J Ind Microbiol Biotechnol (2013) 40:1461–1475
DOI 10.1007/s10295-013-1342-y

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Directed pathway evolution of the glyoxylate shunt in *Escherichia coli* for improved aerobic succinate production from glycerol

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Received: 29 June 2013 / Accepted: 7 September 2013 / Published online: 2 October 2013

Abstract α-Ketoglutarate is accumulated as the main byproduct during the aerobic succinate production from glycerol by *Escherichia coli* BL21(DE3) in minimal medium. To address this issue, here a strategy of directed pathway evolution was developed to enhance the alternative succinate production route—the glyoxylate shunt. Via the directed pathway evolution, the glyoxylate shunt was recruited as the primary anaplerotic pathway in a *ppc* mutant, which restored its viability in glycerol minimal medium. Subsequently, the operon *sdhCDAB* was deleted and the gene *ppc* was reverted in the evolved strain for succinate production. The resulting strain E2-Δ*sdh-ppc* produced 30 % more succinate and 46 % less α-ketoglutarate than the control strain. A G583T mutation in gene *icdA*, which significantly decreased the activity of isocitrate dehydrogenase, was identified in the evolved strain as the main mutation responsible for the observed phenotype. Overexpression of α-ketoglutarate dehydrogenase complex in E2-Δ*sdh-ppc* further reduced the amount of byproduct and improved succinate production. The final strain E2-Δ*sdh-ppc-sucAB* produced 366 mM succinate from 1.3 M glycerol in minimal medium in fed-batch fermentation. The maximum and average succinate volumetric productivities were 19.2 and 6.55 mM h^{-1}, respectively, exhibiting potential industrial production capacity from the low-priced substrate.

Keywords *Escherichia coli* · Glycerol · Succinate · Glyoxylate shunt · Isocitrate dehydrogenase

Introduction

As the biodiesel industry increases significantly worldwide, glycerol has become an abundant and inexpensive feedstock for industrial microbiology as the major by-product of biodiesel [10]. The production of fuels and valuable chemicals through microbial fermentation of glycerol by many microorganisms such as *Citrobacter freundii*, *Klebsiella pneumonia*, *Clostridium pasteurianum*, *Clostridium butyricum*, *Enterobacter aerogenes*, and *Lactobacillus reuteri* [8] is becoming an attractive field, including organic acids, 1,3-propanediol, ethanol, butanol, and so on [11].

Succinate as a valued platform compound, which has significant applications in agricultural, food, and pharmaceutical industries, has been studied for many years in the aspect of microbial production using several different microorganisms [2]. Production of succinate from glycerol fermentation has been primarily focusing on *A. succiniciproducens* [18, 19], *B. succiniciproducens* DD1 [37, 38], *A. succinogenes* [41], *Y. lipolytica* [45], and *E. coli* [3, 47]. In particular, the succinate production performance of *E. coli* from glycerol under anaerobic conditions was examined recently. Metabolically engineered *E. coli* strain (XZ721)

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showed the capability to produce 102 mM succinate with a succinate yield of 0.8 mol/mol glycerol under anaerobic conditions [47]. On the other hand, *E. coli* was engineered to produce succinate from glycerol under microaerobic conditions with a yield of 0.54 mol/mol glycerol [3].

The metabolism of glycerol in *E. coli* under anaerobic conditions in the absence of external electron acceptors was previously reported [10, 13, 28]. However, the growth of *E. coli* is extremely slow with an average specific growth rate of $0.040 \pm 0.003\ h^{-1}$ in glycerol low-supplement minimal medium [28]. In accordance with this, metabolically engineered *E. coli* strain (XZ721) had a long fermentation period of 6 days [47]. Even though the fermentation period was shortened under microaerobic conditions, the productivity was still not sufficient for the industrial application [3]. On the other hand, designing aerobic succinate production system can overcome the drawbacks of anaerobic fermentation, with the advantages of fast growth, no need of rich supplements, and high productivity.

Strategies employed for the construction of aerobic succinate producers primarily focus on the interruption of TCA cycle for accumulation succinate as the end product, adoption of the glyoxylate cycle as alternative succinate route, overexpression of carboxylation enzymes, and blocking competing by-product pathways [21–24, 35, 45]. Although the maximum theoretical yield of 1.0 mol/mol glucose has been achieved by metabolic engineered *E. coli* in rich nutrient medium [23], the succinate yield in defined minimal medium using glycerol as the carbon source has not been reported. Being one of the two potential routes for succinate production under aerobic conditions, the glyoxylate shunt is significantly important for improving succinate production. Several efforts has been made to functionalize the glyoxylate cycle, which is generally active with acetate as the sole carbon source [42], including deletion of the repressor gene *iclR* in *E. coli* [22] and overexpression of the glyoxylate shunt genes in *Corynebacterium glutamicum* [24]. However, due to the existence of some other regulation of intracellular metabolic flux, these efforts had limited effect.

Evolutionary engineering as a widely used tool for strain development in industrial microbiology has many applications including latent pathway activation, phenotype optimization, and environmental adaptation [34]. Instead of rational engineering, evolutionary engineering has the capacity to address issues in a nonintuitive way. Nowadays, the aim of evolutionary engineering generally focused on the improvement of nonnative substrates utility or tolerance to fermentative products. Here we adopted evolutionary engineering to improve the flux of the glyoxylate cycle for improved aerobic succinate production from glycerol in engineered *E. coli*. This was accomplished by deletion of phosphoenolpyruvate carboxylase (PPC) and couple the glyoxylate shunt to cell viability in glycerol minimal medium.

Materials and methods

Strains, plasmids, and primers

The strains and plasmids used in this study are listed in Table 1. The primers used for gene knockout and promoter replacement are listed in Table 2. *E. coli* strain BL21(DE3) was used as a host strain for succinate production and *E. coli* DH5α was used for gene cloning. For plasmids construction and genetic manipulations, Luria–Bertani (LB) broth was used for culture. As appropriate, ampicillin (100 μg/ml), kanamycin (24 μg/ml), and chloramphenicol (4 μg/ml) were supplemented.

Gene deletion, promoter replacement, and allele replacement

Deletion strains were constructed following a one-step inactivation method developed earlier [9]. Knockout of *sdhCDAB*, *aceBA*, *pck*, *maeA*, and *maeB* were performed with homologous regions as long as 50 bp contained in the primers (Table 2). For *ppc* deletion, chloramphenicol resistance gene amplified from pKD3 was firstly cloned into pUC18 to produce plasmid pCM01. Homologous regions of 600 bp positioned upstream and downstream of the *ppc* gene were then cloned into plasmid pCM01, respectively. The resulting plasmid pCMppcHR was used as the template of PCR amplification. Amplified PCR fragments comprised of homogenous regions and antibiotic resistance gene were electroporated into *E. coli* strains harboring plasmid pKD46. The resistance gene flanked by FRT sites was subsequently removed from the chromosome with FLP recombinase using plasmid pCP20 [6].

Replacement of the native promoters of *aceBA*, *sucAB*, and *ppc* with the *trc* promoter was performed also by λ-Red recombination. Firstly, the *trc* promoter was amplified using plasmid pTrc99a as template and cloned into pUC18 to produce plasmid pUCtrc. Secondly, the kanamycin resistance gene or chloramphenicol resistance gene amplified from plasmid pKD4 or pKD3 was cloned into pUCtrc to produce plasmid pKMtrc or pCMtrc. For promoter replacement of *aceBA* and *gltA*, primers containing 50 bp homologous regions (Table 2) were used to amplify PCR fragments comprised of antibiotic marker and *trc* promoter using pKMtrc as template. For promoter replacement of *sucAB*, homologous regions of 500 and 900 bp positioned upstream and downstream of the promoter region of *sucAB* were used through cloned into plasmid pCMtrc,

Table 1 *Escherichia coli* strains and plasmids used in this study

Strains/plasmids	Relevant characteristics	Reference/source
Strains		
DH5α	Cloning host	Lab collection
BL21(DE3)	F' *ompT gal dcm lon hsdSB*(*rB- mB-*) λ(DE3 [*lacI lacUV5*-T7 gene *1 ind1 sam7 nin5*])	Novagen
CP	BL21(DE3), Δ*sdhCDAB, ppc:trc*, *aceBAK:trc*	This study
C1	BL21(DE3), Δ*ppc*	This study
C2	C1, *aceBAK:trc*	This study
E1	Evolved C2	This study
E2	Evolved E1	This study
E2-Δ*aceBA*	E2, Δ*aceBA*	This study
E2-Δ*pck*	E2, Δ*pck*	This study
E2-Δ*maeA*	E2, Δ*maeA*	This study
E2-Δ*maeB*	E2, Δ*maeB*	This study
E2-Δ*sdh*	E2, Δ*sdhCDAB*	This study
E2-Δ*sdh-ppc*	E2, Δ*sdhCDAB, ppc:trc*	This study
E2-Δ*sdh-ppc-sucAB*	E2, Δ*sdhCDAB, ppc:trc*,*sucAB:trc*	This study
C2-583	C2, G583T *icdA*	This study
CP-583	CP, G583T *icdA*	This study
E2-Δ*sdh-ppc*-583	E2-Δ*sdh-ppc*, wild-type *icdA*	This study
C2-583-*gltA*	C2-583, *gltA:trc*	This study
CP-583-*gltA*	CP-583, *gltA:trc*	This study
Plasmids		
pUC18	*bla*, pBR322 ori	Lab collection
pEL04	*cat-sacB* cassette	[17]
pTrc99a	*bla*, pBR322 ori, *trc* promoter, $lacI^q$	Invitrogen
pKD3	*bla*, FRT-*cat*-FRT	[9]
pKD4	*bla*, FRT-*kan*-FRT	[9]
pCP20	*bla*, *cat*, yeast Flp recombinase	[6]
pKD46	*bla*, λ-Red recombinase under *araBAD* promoter, temperature-conditional replicon	[9]
pET-28a(+)	*Kan,* pBR322 ori, T7 promoter, *lacI*	Novagen
pCM01	*Bla, cat, cat* from pKD3 cloned into pUC18	This study
pCMppcHR	*bla*, *cat*, homologous regions of 600 bp positioned upstream and downstream of the gene *ppc* cloned into pCM01	This study
pUCtrc	*Bla*, *trc* promoter from pTrc99a cloned into pUC18	This study
pKMtrc	*Bla*, *kan*, *kan* from pKD4 cloned into pUCtrc	This study
pCMtrc	*Bla*, *cat*, *cat* from pKD3 cloned into pUCtrc	This study
pCMtrcHR	*Bla*, *cat*, homologous regions of 500 bp and 900 bp positioned upstream and downstream of the gene *sucAB* cloned into pCMtrc	This study
pCSHR	*Bla*, *cat-sacB*, homologous regions of 500 bp positioned upstream and downstream of the gene *icdA* and *cat-sacB* cassette cloned into pUC18	This study

respectively. The promoter replacement of *ppc* was performed similar to that of *aceBA*. Once it was done in wild-type *E. coli* BL21(DE3), the antibiotic region, the *trc* promoter, and the coding region of *ppc* were amplified together using primers Pout5/Pout6 and electroporated into *E. coli ppc* mutants for recovering the *ppc* gene.

The allele replacement of *icdA* was accomplished through a two-step homogenous recombination method [39] with the counter-selectable *sacB* gene. Homologous regions of 500 bp positioned upstream and downstream of the gene icdA and the cat-sacB cassette from plasmid pEL04 [17] were cloned into pUC18, respectively. The

Table 2 Primers used in this study

Primer name	Sequence
sdhCDAB knockout	5′ ATGATAAGAAATGTGAAAAAACAAAGACCTGTTAATCTGGACCTAGTGTAGGCTGGAGCTGCTTC 3′
	5′ TTACGCATTACGCTGCAACAACATCGACTTGATATGGCCGATGGCCATATGAATATCCTCCTTAG 3′
aceBA knockout	5′ ATGACTGAACAGGCAACAACAACCGATGAACTGGCTTTCACAAGGCCGTACCTGTGACGGAAGATCACTTCG 3′
	5′ TTAGAACTGCGATTCTTCGGTGGAGCCGGTCAGCGCTGTAACCGACGACGTTACGCCCCGCCCTGCCACT 3′
pck knockout	5′ ATGCGCGTTAACAATGGTTTGACCCCGCAAGAACTCGAGGCTTATGGTATGTGTAGGCTGGAGCTGCTTC 3′
	5′ TTACAGCTTCGGACCAGCCGCTACCAGCGCGGCACCCGCAGGGGTGTCGGCATATGAATATCCTCCTTAG 3′
maeA knockout	5′ ACGATAAAAGCCCCCCAGGGATGGATATTCAAAAAAGAGTGAGTTACATGGTGTAGGCTGGAGCTGCTTC 3′
	5′ CCCGGTAGCCTTCACTACCGGGCGCAGGCTTAGATGGAGGTACGGCGGTACATATGAATATCCTCCTTAG 3′
maeB knockout	5′ ATGGATGACCAGTTAAAACAAAGTGCACTTGATTTCCATGAATTTCCAGTGTGTAGGCTGGAGCTGCTTC 3′
	5′ TTACAGCGGTTGGGTTTGCGCTTCTACCACGGCCAGCGCCACCATGTTGACATATGAATATCCTCCTTAG 3′
aceBA promoter replacement	5′ CACCTTACCTCAGGCACCTTCGGGTGCCTTTTTTATTTCCGAAACGTACCGTGTAGGCTGGAGCTGCTTC 3′
	5′ TACGGCCTTGTGAAAGCCAGTTCATCGGTTGTTGTTGCCTGTTCAGTCATGGTCTGTTTCCTGTGTGAAA 3′
ppc promoter replacement	5′ GGCGATTTTTTAACATTTCCATAAGTTACGCTTATTTAAAGCGTCGTGAGTGTAGGCTGGAGCTGCTTC 3′
	5′ TTGCCGAGCATACTGACATTACTACGCAATGCGGAATATTGTTCGTTCATGGTCTGTTTCCTGTGTGAAA 3′
ppc knockout	5′ GTACAAGCTTATGAATGCCCACCGAATC 3′
	5′ GTACAAGCTTTTGCCGAGCATACTGACA 3′
	5′ GGACGGATCCCCTCTTCTGCAAACCCTC 3′
	5′ GTAGGAGCTCGCGTGTCCACAGCAGAAC 3′
	5′ ATGAATGCCCACCGAATC 3′
	5′ GCGTGTCCACAGCAGAAC 3′
Trc promoter	5′ GTAGGGATCCCAGCTTATCATCGACTGCAC 3′
	5′ GCGCGAATTCCCATGGTCTGTTTCCTGTGT 3′
Kan or Cm amplification	5′ GTCGAAAGCTTGATTGCAGCATTACACGTCT 3′
	5′ ACGTGGTCGACCATATGAATATCCTCCTTAG 3′
sucAB promoter replacement	5′ AGAGAAGCTTCGGGCAAGAAGATTGTGAT 3′
	5′ AGAGAAGCTTTTACGCATTACGCTGCAAC 3′
	5′ AGAGGAATTCCGATGCAGAACAGCGCTTTG 3′
	5′ AGAGGAATTCAGAAGCCCATGTGGTATTTCA 3′
	5′ CGGGCAAGAAGATTGTGAT 3′
	5′ AGAAGCCCATGTGGTATTTCA 3′
icdA allele replacement	5′ GTGTAAGCTTCCTTCGCTATCGCGGTCAA 3′
	5′ GACTAAGCTTAGCGCTACTGGTTTGCTCG 3′
	5′ CTACGTCGACATGTAATCACTACATGTG 3′
	5′ GCAGGGTACCTTGGCACAAAGTAGCATA 3′
	5′ CCTTCGCTATCGCGGTCAA 3′
	5′ TTGGCACAAAGTAGCATA 3′
	5′ CTAGCTGCAGCCTGTGACGGAAGATCACTTCG 3′
	5′ CTAGCTGCAGCTGAGGTTCTTATGGCTCTTG 3′
gltA promoter replacement	5′ GATGTGCGAAGGCAAATTTAAGTTCCGGCAGTCTTACGCAATAAGGCGCAAGATCCCCTCACGCTGC 3′
	5′ CCGTTGAGGGTGAGTTTTGCTTTTGTATCAGCCATTTAAGGTCTCCTTAGTGTGAAATTGTTATCCGC 3′
CD28A	5′ CGATGAGCTCATGGAAAGTAAAGTAGTTGTTCC 3′
CD28B	5′ GACGCTCGAGTTACATGTTCTTGATGATCGC 3′
Verification	
sdhCDAB knockout	5′ ACCTCTGTGCCCGTAGTCC 3′
	5′ AGAAGCCCATGTGGTATTTCA 3′
aceBA knockout	5′ GTAGGGATCCCAGCTTATCATCGACTGCAC 3′
	5′ GCATTTAGCGCCCTCATC 3′

Table 2 continued

Primer name	Sequence
pck knockout	5′ AAAAGTTAGCGTGGTGAATC 3′
	5′ TTAGCGGCACGGGATT 3′
maeA knockout	5′ TCAGTGATATCTACCAGCAAACGA 3′
	5′ GTATCCGGCATTTTTAACTGAACG 3′
maeB knockout	5′ AAAAGTAAAGTGTTAGATGAGTGC 3′
	5′ GATTTTCTTCGCCAGTTCCTCGC 3′
ppc knockout	5′ ATGAATGCCCACCGAATC 3′
	5′ GCGTGTCCACAGCAGAAC 3′
aceBA promoter replacement	5′ TACGGCACATGAATCCAACG 3′
	5′ TTTGCCTGCTTCATTAGTGT 3′
sucAB promoter replacement	5′ CGGGCAAGAAGATTGTGAT 3′
	5′ AGAAGCCCATGTGGTATTTCA 3′
ppc promoter replacement	5′ ACACCTTTGGTGTTATGTATG 3′
	5′ TGATGGTGTCTTCGCTCAGTT 3′
icdA allele replacement	5′ CCTTCGCTATCGCGGTCAA 3′
	5′ TTGGCACAAAGTAGCATA 3′
gltA promoter replacement	5′ AGTTTGTCGGGTTTTATCCT 3′
	5′ CAGGATGCGGTTGAAGTGAA 3′

RT-qPCR	Primer sequences	Reference
aceA	5′ ATCTGATCACCTCCGATTGC 3′	[26]
	5′ CACCAGACCAGGTCAGCATA 3′	
gltA	5′ ATGATTCTTTCCGCCTGATG 3′	[26]
	5′ TGTTTTCCAGCTCCATAGCC 3′	
icdA	5′ TGACGAATGCGCCCTGTTT 3′	This study
	5′ TCAGGTCTGCGGCTTCAGT 3′	
rrsA	5′ TGTAGCGGTGAAATGCGTAG 3′	[26]
	5′ CCTCCAAGTCGACATCGTTT 3′	

resulting plasmid pCSHR was used as the template for amplification of PCR fragments which were used for accomplishing the first homogenous recombination. The second homogenous recombination was achieved using PCR fragments harboring point mutation amplified from the genome of wild type or the mutant. Cells finishing the second homogenous recombination were selected in the presence of 10 % sucrose, and the allele replacement was confirmed by sequencing.

Medium and cultivation conditions

Flask cultures were performed in M9 minimal medium [40] supplemented with 110 mM glycerol at 37 °C and 220 rpm in a rotary shaker. A single colony was inoculated into a 15-ml test tube containing 4 ml LB medium and cultured at 37 °C and 220 rpm for 12 h. One percent of seed culture was transferred into 25 ml of M9 minimal medium with 110 mM glycerol in a 250-ml flask. When the culture grew to mid-exponential phase, 1 % of it was inoculated into 50 ml M9 medium with 110 mM glycerol in a 500-ml flask. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was supplemented to 0.2 mM in the shake flask culture at the beginning of fermentation to induce gene expression. All the fermentation experiments were performed in triplicate.

Fed-batch fermentation was carried out in a 1.3-l reactor (BioFlo 110, New Brunswick Scientific, Enfield, CT, USA) containing 600 ml M9 minimal medium supplemented with 470 mM glycerol initially. The frozen glycerol stock at −80 °C was firstly streaked on a LB plate, incubated at 37 °C for 12 h, and then a colony was inoculated into a 15-ml test tube containing 4 ml LB medium. After incubating in the test tube at 37 °C and 220 rpm for 12 h, the cells were transferred into a 500-ml shake flask containing 50 ml M9 medium supplemented with 110 mM glycerol. When the culture grew to mid-exponential phase in the shake flask, the cells were

inoculated into the reactor with an initial OD_{600} of 0.18. Fed-batch fermentation was performed at 37 °C and the pH was controlled at 7.0 with 30 % ammonium hydroxide and 1 M sulfuric acid. The dissolved oxygen was maintained above 30 % of air saturation by aerating air at 1 l/min and adjusting the agitation speed from 500 to 850 rpm. IPTG was added to a final concentration of 1 mM during inoculation. When the glycerol concentration was below 110 mM, 25 ml 80 % (v/v) glycerol was supplemented into the reactor. Samples were taken periodically to determine cell growth, glycerol and extracellular metabolites concentration.

Adaptive evolution

Adaptive evolution was performed through sequential transfers in M9 minimal medium under aerobic conditions. The starting strain was initially subjected to incubation in a 500-ml flask containing 50 ml M9 minimal medium with 110 mM glycerol at 37 °C and 220 rpm. Several days were needed for the growth to occur and sequential transfers were subsequently carried out during the mid-exponential phase. When the cell density (OD_{600}) grew to approximately 1.0, the cells were transferred into fresh M9 medium with 2 % inoculum. Each transfer was stored at −80 °C. 0.2 mM IPTG was added to induce the expression of *aceBA* at the time of inoculation.

Analytical methods

Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}) with a UV–Vis spectrophotometer. Glycerol and organic acids were quantified by HPLC equipped with a cation exchange column (Aminex HPX 87-H, Bio-Rad, Hercules, CA, USA), an UV absorbance detector (Agilent, G1315D), and a refractive index (RI) detector (Agilent, HP1047A); 5 mM H_2SO_4 was used as the mobile phase at a flow rate of 0.4 ml/min. The column was operated at 65 °C.

Enzyme assays

For preparation of cell extract, all the strains were cultured in 500-ml flasks containing 50 ml M9 minimal medium with 110 mM glycerol at 220 rpm and 37 °C. Cell was harvested by centrifugation at 8,000 × *g* for 10 min at the mid-exponential phase, washed twice with 100 mM Tris–HCl (pH 7.0) containing 20 mM KCl, 5 mM H_2SO4, 2 mM DTT, and 0.1 mM EDTA and then resuspended in the same buffer [33]. All the operations were conducted at 4 °C. Crude extract was prepared through freezing cells with liquid nitrogen and immediately grinding with silica sand at 4 °C for 5 min. Cell debris was removed by centrifugation at 20,000 × *g* for 15 min. The supernatant was immediately used for enzyme assays. The total protein concentration was measured as previously described [4] using bovine serum albumin as standard. Citrate synthase (CS) activity was determined based on the reaction of 5,5-dithiobis (2-nitrobenzoic acid) and CoA as reported [27]. Isocitrate lyase (ICL) activity was measured according to the formation of glyoxylic acid phenylhydrazone from glyoxylate and phenylhydrazine [5]. Isocitrate dehydrogenase (ICDH) activity was detected through monitoring the formation of NADPH at 340 nm [1]. Enzyme activities were measured spectrophotometrically at 30 °C for ICL and 37 °C for CS and ICDH.

For purification of the wild-type and mutated ICDH, the wild-type and mutated *icdA* genes were amplified with primers CD28A/CD28B from the genome of wild-type BL21(DE3) and evolved strain E2, and cloned into plasmid pET-28a(+). The resulting plasmids were then transformed into *E. coli* BL21(DE3). When culture was grown to an OD_{600} of 0.6 in 50 ml of LB medium, 1 mM IPTG was supplemented and after 3 h of induction the cells was harvested and washed. The pellet was resuspended in 5 ml 50 mM NaH_2PO_4 (pH 8.0) containing 0.3 M NaCl. Cells were lysed and the cell debris was removed as mentioned above. Protein was purified using Ni–NTA-Sefinose Column (Sangon, Shanghai, China) according to the manufacturer's protocol. Kinetics parameters of ICDH were determined by altering the concentration of isocitrate while the other substrates were maintained at saturating concentrations. The K_M values were determined from Lineweaver–Burk plots.

Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from mid-exponential phase cultures grown in M9 minimal medium with RNAprep Pure Cell/Bacteria Kit (Tiangen, Beijing, China) according to the manufacturer's protocol. cDNA synthesis was prepared using FastQuant RT Kit (with gDNase) (Tiangen, Beijing, China) with 500 ng total RNA and random primers. Prior to cDNA synthesis, the total RNA was treated with the gDNase in the kit at 42 °C for 3 min to avoid the interference of genome DNA. RT-qPCR was performed in triplicate for each sample in a Light CyclerH 480 II (Roche, Basel, Switzerland) using Real Master Mix (SYBR Green). The 16s rRNA gene was used as the internal control. The relative quantitative method ($2^{-\Delta\Delta C_T}$) [25] was used to calculate the fold change between different samples. The sequences of primers used for RT-qPCR are listed in Table 2.

Results

Engineering *E. coli* BL21(DE3) for aerobic succinate production from glycerol

When *E. coli* was engineered to produce succinate under aerobic conditions, succinate dehydrogenase (SDH) was generally deleted (Fig. 1) to allow the accumulate of succinate as an end product [22, 24, 35]. In addition, overexpression of carboxylation pathways and activation of the glyoxylate shunt has been proved to be effective for increasing aerobic succinate production [23, 24]. Therefore, we firstly engineered *E. coli* BL21(DE3) for succinate production from glycerol through deleting the operon

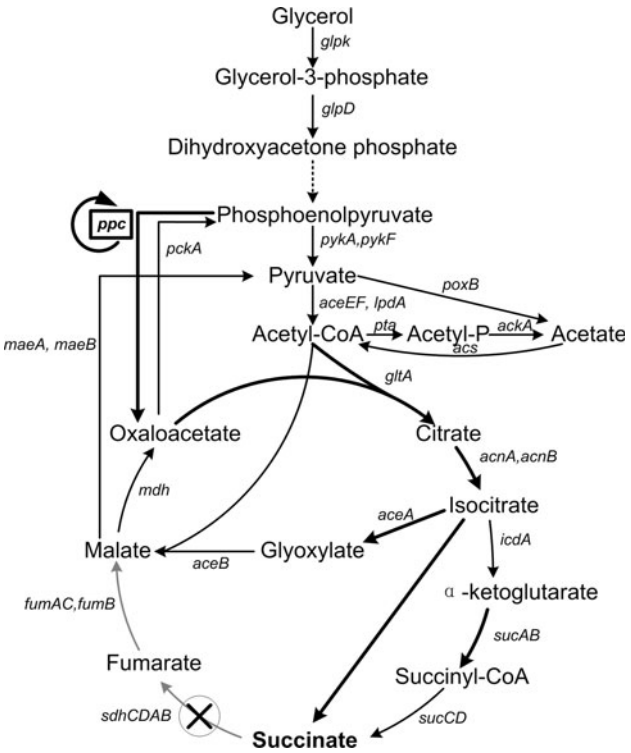

Fig. 1 The central carbon metabolism of *Escherichia coli* BL21(DE3) with glycerol as carbon source and the manipulation strategies for succinate production. The *circular arrow* around the gene *ppc* represents that it was firstly deleted for adaptive evolution and then recovered for succinate production. The *cross in the circle* indicates deleted pathways, which are displayed in *grey*. The *bold arrow* represents the upregulated pathways. *glpK*, glycerol kinase; *glpD*, glycerol-3-phophate dehydrogenase; *pykA*/*pykF*, pyruvate kinase; *aceEF*/*lpdA*, pyruvate dehydrogenase complex; *poxB*, pyruvate oxidase; *pta*, phosphotransacetylase; *ackA*, acetate kinase; *acs*, acetyl-CoA synthase; *gltA*, citrate synthase; *acnA*/*acnB*, aconitate hydratase; *icdA*, isocitrate dehydrogenase; *sucAB*, α-ketoglutarate dehydrogenase; *sucCD*, succinyl-CoA synthase; *sdhCDAB*, succinate dehydrogenase; *fumAC*/*fumB*, aerobic and anaerobic fumarases; *mdh*, malate dehydrogenase; *aceA*, isocitrate lyase; *aceB*, malate synthase; *maeA*/*maeB*, NADH/NADPH malic enzymes; *ppc*, phosphoenolpyruvate carboxylase; *pckA*, phosphoenolpyruvate carboxykinase

sdhCDAB (encoding SDH) and replacing the native promoters of *ppc* (encoding PPC) and *aceBAK* (encoding the glyoxylate shunt enzymes) with *trc* strong promoter. The resulting strain CP was then tested for aerobic succinate production from glycerol in M9 minimal medium by shake-flask cultivations. As shown in Fig. 2, strain CP consumed 110 mM (10 g/l) glycerol within 24 h and produced 23.7 mM (2.8 g/l) succinate with a succinate yield of 0.22 mol/mol (0.28 g/g), which representing 43 % of the maximum theoretical yield. Different from the results reported previously [22, 49] that acetate was the main byproduct during aerobic succinate fermentation in rich nutrient medium, only a small amount of acetate was produced in minimal medium in this study and it was consumed gradually after 12 h (Fig. 2e). However, some intermediates of the TCA cycle were produced as byproducts, in which the main byproduct α-ketoglutarate was accumulated to 10.3 mM (1.5 g/l) (Figs. 2d, 3).

The accumulation of α-ketoglutarate wasted carbon source and resulted in low succinate yield. Redirecting more carbon flux into the glyoxylate bypass and consequently decreasing the flux of the oxidative arm of the TCA cycle were expected to reduce the accumulation of α-ketoglutarate and improve succinate production. Since the transcriptional regulation of key enzymes of the glyoxylate shunt had been removed in strain CP through replacing the native promoter of *aceBAK* with *trc* strong promoter, some other efforts needed to be made to further increase the flux of the glyoxylate shunt.

Directed pathway evolution of the glyoxylate shunt

The reaction catalyzed by PPC and the glyoxylate shunt can both function as anaplerotic pathways for replenishing oxaloacetate (OAA) under different conditions. PPC is the primary anaplerotic enzyme and is essential for growth in minimal medium with glycerol as the sole carbon source in *E. coli* K-12 [15]. However, when acetate is used as the sole carbon source, the glyoxylate shunt becomes essential and functions as the primary anaplerotic pathway for avoiding the loss of carbon as CO_2 [42]. As shown in Fig. 1, one OAA and two acetyl-CoA are converted to one malate and one succinate by one round of the glyoxylate shunt, and then malate and succinate can be converted to two OAA by malate dehydrogenase and SDH, respectively. Therefore, the net effect of the glyoxylate shunt is to replenish one OAA. It has been reported that the glyoxylate shunt is inactive in *E. coli* K-12 but active in *E. coli* BL21 with 22 % flux of the TCA cycle in the presence of glucose [29]. Therefore, *ppc* was deleted in *E. coli* BL21(DE3) to examine if the active glyoxylate shunt could support cell growth in glycerol minimal medium in the absence of PPC. However, the growth of the resulting strain C1 in glycerol

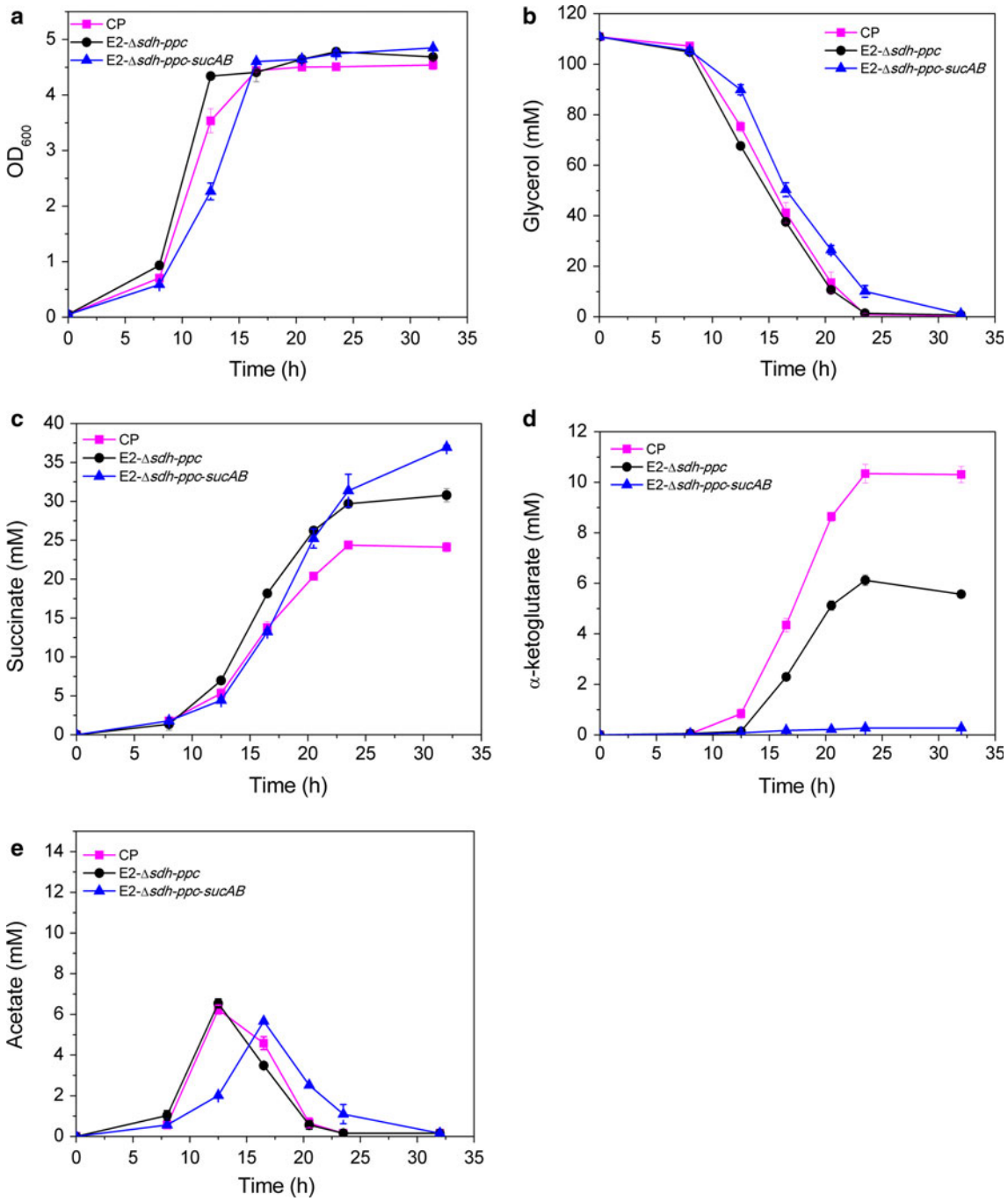

Fig. 2 Time profiles of biomass synthesis (**a**), glycerol consumption (**b**), succinate production (**c**), and byproducts accumulation (**d**, **e**) for strain CP (*square*), E2-Δ*sdh-ppc* (*circle*) and E2-Δ*sdh-ppc-sucAB* (*triangle*) in minimal medium measured from flask cultures

minimal medium was completely abolished (Table 3), similar to *E. coli* K-12 as previously reported [15]. This phenomenon suggested that the flux of the active glyoxylate shunt in *E. coli* BL21(DE3) was not sufficient for cell growth on glycerol minimum medium. We then replaced the native promoter of the operon *aceBAK* with *trc* strong promoter in strain C1 to eliminate the transcriptional regulation by regulators such as *iclR, crp* [7, 44, 48]. The resulting strain C2 was still unable to grow in glycerol minimal medium, indicating that the flux of the glyoxylate shunt was still insufficient for cell growth. The regulation of the key enzymes of the glyoxylate shunt by inhibitors on enzyme kinetics [14, 30] might be responsible for it.

The inviability of C1 and C2 in glycerol minimal medium indicated the low flux of the glyoxylate shunt and the need to increase it for succinate production. It has been

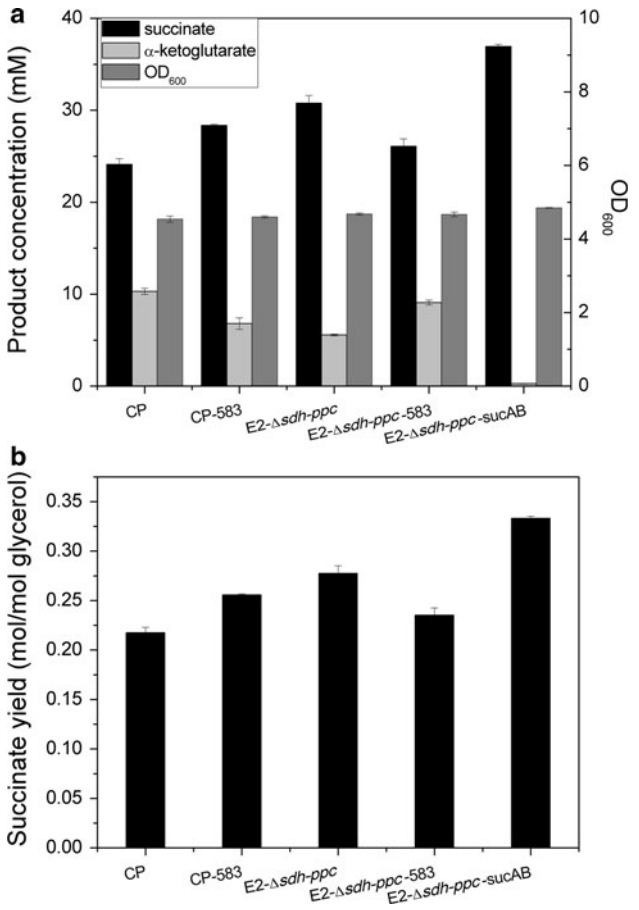

Fig. 3 Comparison of succinate production and byproduct accumulation between different strains developed in this study. **a** Biomass (OD_{600}), succinate and α-ketoglutarate concentrations; **b** succinate yield (mol/mol glycerol). All the strains were cultured in minimal medium with 110 mM glycerol in 500-ml flasks at 37 °C and 220 rpm

reported that the glyoxylate shunt was activated in *E. coli* K-12 *ppc* mutant as alternative anaplerotic pathway in the presence of glucose [32]. In the *ppc* mutant, ICL activity increased by about threefold and 18.9 % of the carbon flux flowed into the glyoxylate shunt [32]. In another study, the glyoxylate shunt was also recruited as the anaplerotic pathway in place of PPC in unevolved and evolved *E. coli* K-12 *ppc* mutants [12]. Based on the facts mentioned above, we attempted to perform directed pathway evolution using strain C2 as the starting strain and hoped to isolate mutant strains with enhanced glyoxylate shunt.

At the beginning of the directed pathway evolution, strain C2 was incubated in glycerol minimum medium for several days, after which culture of C2 began to grow and a clone was isolated through streaking on agar plate and designated as E1. When cultured in glycerol minimal medium, E1 was able to grow at a specific growth rate of 0.18 h^{-1}. Because of the slow growth of E1, serial transfers

Table 3 The growth phenotype and specific growth rate of strains developed in this study in glycerol minimal medium

Strains	Growth (Y, growth; N, non-growth)	Specific growth rate (h^{-1})
C1	N	–
C2	N	–
E1	Y	0.18 ± 0.0039
E2	Y	0.40 ± 0.00505
E2-Δ*aceBA*	N	–
E2-Δ*pck*	Y	0.32 ± 0.0049
E2-Δ*maeA*	Y	0.34 ± 0.0012
E2-Δ*maeB*	Y	0.39 ± 0.00035
C2-583	Y	0.19 ± 0.00045
C2-583-*gltA*	Y	0.26 ± 0.004

The specific growth rate was measured at 37 °C and 220 rpm in 500-ml flask. 'Y' represents the ability to growth under this condition, and 'N' represents that the strain could not grow in glycerol minimal medium

were carried out in minimal medium with glycerol as the sole carbon source to further improve the growth. After adaptive evolution of about 100 generations, a mutant (E2) was obtained that was able to grow in glycerol minimal medium at a specific growth rate of 0.40 h^{-1} (Table 3). When the operon *aceBA* encoding the key enzymes of the glyoxylate shunt was deleted in E2, the mutant E2-Δ*aceBA* lost the ability to grow in glycerol minimal medium. This result indicated that the glyoxylate shunt had become essential for cell growth as the primary anaplerotic pathway through adaptive evolution, which was in accordance with the results of previous studies [12, 32]. In order to examine whether mutations had occurred in ICL or malate synthase, the promoter and coding region of the operon *aceBAK* were sequenced. Unexpectedly, no mutation was observed.

The previously reported results [12, 32] and the above result in this study demonstrated that in the absence of PPC the favored alternative anaplerotic pathway would be the glyoxylate shunt. However, there are three other carboxylation enzymes besides PPC, phosphoenolpyruvate carboxykinase (*pck*) and two malic enzymes (*maeA* and *maeB*), which are involved in gluconeogenesis and physiologically function in reverse direction [36]. In order to confirm that they were not evolved to serve as primary anaplerotic enzymes in this study, they were also deleted individually in strain E2. As depicted in Table 3, all the mutants (E2-Δ*pck*, E2-Δ*maeA* and E2-Δ*maeB*) retained the ability to grow in glycerol minimal medium with slightly decreased growth rates, suggesting that the three pathways were not evolved to function as the primary anaplerotic pathway in the evolved mutant.

Effects of the enhanced glyoxylate shunt on succinate production

In order to explore the effects of the enhanced glyoxylate shunt on succinate production, SDH was deleted in strain E2 (producing strain E2-Δ*sdh*) to allow succinate to accumulate as an end product. Knockout of SDH resulted in the inviability of strain E2-Δ*sdh* in glycerol minimal medium due to the elimination of the ability of the glyoxylate shunt to replenish OAA. So *ppc* was recovered in stain E2-Δ*sdh* and at the same time the native promoter of *ppc* was replaced with *trc* strong promoter. As expected, the resulting strain E2-Δ*sdh-ppc* was able to grow in glycerol minimal medium. Subsequently, the production performance of E2-Δ*sdh-ppc* was examined, using strain CP as the control strain.

As shown in Fig. 2a, the growth rate of strain E2-Δ*sdh-ppc* was a little higher than that of strain CP. After being cultured in glycerol minimal medium under aerobic conditions for 32 h, strain E2-Δ*sdh-ppc* produced 30.7 mM (3.62 g/l) succinate from 110 mM (10 g/l) glycerol. Succinate yield increased by 30 % for strain E2-Δ*sdh-ppc* in comparison with the control strain CP, which represented 55.5 % of the maximum theoretical yield (Figs. 2c, 3). Meanwhile, α-ketoglutarate level reduced to 5.5 mM (0.8 g/l), which was 46 % lower than strain CP (Figs. 2d, 3). These results suggested that the enhanced glyoxylate shunt acquired by directed pathway evolution successfully improved succinate production and reduced the byproduct level as expected.

Relative gene transcription levels and enzyme activities of key steps of central carbon metabolism

In order to figure out the genetic basis for the phenotypes of the evolved strain, several key reactions were investigated in the aspects of gene transcription levels and enzyme activities. Citrate synthase (CS) is the first enzyme of both the TCA cycle and the glyoxylate shunt and plays an important role in controlling the rate of these pathways. ICL and isocitrate dehydrogenase (ICDH) are key enzymes at isocitrate branch point and control the flux distribution between the glyoxylate shunt and the oxidative arm of the TCA cycle. Due to the importance of these enzymes, the relative gene transcription levels and the activities were evaluated for strain CP and E2-Δ*sdh-ppc*.

As shown in Fig. 4a, relative transcription levels of *aceA* and *icdA* were very similar for the two strains. However, the transcription level of *gltA* in E2-Δ*sdh-ppc* more than doubled in comparison with strain CP. With respect to enzyme activities, significant reduction for ICDH was observed in E2-Δ*sdh-ppc* (Fig. 4b), which was not reflected in the transcription levels. In addition, the activity

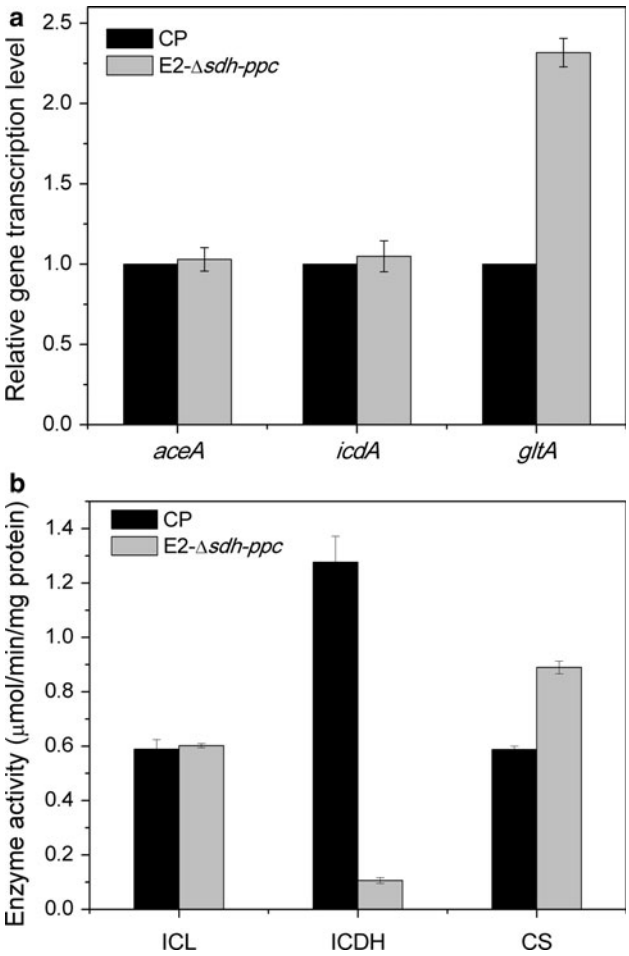

Fig. 4 **a** Changes of gene transcription levels of *aceA*, *icdA*, and *gltA* between strain CP and E2-Δ*sdh-ppc*; **b** Changes of enzyme activities of ICL, ICDH, and CS between strain CP and E2-Δ*sdh-ppc*. The unit of enzyme activity is μmol/min/mg protein. All the measurements were performed in triplicate

of CS increased by 50 % for E2-Δ*sdh-ppc* compared to the control strain, which showed a similar trend to the gene transcription levels. As expected, the activities of ICL for the two strains were almost the same. To determine the genetic basis for the altered activities of CS and ICDH between the two strains, the promoter and coding regions of genes *gltA* and *icdA* were sequenced for the evolved mutant E2. Unfortunately, no mutation was found in *gltA*. However, a missense mutation G583T was found in *icdA*, resulting in G195C amino acid variance.

Effects of the mutated ICDH on succinate production

When acetate is the sole carbon source, the flux of the glyoxylate shunt is increased through dramatically decreasing ICDH activity [16]. Hence, the enhanced glyoxylate shunt in the evolved mutant in this study might also be attributed to the extremely low activity of the mutated

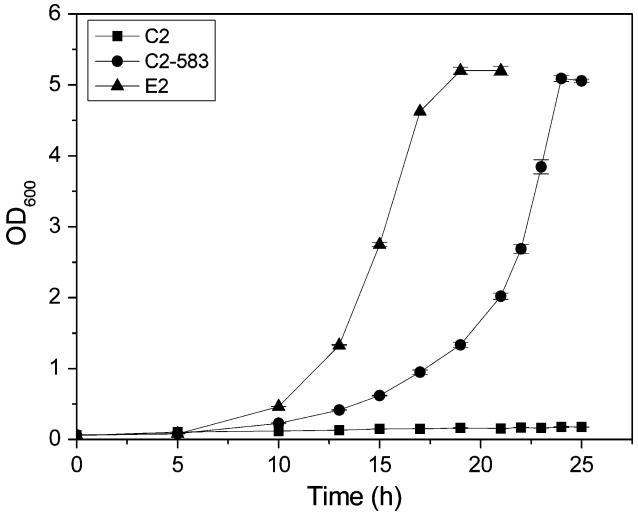

Fig. 5 The growth curves of strain C2, C2-583, and E2 in glycerol minimal medium in 500-ml flasks at 37 °C and 220 rpm

ICDH. In order to validate this hypothesis, the G195C mutation was introduced to the parental strains C2 and CP to explore the role of the mutated ICDH. The growth performance and fermentation products pattern were evaluated for the resulting strains C2-583 and CP-583, respectively.

While the parental strain C2 was unable to grow in glycerol minimum medium, the mutant C2-583 acquired the ability to grow at a specific growth rate of 0.19 h^{-1} as depicted in Fig. 5. The above observed phenomenon suggested that the significantly reduced activity of ICDH caused by G195C mutation forced carbon flow into the glyoxylate shunt and allowed it to function as the primary anaplerotic pathway, which led to the viability of strain C2-583. However, the growth of C2-583 was slower than that of E2 (Fig. 5), indicating the existence of other genetic changes aroused by adaptive evolution. Notably, the specific growth rate of C2-583 was very similar to that of E1, which gave rise to a supposal that the mutation of ICDH had occurred in strain E1 and it rendered E1 viability in glycerol minimum medium. To confirm this, the gene *icdA* of E1 was sequenced and the mutation was indeed present.

As expected, significant differences in the product's pattern were observed after introducing the G195C mutation into ICDH of the control strain CP (Fig. 3). The resulting strain CP-583 produced 20 % more succinate than the control CP. Meanwhile, α-ketoglutarate level of CP-583 showed a 34 % reduction compared to CP. Cell growth was not affected by the mutated ICDH. These results indicated that the enhanced glyoxylate shunt resulting from the mutated ICDH could reduce α-ketoglutarate level and improve succinate production.

In order to better explore the role of the mutated ICDH, the G195C mutation in strain E2-Δ*sdh-ppc* was reverted to the wild type to produce strain E2-Δ*sdh-ppc*-583. As depicted in Fig. 3, succinate production decreased by 15 % and α-ketoglutarate level increased by 63 % for strain E2-Δ*sdh-ppc*-583 compared to E2-Δ*sdh-ppc*. This suggested that the G195C mutation in ICDH was the main reason for the observed phenotype of E2-Δ*sdh-ppc* and further confirmed the role of the mutated ICDH.

The mutated ICDH and wild-type ICDH were both purified and the kinetics of them were measured and compared. The K_M value of wild-type ICDH for isocitrate was 0.035 mM as previously reported [30], whereas that of the mutated ICDH was 0.225 mM. In addition, the k_{cat} value of the mutated ICDH was 9.9 s^{-1}, much lower than that of wild-type ICDH of 57.8 s^{-1}.

Effects of overexpression of CS on cell growth and succinate production

According to the above-mentioned results, CS was upregulated in the evolved strain. In order to explore the effects of the upregulated CS on cell growth and succinate production, the native promoter of gene *gltA* was replaced with *trc* strong promoter in strain C2-583 and CP-583, producing strain C2-583-*gltA* and CP-583-*gltA*. Strain C2-583-*gltA* grew faster with a specific growth rate of 0.26 h^{-1} in glycerol minimal medium, compared to that of strain C2-583 of 0.19 h^{-1}, but still lower than that of the evolved strain E2. Similar phenomenon was also observed for strain CP-583-*gltA* in comparison with CP-583. Although cell growth was improved for strain CP-583-*gltA*, succinate production was not affected unexpectedly.

Further improving succinate production by adopting dual enhanced routes

Although the amount of the main byproduct α-ketoglutarate was significantly reduced by the enhanced glyoxylate shunt, it was still accumulated to a concentration of 5.5 mM. With the aim to further improve succinate production, continuing to reduce the level of α-ketoglutarate would probably be effective. α-ketoglutarate dehydrogenase complex is a rate-limiting enzyme of the TCA cycle which catalyzes the dehydrogenation of α-ketoglutarate to generate succinyl-CoA. Overexpression of this enzyme might be able to reduce the accumulation of α-ketoglutarate. Therefore, the native promoter of *sucAB* was replaced by the strong promoter *trc* in strain E2-Δ*sdh-ppc* to produce strain E2-Δ*sdh-ppc-sucAB*. As shown in Figs. 2 and 3, E2-Δ*sdh-ppc-sucAB* showed a 20 % increase in succinate yield as compared with E2-Δ*sdh-ppc*, and a 55.8 % increase as compared to the original strain CP, which represented 67 % of the maximum theoretical yield. As expected, the amount of α-ketoglutarate decreased significantly to 0.27 mM (0.04 g/l) for strain E2-Δ*sdh-ppc*-

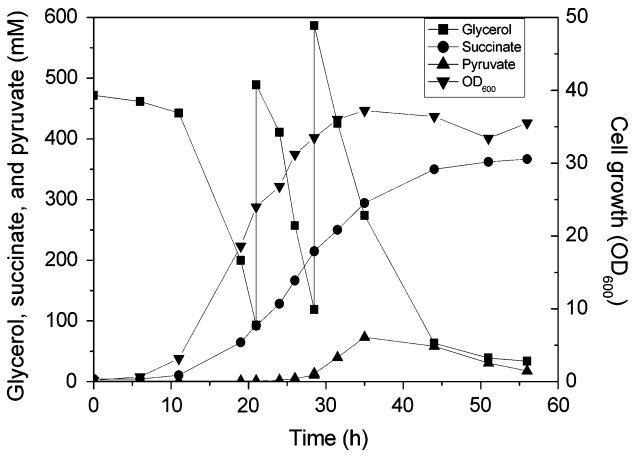

Fig. 6 Fed-batch fermentation profile of strain E2-Δ*sdh-ppc-sucAB* for succinate production under aerobic conditions in defined minimal medium

sucAB. Meanwhile, cell growth of E2-Δ*sdh-ppc-sucAB* was retarded by overexpression of α-ketoglutarate dehydrogenase complex (Fig. 2a). Eventually, E2-Δ*sdh-ppc-sucAB* gained two enhanced routes to produce succinate.

Fed-batch fermentation for aerobic succinate production

Strain E2-Δ*sdh-ppc-sucAB* was further evaluated for succinate production potential via fed-batch fermentation in a 1.3-l fermenter in glycerol minimum medium under fully aerobic conditions. As depicted in Fig. 6, cell growth reached the maximum OD_{600} of 37.2 at 35 h and then began to decrease gradually. After the initial 470 mM (43 g/l) glycerol was almost depleted, feedings were performed twice at 21 and 28 h, respectively. Glycerol consumption was rapid at the first 44 h at a maximum rate of 61.5 mM h^{-1} (5.6 g/l/h), which began to slow and finally stopped at 56 h. Ultimately, about 1.3 M (120 g/l) glycerol was consumed at an average rate of 23 mM h^{-1} (2.1 g/l/h). Succinate was produced promptly during the exponential phase and the early stationary phase. After the biomass began to decline, the rate of succinate production slowed down. At the end of fermentation, 366 mM (43.2 g/l) succinate was produced, and the maximum and average volumetric productivities were 19.2 mM h^{-1} (2.26 g/l/h) and 6.55 mM h^{-1} (0.77 g/l/h), respectively. The amount of α-ketoglutarate and acetate were extremely low, which was in agreement with the results of the flask fermentation. Unfortunately, a large amount of pyruvate was produced as the main byproduct that was not accumulated during the flask fermentation. Pyruvate was accumulated to a maximal concentration of 73 mM (6.4 g/l) at 35 h and then it was gradually consumed by the strain. The overall succinate yield of fed-batch fermentation was 0.284 mol/mol (0.364 g/g) glycerol representing 57 % of the maximum theoretical yield, which was lower than that of the flask fermentation. Although the succinate yield from glycerol under aerobic conditions was lower than that of the microaerobic or anaerobic fermentation [3, 47], the succinate volumetric productivity achieved in this work was much higher due to faster cell growth and higher cell density.

Discussion

For some desired phenotypes that are difficult to acquire through rational design, evolutionary engineering provides us an effective alternative. The flux ratio between the glyoxylate shunt and the oxidative arm of the TCA cycle, which we attempted to alter in this study, is controlled by several factors and is difficult to change. Therefore, a strategy of directed pathway evolution of the glyoxylate shunt was developed in *E. coli* for improved production of succinate from glycerol under aerobic conditions. The selection pressure of the evolution was provided by the knockout of PPC, which functioned as the primary anaplerotic pathway. The glyoxylate shunt was successfully recruited as the essential anaplerotic pathway in place of PPC for growth in glycerol minimum medium through adaptive evolution, as evidenced by the inviability of the evolved mutant after deletion of the operon *aceBA*. This result further confirmed the results reported previously that the glyoxylate shunt would be activated as the anaplerotic pathway in response to knockout of PPC [12, 32]. However, in another study, the energy-conserving phosphoenolpyruvate carboxykinase was evolved to function as the primary carboxylation pathway for succinate production in the presence of PPC [46]. Different from our work, the selection pressure of evolution in that study was not the absence of the anaplerotic pathway, but the insufficient supply of ATP for cell growth, which resulted in the recruitment of phosphoenolpyruvate carboxykinase.

When acetate is used as the sole carbon source, the inactivation of ICDH by ICDH kinase allows isocitrate lyse (ICL), whose affinity for isocitrate is relatively lower than ICDH [29], to acquire more substrate isocitrate, directing more carbon flux into the glyoxylate bypass [16]. In this study, the G195C mutation of ICDH discovered in strain E2 could also significantly decrease the activity of ICDH and consequently increase the flux of the glyoxylate shunt. This was evidenced by the viability of C2-583 in glycerol minimal medium rendered by the mutated ICDH. In addition, the G195C mutation lowered the affinity of ICDH for isocitrate, which was also beneficial for ICL to acquire more substrate. However, the growth of C2-583 was much slower than that of strain E2, and succinate production of CP-583 was lower than that of E2-Δ*sdh-ppc*. These results

indicated that not all the phenotypes of E2 and E2-Δ*sdh-ppc* were attributed to the mutated ICDH and some other genetic changes might be present.

It was observed that the transcription level and activity of citrate synthase (CS) both increased for strain E2-Δ*sdh-ppc* compared to strain CP. The increased activity of CS was also reported previously in *E. coli ppc* mutant [32]. In that study, the flux of the TCA cycle was increased by the upregulation of CS for channeling more carbon to replenish OAA. In another study, the flux of the TCA cycle was also increased in unevolved and evolved *ppc* mutants [12]. As the first enzyme of the TCA cycle and the glyoxylate bypass, CS is subjected to tight regulation [31] and plays a key role in controlling cell growth and carbon flow through the TCA cycle under certain conditions [43]. This probably could explain the faster growth of strain C2-583-*gltA* and CP-583-*gltA* observed in this study. However, succinate production was not improved by overexpression of CS. On the contrary, the high CS activity has been previously reported in the efficient aerobic succinate producer HL27659K [20], and succinate production was improved by overexpression of CS in *Corynebacterium glutamicum* under aerobic conditions [49]. The reason for the different results observed between this work and others' was not clear. Probably the degree of overexpression was still not sufficient for improving succinate production in this study and this would be examined in future study.

Under oxygen-limited conditions, poor growth and low cell density were commonly observed when glycerol was used to produce succinate by metabolic engineered *E. coli*, which resulted in long fermentation periods and low volumetric productivities [3, 47]. As an alternative strategy, engineering *E. coli* for aerobic production of succinate from glycerol would solve the issue mentioned above. The biocatalyst developed in this study achieved relative high cell density and exhibit a maximum glycerol consumption rate of 61.5 mM h^{-1} during fed-batch fermentation under aerobic conditions. These advantages led to the high succinate volumetric productivity of 6.55 mM h^{-1}, which exhibited potential industrial production capacity from the low-priced substrate.

Rational engineering based on the information obtained from evolutionary engineering would be greatly meaningful. Regulating the expression of key genes which exhibited different patterns between the parental and the evolved strain with promoter libraries or RBS libraries would probably have striking effects on succinate production. In addition, more genetic changes need to be figured out through genome sequencing to discover the underlying reasons of the phenotype of the evolved strain. Moreover, metabolic flux analysis needs to be performed to explore the different flux distribution pattern of the developed strains in this study, to figure out the bottleneck of carbon flow and guide us to further improve succinate production.

Acknowledgments This work was supported by National 973 Project (2011CBA00804, 2012CB725203), National Natural Science Foundation of China (NSFC-21176182, NSFC-21206112) and National High-tech R&D Program of China (2012AA02A702, 2012AA022103).

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