739	Wild type
Suc-T110	ATCC 8739, Δ*ptsI*, Δ*ldhA*, Δ*pflB*, *Ppck*-galP*, *Ppck*-pck*
NZ-035	Suc-T110, Δ*ackA-pta*
NZ-036	Suc-T110, Δ*ackA-pta*, *Ppck*-aceBA*
NZ-037	Suc-T110, Δ*ackA-pta*, *Ppck*-aceBA*, *Ppck*-dcuC*
HX021	Metabolic evolution of NZ-037 for 1080 generations
HX023	HX021, Δ*mgsA*
HX024	Metabolic evolution of HX023 for 360 generations
HX041	HX024, Δ*pck*
HX042	HX024, Δ*sfcA*
HX043	HX024, Δ*maeB*
HX044	HX024, Δ*ppc*
NZ-038	Suc-T110, Δ*ackA-pta*::*M1-93-lpdA**
NZ-041	Suc-T110, *M1-93-aceEF*, Δ*ackA-pta*::*M1-93-lpdA**
ZT-251	Suc-T110, *M1-37-tktA*
ZT-252	Suc-T110, *M1-37-sthA*
ZT-253	Suc-T110, *M1-37-tktA*, *M1-37-sthA*
ZT-273	ZT-253, *M1-93-aceEF*, Δ*ackA-pta*::*M1-93-lpdA**

[a] *Ppck** represented mutated *pck* promoter (included −378 to −1 and G to A transition at position −64 relative to the ATG start codon) (Zhang et al., 2009a). M1-93 and M1-37 were artificial regulatory parts constructed previously, and their strengths were 5 and 2.5 times of induced *E. coli lacZ* promoter (Lu et al., 2012).

2.3. Fermentation

Fresh colonies were picked from New Brunswick Scientific (NBS) (Zhang et al., 2009a) or AM1 (Jantama et al., 2008a) mineral salts plates containing 20 g liter^{-1} glucose, inoculated into 250 ml flasks containing 100 ml mineral salts medium with 50 g liter^{-1} glucose, and grown at 37 °C and 100 rpm for 12 h. Cultures were then transferred to a 500-ml fermentation vessel containing 250 ml mineral salts medium with different concentrations of glucose. The initial OD_{550} was 0.1.

For strains Suc-T110, NZ-035, NZ-036 and NZ-037, fermentations were carried out in NBS mineral salts medium containing 5% glucose and 100 mM potassium bicarbonate. The pH was maintained at 7.0 by automatic addition of a base containing 2.4 M potassium carbonate and 1.2 M potassium hydroxide. It should be noted that a higher succinate titer of strain Suc-T110 was obtained in this work (280 mM) compared with that reported previously (226 mM) (Tan et al., 2013). This might be caused by different water qualities. In our previous study, deionized water provided by our institute was used for fermentation. In this study, water was made using Ultrapure Water Purification System (MERCK MILLIPORE, Milli Q Reference).

For metabolic evolution of strain NZ-037, AM1 mineral salts medium containing 10% glucose and 35 mM sodium bicarbonate were used during 1–780 generations. Sodium salt (2.4 M Na_2CO_3 and 1.2 M NaOH) was used as the neutralizer to maintain the pH and provide CO_2 for succinate production. The initial glucose concentration increased to 12% during 781–1080 generations. For metabolic evolution of strain HX023, AM1 mineral salts medium containing 12% glucose and 35 mM sodium bicarbonate were used, and strain HX024 was obtained after 360 generations.

To compare the succinate-producing capacities of strains NZ-037 and HX024, fermentations were carried out in AM1 mineral salts medium containing 12% glucose and 35 mM sodium bicarbonate. The pH was maintained at 7.0 by automatic addition of a base containing 2.4 M sodium carbonate and 1.2 M sodium hydroxide.

2.4. Genome sequencing

The genomic DNA of strain HX024 was extracted using Bacterial gDNA Kit (Biomiga, US). DNA quality was determined using

ND-1000 (NanoDrop Technologies). Genome sequencing was performed by BGI (Beijing, China).

2.5. Transcriptome sequencing

HX024 and *E. coli* ATCC 8739 cells were collected at the mid-log phase. Cultures were fixed with 2:1 volumes of RNAprotect Bacteria (Qiagen) and harvested. RNA was isolated from the pellet using RNeasy Mini kit (Qiagen). Genomic DNA was removed using DNAse I (Qiagen). RNA quality was determined using Agilent 2100 Bioanalyser (Agilent) and quantified using ND-1000 (NanoDrop Technologies). Transcriptome sequencing was performed by BGI (Beijing, China).

2.6. Enzyme assay

The activities of PCK, PDH, MDH, GltA, AceA, AceB, TktA, SthA, aconitate hydratase and fumarase were determined as described previously (Van der Werf et al., 1997; Chin et al., 2009; Honer Zu Bentrup et al., 1999; Iida et al., 1993; Kennedy et al., 1983; Kim et al., 2008; Roucourt et al., 2009; Samuelov et al., 1991; Underwood et al., 2002).

Crude extracts were prepared from cells harvested during the mid-log phase of fermentation. The collected cells were washed thrice with cold 50 mM potassium phosphate buffer with 1 mM dithiothreitol (DTT; pH 7.0) and re-suspended in the same buffer. The cells were disrupted with a Bead-Beater (Biospec, Bartlesville, Okla.), and the supernatant was harvested after centrifugation for 20 min at 30,000*g* at 4 °C. The concentration of crude enzyme was determined using the Bio-Rad Protein Assay Kit (Bio-Rad, USA). One unit (U) of enzyme activity represents the amount of enzyme catalyzing the conversion of 1 μmol of substrate per min into specific products. The U/mg protein refers to the mg crude protein extract. The details for determining the activity of each enzyme are described in Text S1.

2.7. Analysis

The dry weight of cells was calculated by measuring the optical density value at 550 nm (OD_{550}). Organic acids and residual glucose in the fermentation broth were measured by high-performance liquid chromatography (Zhang et al., 2009a). The product titers were normalized by arithmetically factoring out the volume of base solution that was added to the fermentors for pH control. The product titer=(the real product titer) × (starting volume+added volume)/(starting volume).

3. Results

3.1. Metabolic engineering of E. coli for succinate production

E. coli ATCC 8739 was previously engineered for succinate production through (1) the deletion of *ldhA* and *pflB* genes to inactivate competitive fermentation pathways, (2) the deletion of *ptsI* gene and activation of an alternative glucose utilization pathway (Galactose permease, GalP) to increase PEP supply, and (3) the activation of PCK to increase energy supply (Tan et al., 2013). The resulting strain, Suc-T110 (Table 1), produced 280 mM succinate with a yield of 1.12 mol/mol (Table 2). In addition, 96 mM acetate was produced. The acetate kinase (*ackA*) and phosphate acetyltransferase (*pta*) genes were then deleted (Fig. 1), resulting in strain NZ-035, and the acetate production decreased 46% to 44 mM (Table 2). The remaining acetate might have been produced by either pyruvate oxidase (PoxB) or 2-ketobutyrate formate-lyase/pyruvate formate-lyase (TdcE) and propionate kinase/acetate kinase (TdcD) (Jantama et al., 2008b).

Table 2
Production of succinate by engineered *E. coli* strains.

Strain	Cell mass (g liter^{-1})	Suc yield (mol/mol)	Fermentation products (mM)[c]	
			Suc	Ace
Suc-T110[a]	1.53	1.12 ± 0.03	280 ± 10	96 ± 10
NZ-035[a]	1.51	1.16 ± 0.03	286 ± 7	44 ± 6
NZ-036[a]	1.48	1.19 ± 0.03	298 ± 6	27 ± 4
NZ-037[a]	1.50	1.28 ± 0.03	357 ± 7	25 ± 3
NZ-037[b]	0.88	1.25 ± 0.01	181 ± 13	37 ± 4
HX021[b]	2.43	1.27 ± 0.02	630 ± 5	19 ± 3
HX023[b]	2.12	1.28 ± 0.01	605 ± 29	17 ± 1
HX024[b]	2.75	1.36 ± 0.04	813 ± 28	21 ± 2

[a] Fermentations were carried out in NBS mineral salts medium containing 5% glucose and 100 mM potassium bicarbonate. The pH was maintained at 7.0 by automatic addition of a base containing 2.4 M potassium carbonate and 1.2 M potassium hydroxide.
[b] Fermentations were carried out in AM1 mineral salts medium containing 12% glucose and 35 mM sodium bicarbonate. The pH was maintained at 7.0 by automatic addition of a base containing 2.4 M sodium carbonate and 1.2 M sodium hydroxide.
[c] Three repeats were performed and the error bars represent standard deviation. Abbreviations: Suc, succinate; Ace, acetate.

Activating glyoxylate bypass had been suggested to increase succinate yield (Sanchez et al., 2005; Vemuri et al., 2002). The isocitrate lyase (*aceA*) and malate synthase (*aceB*) genes were thus activated by changing their native promoter to a mutated *pck* promoter (*Ppck**) (Fig. 1) (Zhang et al., 2009a). However, the succinate titer and yield of the resulting strain NZ-036 increased only 4% and 3% compared with the parent strain NZ-035 (Table 2). Dicarboxylate transporter DcuC was suggested to act primarily in succinate efflux during glucose fermentation (Chen et al., 2014; Zientz et al., 1999). Thus, this enzyme was activated by changing its native promoter with *Ppck** to improve succinate production (Fig. 1). The resulting strain NZ-037 produced 357 mM succinate with a yield of 1.28 mol/mol (Table 2). The succinate titer and yield increased 20% and 8% compared with the parent strain NZ-036.

3.2. Metabolic evolution to improve succinate production

Metabolic evolution had been successfully used to improve the production of many fermentation products, such as ethanol (Yomano et al., 2008), D-lactate (Zhou et al., 2006), L-lactate (Grabar et al., 2006), L-alanine (Zhang et al., 2007) and succinate (Jantama et al., 2008a). Succinate synthesis is the only fermentation pathway in strain NZ-037 to oxidize NADH. The cell growth of this strain was thus coupled with succinate production, as this was the only way to regenerate NAD^+ for continuous glycolysis to provide ATP for cell growth. Metabolic evolution was performed in strain NZ-037 to improve cell growth and succinate production. Because potassium salt is expensive for large-scale commercial production, sodium salt (2.4 M Na_2CO_3 and 1.2 M NaOH) was used as the neutralizer to maintain the pH and provide CO_2 for succinate production. In addition, the initial glucose concentration increased from 50 to 120 g/L. The starting strain NZ-037 produced 181 mM succinate with a yield of 1.25 mol/mol (Table 2). After evolution for 1080 generations, strain HX021 was obtained (Fig. 2). This strain produced 630 mM succinate with a yield of 1.27 mol/mol (Table 2). The methylglyoxal synthase gene (*mgsA*) was further deleted in HX021 to eliminate lactate production (Grabar et al., 2006). The resulting strain HX023 produced 605 mM succinate with a yield of 1.28 mol/mol (Table 2). Deletion of the *mgsA* gene had nearly no effect on succinate production. Strain HX023 was

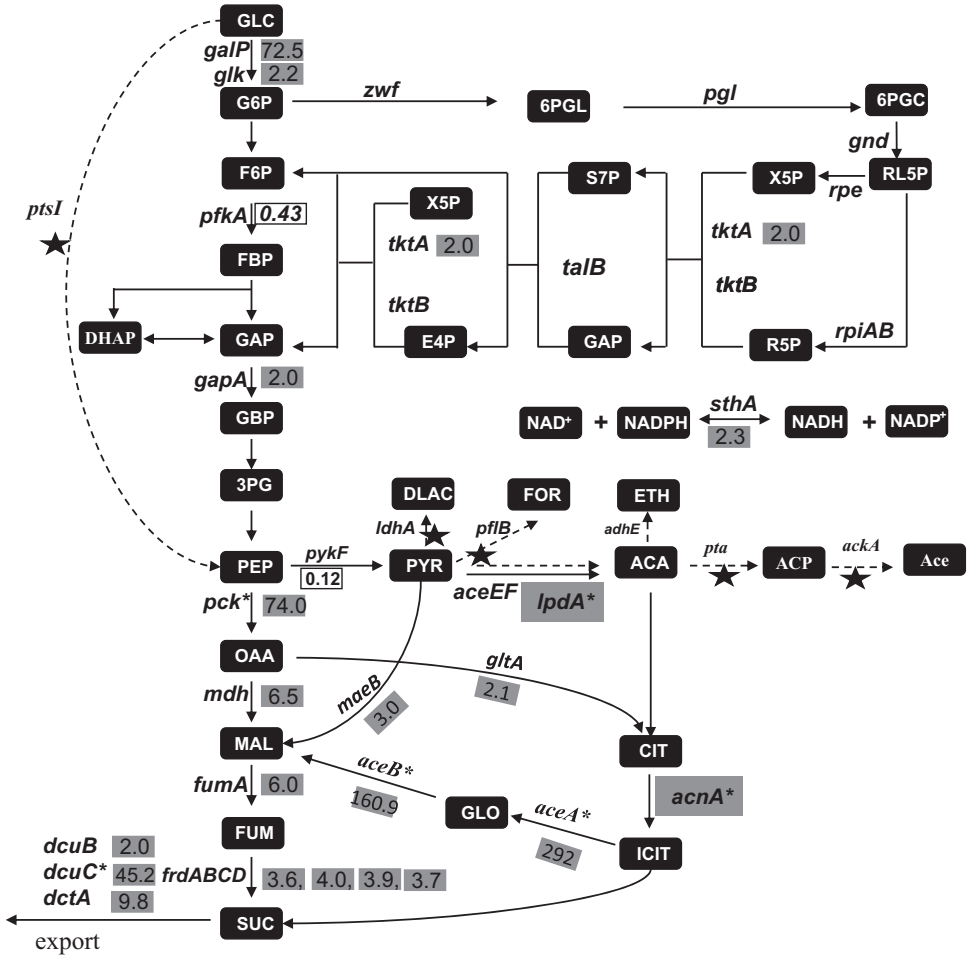

Fig. 1. Results of comparative transcriptome profiling. The numbers are the ratios of the expression levels in HX024 vs. ATCC 8739. The shaded and boxed numbers indicate significantly up- and down-regulated genes, respectively, in strain HX024. GLC, glucose; G6P, glucose-6-phosphate; 6PGL, gluconolactone-6-phosphate; 6PGC, 6-phospho gluconate; RL5P, ribulose-5-phosphate; X5P, xylurose-5-phosphate; R5P, ribose-5-phosphate; S7P, sedoheptulose-7-phosphate; E4P, erythrose-4-phosphate; F6P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetonephosphate; GBP, 1,3-bisphosphoglycerate; 3PG, 3-phosphoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate; ACA, acetyl-CoA; ACP, acetylphosphate; Ace, acetate; DLAC, D-lactate; FOR, formate; ETH, ethanol; OAA, oxaloacetate; CIT, citrate; ICIT, isocitrate; GLO, glyoxylate, MAL, malate; FUM, fumarate; SUC, succinate; NAD^+, oxidized nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NADH, reduced nicotinamide adenine dinucleotide; $NADP^+$, oxidized nicotinamide adenine dinucleotide phosphate.

further metabolically evolved for another 360 generations (Fig. 3). The resulting strain HX024 produced 813 mM succinate with a yield of 1.36 mol/mol (Table 2). The succinate titer and yield of strain HX024 increased 350% and 9% compared with parent strain NZ-037 (Table 2).

3.3. Genome sequencing analysis of strain HX024

To characterize genetic mechanisms for improved succinate production in strain HX024, genome sequencing and transcriptome and enzyme assay analysis were performed. Multiple genes exhibit mutations in strain HX024 compared with wild type ATCC 8739 (Table S4). Among these genes, *lpdA* and the aconitate hydratase (*acnA*) genes were directly related to succinate production (Fig. 1). LpdA is the E3 component of pyruvate dehydrogenase (PDH), and activation of PDH can provide additional NADH for succinate production (Vemuri et al., 2002). Three nucleotide mutations were observed in the coding region of *lpdA* gene (C242T, C823T, and C1073T), leading to three changed amino acids (T81I, P275S and A358V). AcnA is the second enzyme of the TCA cycle. Activation of the oxidative TCA cycle and glyoxylate bypass is an effective strategy to increase succinate yield (Sanchez et al., 2005; Vemuri et al., 2002). A single nucleotide mutation was observed in the coding region of the *acnA* gene (T209G), leading to a changed amino acid (L70R).

3.4. Characterization of the mutated LpdA

The PDH activity in anaerobic *E. coli* cultures is either very low or undetectable due to inhibition by NADH (Kim et al., 2007; Snoep et al., 1993). The NADH sensitivity of the PDH complex had been demonstrated to reside in the LpdA subunit, which interacts with NAD^+ as a substrate (Wilkinson and Williams, 1981). Recently, a mutated LpdA (E354K) was identified that was significantly less sensitive to NADH inhibition than the native one (Kim et al., 2008). This reduced NADH sensitivity of the mutated LpdA was translated into the lower sensitivity of the appropriate PDH complex to NADH inhibition (Kim et al., 2008).

The pyruvate dehydrogenase activity of strain HX024 under anaerobic glucose fermentation conditions was 0.34 U/mg protein, which was 26-fold higher than wild type *E. coli* ATCC 8739 (Table 3). When over-expressing mutated *lpdA* gene identified in this work (*lpdA**) using a low-copy plasmid, the PDH activity was 80% and 40% higher than the wild-type *lpdA* and *lpdA* (E354K), respectively (Fig. 4A). It was also significantly less sensitive to NADH inhibition (Fig. 4B). When overexpressing *lpdA**, PDH

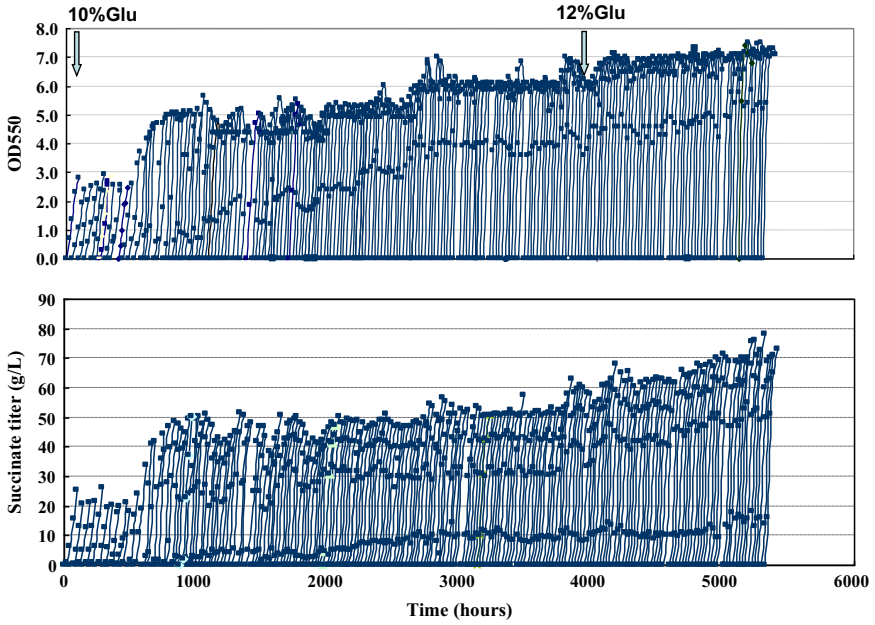

Fig. 2. Metabolic evolution of strain NZ-037 to produce HX021. Strain NZ-037 was sequentially transferred in AM1 medium containing 10% (w/v) and 12% (w/v) glucose to produce HX021. 2.4 M Na_2CO_3 and 1.2 M NaOH were used as the neutralizer to maintain pH and provide CO_2 for succinate production.

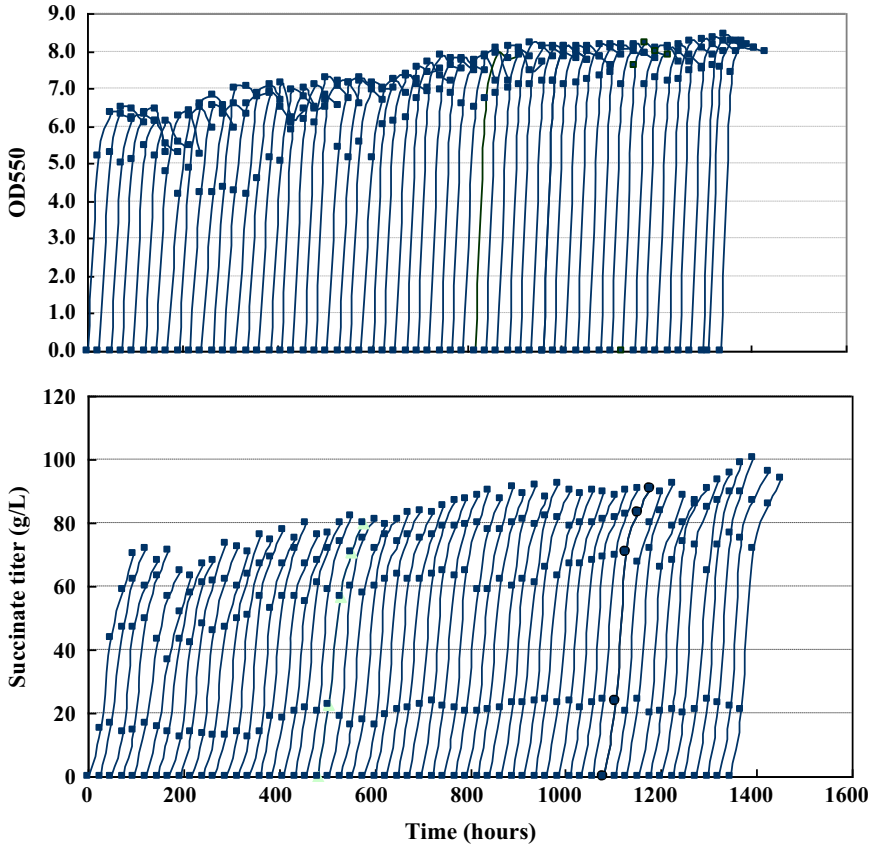

Fig. 3. Metabolic evolution of strain HX023 to produce HX024. Strain HX023 was sequentially transferred in AM1 medium containing 12% (w/v) glucose to produce HX024. 2.4 M Na_2CO_3 and 1.2 M NaOH were used as the neutralizer to maintain pH and provide CO_2 for succinate production.

activity with a NADH/NAD^+ ratio of 0.14 was 67% of the control (NADH/NAD^+ ratio was 0). In contrast, PDH activities with a NADH/NAD^+ ratio of 0.14 were 0% and 1% of the control when overexpressing wild-type *lpdA* and *lpdA* (E354K), respectively (Fig. 4B).

To characterize whether single nucleotide mutations could eliminate the NADH sensitivity of PDH, three plasmids containing each single nucleotide mutation were constructed. The PDH activities of strains over-expressing *lpdA* (T81I), *lpdA* (P275S) and *lpdA* (A358V) were 35%, 83% and 113% of the strain over-expressing *lpdA** (Fig. 5A). The PDH activities of strains over-expressing *lpdA* (T81I) and *lpdA* (P275S) decreased to zero, with a NADH/NAD^+ ratios of 0.04 and 0.12, respectively (Fig. 5B). In contrast, the PDH activities of the strain over-

Table 3
Enzyme assay analysis of strain ATCC 8739 and HX024.

	Enzyme activity (μmol/mg min)[a]		Relative activity[b]
	ATCC 8739	HX-024	
Pyruvate dehydrogenase	0.013 ± 0.002	0.34 ± 0.02	26
Transketolase	0.12 ± 0.01	1.32 ± 0.06	11
Transhydrogenase	0.03 ± 0.00	0.09 ± 0.01	3
PEP carboxykinase	0.10 ± 0.02	2.10 ± 0.08	21
Malate dehydrogenase	0.22 ± 0.03	1.77 ± 0.07	8
Citrate synthase	0.07 ± 0.01	0.49 ± 0.02	7
Aconitate hydratase	0.86 ± 0.05	3.71 ± 0.09	4.3
Isocitrate lyase	0.37 ± 0.04	0.45 ± 0.03	1.22
Malate synthase	0.30 ± 0.02	0.29 ± 0.01	0.97

[a] Three repeats were performed and the error bars represent standard deviation.
[b] Relative activity represented enzyme activities of strain HX024 compared to wild type *E. coli* ATCC 8739.

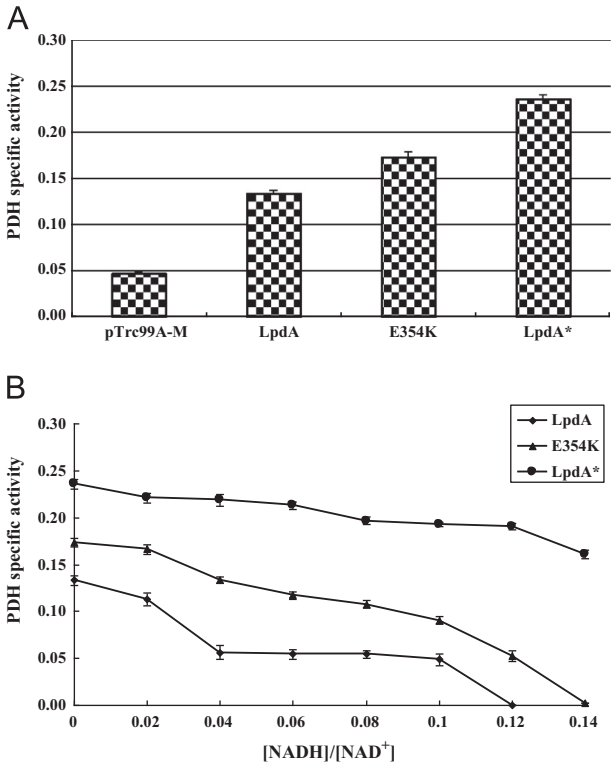

Fig. 4. Characterization of the mutated LpdA. Three repeats were performed, and the error bars represented standard deviation. (A) Comparison of the PDH activities of strains containing the wild-type and two mutated LpdA. (B) The NADH sensitivity of the PDH complexes of strains containing the wild-type and two mutated LpdA.

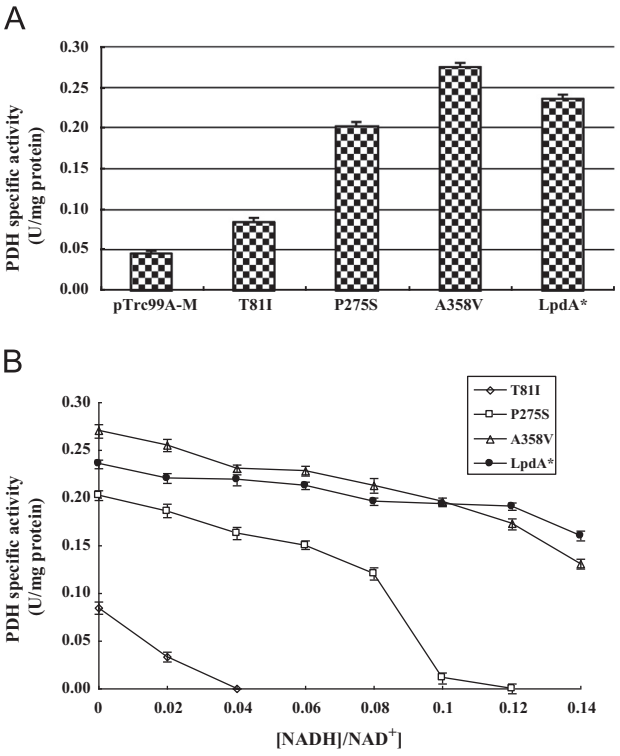

Fig. 5. Analysis of the three nucleotide mutations of *lpdA**. Three repeats were performed, and the error bars represent standard deviation. (A) Comparison of the PDH activities of strains each containing single nucleotide mutation and all three mutations. (B) The NADH sensitivity of the PDH complexes of strains each containing single nucleotide mutation and all three mutations.

expressing *lpdA* (A358V) retained 63% and 48% activity, with a NADH/NAD^+ ratios of 0.12 and 0.14, respectively (Fig. 5B). These data suggested that A358V was the most important mutation site for elimination of the NADH sensitivity of PDH.

3.5. Transcriptome and enzyme assay analysis of strain HX024

Transcriptome analysis was performed between strain HX024 and wild type *E. coli*. The expression levels of 18 genes within nine modules closely related to succinate production had changed (Fig. 1, Table S3). (a) The expression levels of the *galP* and glucose kinase (*glk*) genes (Glucose utilization module) increased 72.5 and 2.2-fold, respectively. (b) The expression levels of the *pck* and NADPH-dependent malic enzyme (*maeB*) genes (Carboxylation module) increased 74.0 and 3.0-fold, respectively. (c) The expression levels of the malate dehydrogenase (*mdh*), fumarase A (*fumA*), fumarate reductase (*frdA*, *frdB*, *frdC* and *frdD*) genes (Reductive TCA module) increased 6.5, 6.0, 3.6, 4.0, 3.9 and 3.7-fold, respectively. (d) The expression level of the citrate synthase (*gltA*) gene (Oxidative TCA module) increased 2.1-fold. (e) The expression levels of the *aceB* and *aceA* genes (Glyoxylate bypass module) increased 160.9 and 292.0-fold, respectively. (f) The expression level of the transketolase I (*tktA*) gene (Pentose phosphate module) increased 2.0-fold. (g) The expression levels of the 6-phosphofructokinase I (*pfkA*) and pyruvate kinase I (*pykF*) genes decreased 57% and 88%, respectively, whereas the expression level of the glyceraldehyde 3-phosphate dehydrogenase A (*gapA*) gene (Glycolysis module) increased 2.0-fold. (h) The expression level of the transhydrogenase (*sthA*) gene (Transhydrogenase module) increased 2.3-fold. (i) The expression levels of the *dcuB*, *dcuC* and *dctA* genes (Succinate export module) increased 2.0, 45.2 and 9.8-fold, respectively.

The activities of several important enzymes within the pentose phosphate, transhydrogenase, PEP carboxylation, reductive TCA, TCA and glyoxylate bypass modules were also analyzed (Table 3). The transketolase, transhydrogenase, PCK, malate dehydrogenase (MDH), citrate synthase (GltA) and aconitate hydratase (ACN) activities in strain HX024 were 11, 3, 21, 8, 7 and 4.3-fold higher, respectively, than in strain ATCC 8739. The isocitrate lyase (AceA) and malate synthase (AceB) activities exhibited nearly no changes.

The data suggested that succinate productivity increased in strain HX024 due to the increased activities of glucose utilization, PEP carboxylation, reductive TCA and succinate export. On the other hand, two reducing equivalent-conserving pathways were activated that were responsible for increased succinate yield. The activation of PDH produced additional NADH when converting pyruvate to acetyl-CoA. The activation of PPP and the repression of the upstream glycolysis pathway provided more NADPH, which was then converted to NADH through transhydrogenase SthA.

Table 4
Activation of PDH, PPP and transhydrogenase in strain Suc-T110 to increase succinate yield.

Strains[a]	Genetic modification	PDH activity (μmol/mg min)	Cell mass (g/L)	Suc yield (mol/mol)	Fermentation products (mM) [b]	
					Suc	Ace
Suc-T110	Parent strain	0.013 ± 0.002	1.53	1.12 ± 0.03	280 ± 10	96 ± 10
NZ-035	Suc-T110, Δ*ackA-pta*	0.015 ± 0.001	1.51	1.16 ± 0.03	286 ± 7	44 ± 6
NZ-038	Suc-T110, *ackA*::*M1-93-lpdA**	0.29 ± 0.02	1.36	1.22 ± 0.04	304 ± 10	40 ± 5
NZ-041	Suc-T110, *ackA*::*M1-93-lpdA**, *M1-93-aceEF*	0.8 ± 0.02	1.40	1.31 ± 0.03	319 ± 6	35 ± 6
ZT-251	Suc-T110, *M1-37-tktA*	–	1.36	1.26 ± 0.02	290 ± 11	74 ± 6
ZT-252	Suc-T110, *M1-37-sthA*	–	1.24	1.27 ± 0.02	293 ± 13	64 ± 2
ZT-253	Suc-T110, *M1-37-tktA*, *M1-37-sthA*	–	1.22	1.33 ± 0.01	307 ± 4	56 ± 7
ZT-273	ZT253, *ackA*::*M1-93-lpdA**, *M1-93-aceEF*	–	1.52	1.50 ± 0.02	346 ± 10	18 ± 2

[a] Fermentations were carried out in NBS mineral salts medium containing 5% glucose and 100 mM potassium bicarbonate. The pH was maintained at 7.0 by automatic addition of a base containing 2.4 M potassium carbonate and 1.2 M potassium hydroxide. Three repeats were performed and the error bars represent standard deviation.
[b] Abbreviations: Suc, succinate; Ace, acetate.

3.6. Verification of the genetic mechanisms for high-yield succinate production

To verify that activation of PDH, PPP and transhydrogenase was responsible for increased succinate yields, the two pathways were activated in the parent strain Suc-T110, which was not metabolically evolved. The mutated *lpdA** gene was integrated into the *ackA* site, followed by modulation of its expression with regulatory part M1-93 (Lu et al., 2012; Zhao et al., 2013). The resulting strain, NZ-038, produced 304 mM succinate with a yield of 1.22 mol/mol (Table 4). The *aceEF* gene of strain NZ-038 was further modulated with M1-93. The resulting strain, NZ-041, exhibited a PDH activity of 0.8 U/mg protein, which was 62-fold higher than parent strain Suc-T110. Strain NZ-041 produced 319 mM succinate with a yield of 1.31 mol/mol (Table 4). The activation of PDH increased the succinate titer and yield by 12% and 13%, respectively.

Modulation of *tktA* gene individually in strain Suc-T110 with regulatory part M1-37 (Lu et al., 2012; Zhao et al., 2013) led to a 10-fold increase of transketolase activity (from 0.12 to 1.2 U/mg protein). The resulting strain, ZT-251, produced 290 mM succinate with a yield of 1.26 mol/mol, which was 4% and 13% higher than the parent strain, respectively. Modulation of the *sthA* gene individually in strain Suc-T110 with regulatory part M1-37 led to a 1.5-fold increase of transhydrogenase activity (from 0.03 to 0.045 U/mg protein). The resulting strain ZT-252 produced 293 mM succinate with a yield of 1.27 mol/mol, which was 5% and 13% higher than the parent strain, respectively. Modulation of the *tktA* and *sthA* genes in combination with regulatory part M1-37 resulted in strain ZT-253. This strain produced 307 mM succinate with a yield of 1.33 mol/mol, which was 10% and 19% higher than the parent strain, respectively (Table 4).

PDH was further activated in strain ZT-253 through the activation of the *lpdA** and *aceEF* genes. The resulting strain, ZT-273, produced 346 mM succinate with a yield of 1.5 mol/mol, which was 24% and 34% higher than parent strain Suc-T110, respectively (Table 4). Our results suggested that activating these two reducing equivalent-conserving pathways had a synergistic effect for improving succinate yield.

4. Discussion

4.1. Utilization of the pentose phosphate pathway for succinate production

A sufficient reducing equivalent supply is essential to obtain the maximum yield of target products. It has been suggested that the activation of pyruvate dehydrogenase, oxidative TCA cycle and glyoxylate bypass can lead to the theoretical maximum yield for succinate production (1.71 mol/mol) (Sanchez et al., 2005; Vemuri et al., 2002). In this work, a novel reducing equivalent-conserving pathway (PPP plus transhydrogenase) was identified that can provide more reducing equivalents for succinate production compared with the traditional glycolysis pathway. Increased activities of transketolase and soluble transhydrogenase suggested that more carbon flux went through PPP and SthA. In addition, because PPP also has a downstream glycolysis module, increased expression of the *gapA* gene would not influence the carbon flux ratio between glycolysis and PPP but would increase the glucose utilization rate. Consumption of one mol glucose through PPP can provide 1.67 NADH and 2 NADPH, while through glycolysis can provide only 2 NADH. The theoretical maximum succinate yield can be obtained if the carbon flux ratio between glycolysis and PPP is 14.3:85.7, which would produce 171.4 NADH and 171.4 NADPH (Fig. 6). NADPH can be converted to NADH through transhydrogenase SthA, thus leading to the production of 342.8 NADH and satisfying the requirement for producing 171.4 mol succinate.

The other benefit of using the PPP for succinate production is that less exogenous CO_2 is required. If all carbon flux goes through glycolysis and the reductive TCA cycle, one CO_2 is required for the synthesis of one succinate. By contrast, if 85.7% carbon flux goes through PPP, only 85.7 mol CO_2 is required for the synthesis of 171.4 mol succinate (Fig. 6). Less sodium carbonate would be required for succinate production, thus reducing the fermentation cost.

4.2. Pyruvate dehydrogenase for anaerobic production of other chemicals

The *lpdA* mutation identified in this work resulted in a decreased sensitivity of PDH to NADH inhibition, which might have wide applications for the production of other reduced chemicals, such as ethanol, butanol, 1,3-propanediol and so on. Activating PDH by increasing *aceEF* gene expression has been demonstrated to improve ethanol production in *E. coli* using the native ethanol synthetic pathway (Zhou et al., 2008). Four molecules NADH can be produced from one glucose molecule, which can satisfy the reducing equivalent requirement for the production of two ethanol molecules. Four NADH molecules are also needed for the production of one molecule butanol. Formate dehydrogenase has been overexpressed to increase NADH supply to improve butanol production (Shen et al., 2011). Activating PDH can also solve the redox balance problem for anaerobic butanol production. In addition, the theoretical maximum yield for anaerobic 1,3-propanediol production from glycerol is 0.67 mol/mol, as 2 NADH molecules are produced when converting one glycerol molecule to one pyruvate molecule, whereas one NADH molecule is required for converting one glycerol molecule to one 1,3-propanediol molecule. After activating PDH, the theoretical maximum yield

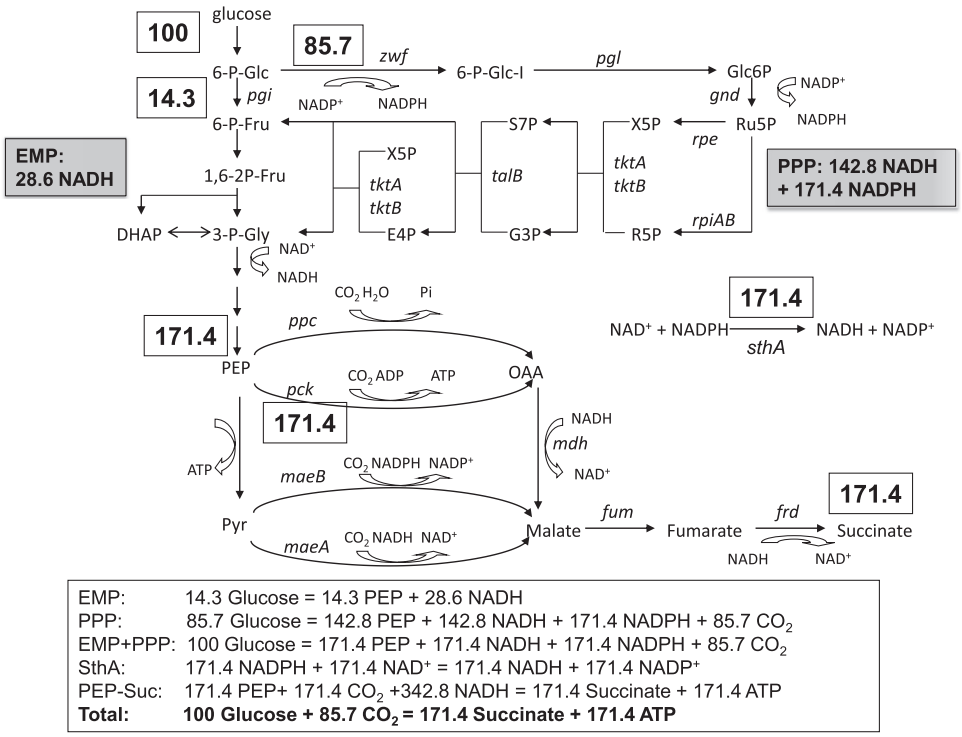

Fig. 6. Flux through biochemical pathways corresponding to the maximum theoretical yield of succinate using both glycolysis (EMP) and PPP modules. The data are based on 100 molar U of glucose uptake.

can increase to 0.75 mol/mol, as 3 NADH molecules can be produced from one glycerol molecule to one acetyl-CoA molecule.

4.3. Carboxylation pathways for succinate production in strain HX024

There are four carboxylation pathways in *E. coli* for succinate production (Zhang et al., 2009a). PEP can be carboxylated to oxaloacetic acid by either PCK or PEP carboxylase (PPC) (Tan et al., 2013; Zhang et al., 2009a). On the other hand, pyruvate can be directly carboxylated to malate by either NADH-dependent (SfcA) or NADPH-dependent (MaeB) malic enzyme (Kao et al., 2005; Oh et al., 2002; Zhang et al., 2009b). The PPC-catalyzed reaction would lose the high energy contained in PEP with the release of inorganic phosphate, whereas the other three carboxylation reactions can conserve the energy (Zhang et al., 2009a). A high succinate-producing strain, KJ073, was previously obtained through genetic engineering and metabolic evolution (Jantama et al., 2008a). Deleting the *ppc*, *sfcA* or *maeB* genes had nearly no effect on succinate production, whereas deleting the *pck* gene dramatically reduced cell growth, sugar metabolism and succinate production of strain KJ073 (Zhang et al., 2009a). Thus, PCK was suggested to be the main carboxylating enzyme for succinate production in strain KJ073, whereas the other three enzymes had nearly no contribution to succinate production (Zhang et al., 2009a).

To investigate which enzyme exhibited the major carboxylation function in strain HX024, the *pck*, *sfcA*, *maeB* and *ppc* genes were deleted individually (Table 5). Deletion of the *pck*, *sfcA* and *maeB* genes of strain HX024 resulted in a 38%, 29% and 49% decrease of the succinate titer, respectively (Table 5). In addition, surprisingly, the strain with the *ppc* gene deleted (HX044) was unable to grow in the fermentor if seeds were prepared in mineral salts medium. Although preparing seed with rich medium recovered cell growth in the fermentor, the succinate titer was decreased by 70% (Table 5). These data suggested that PPC is the main carboxylating enzyme for succinate production in strain HX024, although the other three enzymes also contributed to succinate production.

Table 5
Production of succinate by *E. coli* strains having inactivated carboxylation enzymes.

Strain[a]	Genetic modifications	Cell mass (g liter^{-1})	Suc yield (mol/mol)	Fermentation products (mM)[b]	
				Suc	Ace
HX024[c]	Parent strain	2.72	1.33 ± 0.02	798 ± 21	23 ± 2
HX041[c]	HX024, Δ*pck*	2.00	1.31 ± 0.03	492 ± 18	22 ± 2
HX042[c]	HX024, Δ*sfcA*	1.92	1.31 ± 0.02	405 ± 44	25 ± 3
HX043[c]	HX024, Δ*maeB*	2.18	1.33 ± 0.01	566 ± 31	20 ± 1
HX044[c]	HX024, Δ*ppc*	–	–	–	–
HX044[d]	HX024, Δ*ppc*	1.49	1.21 ± 0.04	241 ± 19	10 ± 1

[a] Fermentations were carried out in AM1 mineral salts medium containing 12% glucose and 35 mM sodium bicarbonate. The pH was maintained at 7.0 by automatic addition of a base containing 1.5 M sodium carbonate and 3 M sodium hydroxide. Three repeats were performed and the error bars represent standard deviation.
[b] Abbreviations: Suc, succinate; Ace, acetate.
[c] Seeds were prepared in mineral salts medium.
[d] Seeds were prepared in LB medium.

Strain KJ073 was obtained through metabolic evolution of KJ012. This initial strain grew very slowly in mineral salts medium because PCK was not activated and there was not sufficient ATP supply for succinate synthesis (Jantama et al., 2008a). The main selection pressure during evolution was to increase the ATP supply for better cell growth. Under this condition, PCK activity increased significantly, and PCK became the main carboxylating enzyme. In contrast, strain HX024 was obtained through metabolic evolution of NZ-037. This initial strain already had high PCK activity and grew well in mineral salts medium. The selection pressure during evolution was to increase cell growth by increased succinate productivity, which might be caused by enhanced capabilities of glucose transport, carboxylation, succinate transport and so on. Under this condition, all four carboxylases were utilized to

enhance the carboxylating capability and improve succinate production and cell growth.

4.4. Other genetic modifications in strain HX024 to improve succinate production

In addition to the two reducing equivalent-conserving pathways, several other genes were activated during metabolic evolution, leading to increased succinate productivity. Increased *galP* and *glk* genes expression activated an alternative non-PTS glucose utilization module, thus increasing the PEP supply and glucose utilization rate (Tang et al., 2013). Decreased *pykF* gene expression reduced carbon flux from PEP to pyruvate, thus also increasing the PEP supply. Increased *gapA* gene expression activated the downstream glycolysis module, thus increasing the glucose utilization rate. The increased expression of the *mdh*, *fum* and *frd* genes activated the reductive TCA module, which was the main succinate synthetic module. The increased expression of the *dcuB*, *dcuC* and *dctA* genes suggested that the succinate export capability might increase in the evolved strain. In addition, although the *aceB* and *aceA* genes expression level increased significantly, these two enzyme activities exhibited nearly no change, which might be due to the tight regulation of these enzymes at the protein level.

Although the activities of transketolase and transhydrogenase increased, no mutation was observed in the coding regions of the *tktA* and *sthA* genes or in their surrounding promoters and terminators. A single nucleotide mutation was observed in the coding region of *rpoB* gene (β-subunit of RNA polymerase, Table S4), which was suggested to lead to the global regulation of multiple genes. Transcriptome analysis will be performed in our future work to investigate the effect of this *rpoB* mutation on global gene expression.

5. Conclusion

An *E. coli* strain for efficient succinate production was developed through combined metabolic engineering and metabolic evolution. Genetic mechanisms for high productivity and yield were then identified through genome sequencing and transcriptome and enzyme assay analysis. Increased activities of glucose utilization, PEP carboxylation, reductive TCA and succinate export were responsible for the increased succinate productivity, whereas the increased activities of PDH, PPP and transhydrogenase were responsible for the increased succinate yield. Reverse metabolic engineering was further performed in the parent strain and increased succinate yield from 1.12 to 1.5 mol/mol. The strategies for increasing reducing equivalent supply identified in this work will also be useful for the synthesis of other products.

Competing interests

This work has been included in patent applications by Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2014.05.003.

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