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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Evolutionary engineering of *Escherichia coli* for improved anaerobic growth in minimal medium accelerated lactate production

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Abstract

Anaerobic fermentation is a favorable process for microbial production of bulk chemicals like ethanol and organic acids. Low productivity is the bottleneck of several anaerobic processes which has significant impact on the technique competitiveness of production strain. Improving growth rate of production strain can speed up the total production cycle and may finally increase productivity of anaerobic processes. In this work, evolutionary engineering of wild-type strain *Escherichia coli* W3110 was adopted to improve anaerobic growth in mineral medium. Significant increases in exponential growth rate and stationary cell density were achieved in evolved strain WE269, and a 96.5% increase in lactate productivity has also been observed in batch fermentation of this strain with M9 minimal medium. Then, an engineered strain for lactate production (BW100) was constructed by using WE269 as a platform and 98.3 g/L lactate (with an optical purity of D-lactate above 95%) was produced in a 5-L bioreactor after 48 h with a productivity of 2.05 g/(L·h). Finally, preliminary investigation demonstrated that mutation in *sucD* (*sucD M245I*) (encoding succinyl-CoA synthetase); *ilvG (ilvG Δ1bp)* (encoding acetolactate synthase 2 catalytic subunit), and *rpoB (rpoB T1037P)* (encoding RNA polymerase β subunit) significantly improved anaerobic growth of *E. coli*. Double-gene mutation in *ilvG* and *sucD* resumed most of the growth potential of evolved strain WE269. This work suggested that improving anaerobic growth of production host can increase productivity of organic acids like lactate, and specific mutation-enabled improved growth may also be applied to metabolic engineering for production of other bulk chemicals.

Keywords Evolutionary engineering · *Escherichia coli* · Anaerobic growth · Productivity · *sucD* · *rpoB* · Lactate

Introduction

Anaerobic fermentation is a favorable process for microbial production of bulk chemicals like ethanol and organic acids (Morris 1983; Wang et al. 2016; Zeikus 1980). Facilitated by progress in metabolic engineering and synthetic biology investigation, many bulk chemicals and high-value chemicals have been produced via anaerobic fermentation by the biotechnology workhorse, *Escherichia coli*, these including alcohols, organic acids, biofuels, and polymers (Fig. 1) (Clark 1989; Forster and Gescher 2014; Ingram et al. 1987; Tan et al. 2016; Zhou et al. 2003). Anaerobic process usually has higher yield and lower energy consumption as compared with aerobic process, for example, anaerobic production of succinate from glucose has been reported to have a theoretical yield of 1.714 mol/mol, while this value of aerobic process via TCA cycle was only 1 mol/mol (Tan et al. 2016; Weusthuis et al. 2011; Xu et al. 2018). Production of lactate and ethanol

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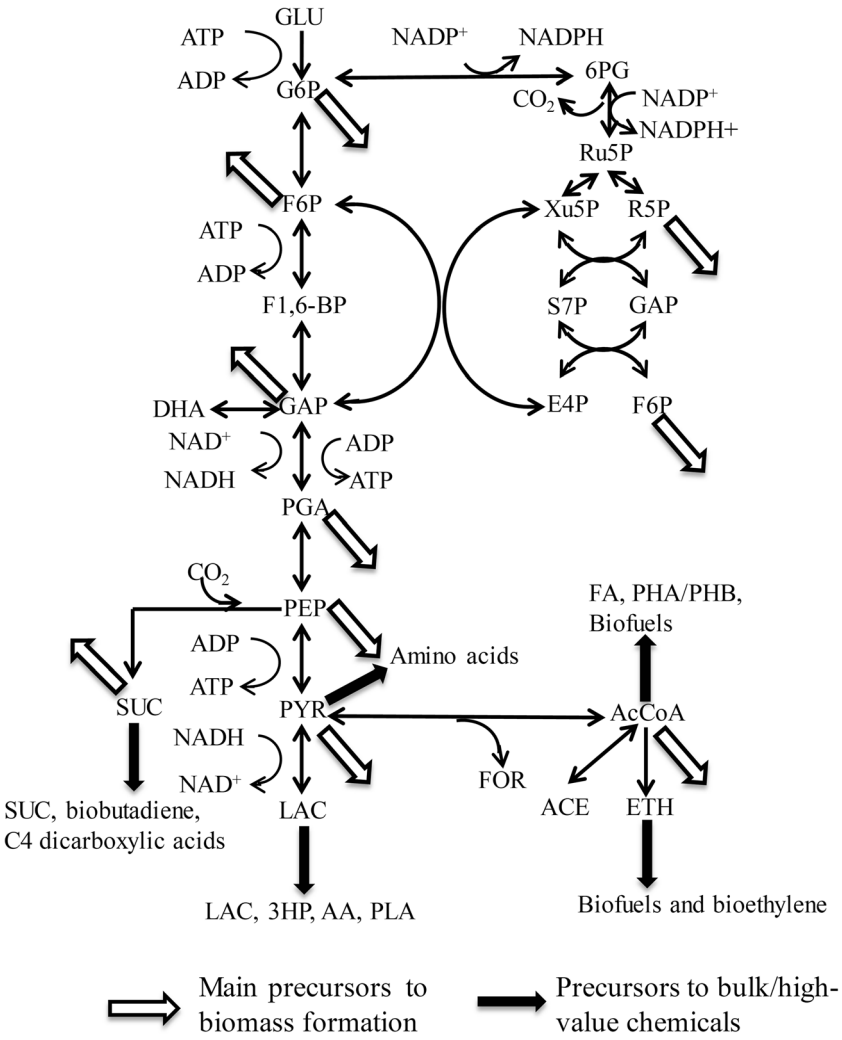

Fig. 1 Sample model of anaerobic metabolic network of *E. coli* and potential industrial application. Main precursors to biomass formation (hollow arrow) and bulk/high-value chemicals formation (solid arrow) have also been indicated. GLU, glucose; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; F1,6-BP, fructose 1,6-bisphosphate; DHA, Dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate; PGA, 3-phosphoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate; SUC, succinate; LAC, lactate; 3HP, 3-hydroxypropionic acid; AA, acrylic acid; PLA, polylactic acid; 6PG, 6-phosphogluconate; Ru5P, ribulose-5-phosphate; Xu5P, xylulose-5-phosphate; R5P, ribose-5-phosphate; E4P, erythrose-4-phosphate; S7P, sedoheptulose-7-phosphate; AcCoA, acetyl coenzyme A; FOR, formate; ACE, acetate; ETH, ethanol; FA, fatty acid; PHA/PHB, polyhydroxyalkanoates/poly-β-hydroxybutyrate

from glucose is the most proficient processes with a yield above 0.9 g/g (Weusthuis et al. 2011). Furthermore, no aeration is incorporated in the anaerobic process which further reduced equipment investment and operation cost (Weusthuis et al. 2011; Xu et al. 2018). However, anaerobic processes also suffer from some drawbacks; a prominent one of them is the low productivity of some production host under anaerobic process (Zhou et al. 2003). Incorporation of an aerobic growth phase at the early stage of fermentation was adopted to increase productivity of some anaerobic processes; however, this not only led to carbon lose and a lower product yield but also suffered from difficulties of selecting the aerobic-anaerobic turning point during the fermentation process (Niu et al. 2014; Tian et al. 2016; Zhou et al. 2003). Improving anaerobic growth of production host can speed up the total production cycle, which may finally increase productivity of anaerobic processes and provide another approach of getting rid of this drawback.

Evolutionary engineering, also called adaptive laboratory evolution, is a systematic approach of employing biology evolution in a controlled laboratory circumstances for a specific purpose; it has the advantage of unbiased and systematic mutation of the experimental microbes with a predetermined selection purpose (Portnoy et al. 2011; Sauer 2001; Winkler and Kao 2014). Evolutionary engineering has been widely used in biotechnology investigation to expand range of substrate utilization such as assimilation of xylose, activation of cryptic metabolic pathways, improving tolerance to environmental stress, improving product formation, and selection of synthetic biology devices (Guzman et al. 2015; He et al. 2017; Horinouchi et al. 2017; Qi et al. 2015; Shui et al. 2015). Facilitated by the next-generation sequencing technology, evolutionary engineering has become a growing field of importance in understanding the genetic basis of complex phenotypes in basic biology research (Barrick et al. 2009; Maddamsetti et al. 2017). Previously, researchers investigated on evolution of *E. coli* for rapid aerobic growth in rich medium or minimal medium with various carbon sources (glycerol, lactate, and glucose), and rapid aerobic growth was enabled by some key mutations those including in *rpoB*, *rpoC*

encoding RNA polymerase, and intergenic region of *pytE/rph* (Conrad et al. 2010; Conrad et al. 2009; Herring et al. 2006; LaCroix et al. 2015). These mutated RNA polymerase genes caused global transcriptional changes in diverse cellular process including cell motility, acid resistance, and expression of fimbria and curlin genes (Conrad et al. 2010). In another work, investigators reported that *E. coli* achieves faster growth by increasing catalytic and translation rates of proteins under aerobic growth conditions (Valgepea et al. 2013). In a recent work, researchers reported investigation on adaptation of *E. coli* to aerobic, anaerobic, or fluctuating oxygenic environment; altered metabolism and disruption of redundant functions were suggested to have caused improved fitness under anaerobic conditions (Finn et al. 2017). Evolutionary engineering has also been often used in strain development for lactate production, combined with the application of genome scale metabolic models; evolutionary engineering was used to confirm in-silicon strain design for lactate production; metabolic flux analysis has also been applied to reveal changes in the evolved strain (Fong et al. 2005; Hua et al. 2006; Niu et al. 2014). However, still little is known about specific mutations which may enable rapid growth of *E. coli* under anaerobic conditions, and the possible application of those strains as platform for metabolic engineering needs to be further explored.

With the aim of increasing productivity of anaerobic process such as lactate production, improving anaerobic growth of *E. coli* in minimal medium via evolutionary engineering was conducted. Preliminary investigation was also carried out to identify genomic mutations which may have caused this phenotype. Evolved strain with significant increase in exponential growth rate and stationary cell density has been obtained. Metabolic engineering of the evolved strain as a platform for lactate production revealed advantages of the evolved strain for anaerobic production of organic acids. Whole genome resequencing and analysis of site-directed mutagenesis of wild-type strain have identified important mutations which has caused improved anaerobic growth in *E. coli*.

Materials and methods

Strains, plasmids, and media

Wild-type strain *E. coli* W3110 (gifted from Prof. Qinhong Wang, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Science) was adopted as parent strain in this study; plasmid pTKRed, and pTKS/CS (laboratory collection) were used for site-directed mutagenesis of *E. coli* genome. Detailed information about strains and plasmids used in this study were listed in Table S1. Lysogeny Broth (LB) medium used for strain recovery and other routine cultivation processes contained (per liter) 10 g peptone, 5 g yeast extract, and 10 g NaCl. Agar plates were prepared with LB medium or M9 medium by adding 1.5% (*w*/*v*) agarose. Appropriate concentrations of antibiotics and inducers were used in gene recombination experiments, precisely, tetracycline (20 μg/L), spectinomycin (100 μg/L), IPTG (2 mM), and arabinose (0.2% (*w*/*v*)). M9 minimal medium used in evolutionary engineering experiment, growth tests, and fermentation assays contained (per liter) 6.78 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1.0 g NH_4Cl, 0.24 g $MgSO_4$, 0.011 g $CaCl_2$, and 0.1% (*v*/*v*) trace element solution (Zhang et al. 2009). Modified NBS medium I used for seed culturing in fed-batch fermentation contained (per liter) 3.5 g KH_2PO_4, 5 g K_2HPO_4, 3.5 g $(NH_4)_2HPO_4$, 0.25 g $MgSO_4{\cdot}7H_2O$, 15 mg $CaCl_2$, 0.5 mg thiamine, 0.1% (*v*/*v*) trace element solution, 5 g yeast extract, and 20 g glucose (Zhou et al. 2006). Modified NBS medium II used as fermentation media in fed-batch fermentation contained (per liter) 7 g KH_2PO_4, 10 g K_2HPO_4, 7 g $(NH_4)_2HPO_4$, 0.5 g $MgSO_4{\cdot}7H_2O$, 30 mg $CaCl_2$, 1 mg thiamine, 0.1% (*v*/*v*) trace element solution, 5 g yeast extract, and 50 g glucose. Yeast extract and peptone used for preparation of LB medium were purchased from Oxoid Ltd., (Basingstoke, Hampshire, UK), while other chemicals used were purchased from Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China).

Evolutionary engineering and screening of evolved strains with improved growth

Evolutionary engineering was carried out via serial transfer cultivation as those described previously (Sauer 2001). Precisely, cells were recovered from frozen stocks with LB rich medium and inoculated into 25 mL scale screw-capped anaerobic bottles filled with 22.5 mL M9 minimal medium containing 2.5% (*w*/*v*) glucose. The initial inoculation ratio was 10% (*v*/*v*), with an OD_{600nm} of 0.1. Cells were incubated at 37 °C in a rotary shaker with a rotation speed of 200 rpm. After 24 h of incubation, 2.5 mL of culture was retransferred to new anaerobic bottles filled with the same volume of M9 minimal medium for another round of cultivation. After 132 rounds of cultivation, glucose concentration was increased to 5.0% (*w*/*v*). The evolution experiment was carried on for about 400 rounds of transfer cultivation, and each round had three parallel replicates.

Screening of evolved strains with improved growth included two stages. At the first stage, frozen stocks of strain collections from 51, 99, 149, 209, 269, 299, 319, 349, 379, and 399 rounds of transfer cultivation were streaked upon M9 minimal medium plates and single colony appeared after 24 h of incubation at 37 °C. Individual colony was picked and inoculated into 2 mL 96 well deep well plates filled with 1.5 mL M9 minimal medium in each well and sealed with airtight membranes to block aeration. Then, plates were incubated in a plate shaker at 37 °C with a rotation speed of 500 rpm. Ninety-six individual colonies of each strain collection were picked for

the screening experiment. After 24 h of incubation, optical absorbance at 570 nm of cultures in each well was determined with a plate reader to evaluate biomass accumulation. Cultures with the top five optical density readings were taken for next round of evaluation. At the second stage, strains screened from the first stage were recovered from frozen stock by streaking upon M9 minimal medium plates and individual colony was picked and inoculated into 25 mL scale screw-capped anaerobic bottles filled with 25 mL M9 minimal medium. Cultures were incubated in a rotary shaker at 37 °C with a rotation speed of 200 rpm for 24 h. Then, optical absorbance at 570 nm was determined. Tested cultures with the top five optical density readings were frozen stocked for further investigation.

Growth test and evaluation of tolerance to acid stress

Growth test of evolved strain WE169, WE269, and WE319 selected from the evolution and screening experiment was carried out in 100 mL scale screw-capped anaerobic bottles (the most important strain WE269 has been stocked at the CGMCC with a primary title: *Escherichia coli* WE269). Test strains were recovered from frozen stock by streaking upon LB plates and incubated at 37 °C for 12 h, then individual colony was picked and inoculate into 5 mL LB medium and incubated in a rotary shaker at 37 °C with a rotation speed of 200 rpm for 12 h. OD_{600nm} of the cultures were determined and proper volumes of cultures were inoculated into M9 minimal medium to obtain an initial OD_{600nm} of 0.2. All strains were anaerobically cultured in 100 mL M9 minimal medium containing 5% (*w*/*v*) glucose at 37 °C, with a rotation speed of 200 rpm. Then, samples were taken at specific time intervals to evaluate biomass formation and extracellular metabolites accumulation.

To evaluate tolerance of evolved strain to acid stress, growth test of evolved strains was assayed in M9 minimal medium with an initial pH ranged from 4.0 to 7.0 tailored with 6 M HCl. Procedures of strain recovery, culture preparation, media, and growth conditions used were the same as those described in the previous growth test experiment. Samples were taken at specific time intervals, and OD_{600nm} was determined to evaluate biomass accumulation. Tolerance of evolved strain to acid stress was also carried out with a lactate isomer, 3-hydroxypropionic acid (3HP). 0.2% (*v*/*v*) 3HP was incorporated in M9 minimal medium (with a pH around 5.0); procedures and other parameters were the same as those described in the growth test experiment.

Fermentation assay

Fermentation assay of test strains was carried out both in 100 mL scale screw-capped anaerobic bottles and 5-L bioreactors. For lactate production in 100 mL scale anaerobic bottles, strains were recovered from frozen stock via streaking on LB plates and incubated at 37 °C overnight. Single colony was picked and inoculated into 5 mL LB medium and then incubated in a rotary shaker at 37 °C with a speed of 200 rpm. OD_{600nm} of cultures were detected and inoculation volumes were tailored to obtain an initial OD_{600nm} of 0.25. Fermentation was carried out with 100 mL M9 minimal medium containing 5% (*w*/*v*) glucose. Cultures were incubated at a rotary shaker at 37 °C with a speed of 200 rpm, each with three replicates. Cultures were sampled at specific time intervals to determine biomass formation, residual glucose, and main extracellular metabolites concentration.

Lactate production via batch fermentation in 5-L bioreactor was carried out as follows; test strains were recovered from frozen stock by streaking on LB plates and incubated at 37 °C overnight. Then, single colony was picked and resuspended into 3 mL LB medium and incubated at 37 °C with a rotation speed of 200 rpm for 12 h. After that, cultures were transferred into 500 mL flasks containing 100 mL LB medium with an inoculation ratio of 1% (*v*/*v*) and incubated at 37 °C with a rotation speed of 200 rpm for 12 h. Then, 187.5 mL of culture with an OD_{600nm} of 5 was inoculated into a 5-L bioreactor filled with 3.56 L M9 minimal medium containing 5% (*w*/*v*) glucose; the final working volume was 3.75 L. Temperature of fermentation and speed of stirring were set at 37 °C and 200 rpm respectively; pH was stabilized at 7.0 with 5 M NaOH solution. No aeration was incorporated during the whole fermentation process.

Fed-batch fermentation in 5-L bioreactor was also carried out for lactate production. Fed-batch fermentation was carried out as follows; test strains were recovered from frozen stock by streaking on LB plates and incubated at 37 °C overnight. Then, single colony was picked and resuspended into 500 mL flask containing 100 mL modified NBS medium I and incubated at 37 °C with a rotation speed of 200 rpm for 12 h. Then, 375 mL of seed culture with an OD_{600nm} 0f 10 was inoculated into 5-L bioreactor filled with 3.375 L modified NBS medium II. Seventy-five percent glucose solution (125 mL as a feeding unit) was fed at specific time intervals when glucose concentration was lower than 20 g/L. Temperature of fermentation and speed of stirring were set at 37 °C and 200 rpm respectively; pH was stabilized at 7.0 with 10 M NaOH solution. Fermentation ended when pumping of base for neutralization stopped.

Whole genome resequencing

Strains WE169, WE269, and WE319 were chosen for whole genome resequencing to identify important mutations occurred in the evolved strain (Table S1). Strains were recovered with LB medium from frozen stock for genomic DNA extraction, with wild-type strain W3110 as a control. Genomic DNA was prepared via a standard phenol-chloroform method. Then, genome DNA prepared was sent to Genewiz (Suzhou, China) for library

construction for next-generation sequencing according to the standard procedure of genome resequencing pipeline. Detailed procedures were carried out as described below. Firstly, genomic DNA was quantified and agarose gel electrophorized before library construction. Qualified genomic DNA was then sheared into smaller fragments with a desired size. Then overhangs generated from the prepared fragmentation were repaired and an "A" base was added to the 3′ end of the blunt phosphorylated DNA fragments; adapters were then ligated to the ends of the DNA fragments. The desired fragments were gel-purified and selectively enriched by PCR amplification. Indexed tags were introduced into the adapters at the PCR stage. Then, the qualified libraries were used for next-generation sequencing via the Illumina HiSeq platform (San Diego, CA, USA). Clean reads were purified from raw data by removing low-quality reads and then used for alignment to the reference genome (*E. coli* W3110, (ref|AC_000091.1|)). Mapping of clean reads to the reference genome was conducted via Breseq (version 0.30.0 revision d61745a1e512 (Barrick et al. 2014; Deatherage and Barrick 2014). Bioinformatics analysis included single nucleotide variation (SNV)/InDel calling, structure variation, gene copy number variation, and gene function annotation with the KEGG database and Uniport protein database. Specific mutations found via whole genome resequencing were confirmed via Sanger sequencing. To determine the chronological order of key mutations taken places during the evolution process in evolved strain, Sanger sequencing of target genes from the intermediate stocking strains and the final evolved strain from the evolution experiments were determined. Then the chronological order of key mutations was predicted based on the first time of appearance of mutation during the evolution process.

Construction of single-gene and multiple-gene mutants of wild-type strain

Construction of single-gene and multiple-gene mutants of wild-type strain was achieved via a marker-less recombination approach as those described previously (Kuhlman and Cox 2010). Plasmid pTKRed and Ptks/CS were used in this marker-less recombination system. Recombination fragments for mutants' construction were prepared by site-directed mutagenesis of each gene cloned from the wild-type strain W3110 genomic DNA via fusion PCR. Recombination fragments were electro-transformed into prepared competent cells, and then positive colonies were selected with LB plates containing appropriate antibiotics. Positive colonies were confirmed by colony-PCR and Sanger sequencing.

Analytic methods

Biomass accumulation was determined via photometric analysis of culture optical density at wavelength of 600 nm using a TU1901 UV-visible spectrophotometry photometer (PERSEE Corp., Beijing, China). Glucose and extracellular metabolite concentrations were determined by high performance liquid chromatography (HPLC) analysis. Aliquots of samples were spun down at 14000×*g* for 5 min and supernatants were filtered and then diluted with 5 mM H_2SO_4 before injecting into an Agilent 1200 system equipped with a reflect index detector (RID) (Agilent Technologies, Santa Clara, CA, USA). A Phenomenex column (Torrance, CA, USA) fitted with a guard column was equilibrated with 5 mM H_2SO_4 mobile phase at 55 °C with a flow rate of 0.6 mL/min. The injection volume of samples was 5 μL; temperature of RID detector was set at 35 °C. Standard solutions of glucose and main components of extracellular metabolites were used to calibrate the measurements. Optical purity of lactate was determined as those described previously (Liang et al. 2018).

Results

Evolutionary engineering improved anaerobic growth and acid tolerance of *E. coli* in minimal medium

Evolutionary engineering is an effective approach for improving various phenotypes of microbes; here, it was used to improve anaerobic growth of wild-type strain *E. coli* W3110 in M9 minimal medium via serial transfer cultivation. Evolution experiment was conducted as those described in the methods section with 25 mL scale screw-capped anaerobic bottles; glucose was adopted as the sole carbon source and no neutralization agent was incorporated in medium used for evolution. After about 400 rounds of serial transfer cultivation, equivalent to around 1300 generations, evolved strains with improved growth of various degree (WE169, WE269, WE319) were selected and confirmed via growth test according to the methods section (Table S1). Under test conditions, evolved strain WE269 and WE319 entered stationary phase after 6 h of growth with an average cell density of OD_{600nm} of 1.44 and 1.39 respectively, while wild strain W3110 only achieved an average cell density of OD_{600nm} of 0.85 during the same time (a cell density of OD_{600nm} of 1.0 equivalented to 0.33 g DCW/L) (Fig. 2). Evolved strain WE169 entered stationary phase after 8 h of growth with an average cell density of OD_{600nm} of 1.31, and wild-type strain went into stationary phase at 10 h with an average cell density of OD_{600nm} of 1.22. Evolved strains had shown various degree of improved average growth rate during the exponential growth phase as compared with wild-type strain ($\mu_{MAX} = 0.18$), with WE269 ($\mu_{MAX} = 0.31$) had the highest growth.

With the aim of developing a platform for anaerobic production of bulk chemicals like lactate and other organic acids, we further evaluated growth potential of evolved strain under acid stress and low concentration of 3HP, an isomer of lactate. In the first test, initial pH of the M9 minimal medium were tailored to

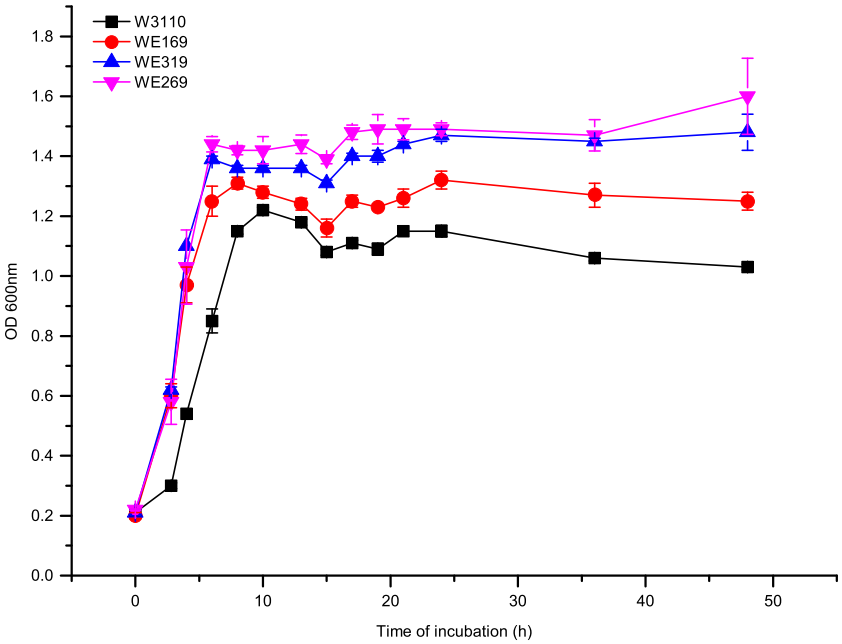

Fig. 2 Growth curves of evolved strains selected from evolutionary engineering experiment. All *E. coli* strains were anaerobically cultured in 100 mL M9 minimal medium containing 5% (*w*/*v*) glucose at 37 °C. WE169 (solid circles), WE319 (up triangles), and WE269 (down triangles) were all evolved strains selected from different generations of frozen stocks of the evolution experiment. Wild-type strain W3110 (squares) served as a control. Data are presented as the mean and standard deviation of triple replicates

7.0, 6.0, 5.0, and 4.0 with 6 M HCl solution, then inoculated with an initial cell density of OD_{600nm} of 0.2 for both wild type and evolved strain. For both strains, there was significant inhibition in growth when pH decreased from 7.0 to 4.0 (Fig. 3a, b). As for growth with an initial pH of 6.0, a cell density of OD_{600nm} of 0.91 was observed in strain W3110 at 72 h, while a 35% increase in OD_{600nm} was observed in strain WE269 at the same time point. Growth rate of WE269 ($\mu_{MAX} = 0.27$) is 12.5% higher than that of W3110 ($\mu_{MAX} = 0.24$) under test conditions. When growth was tested with an initial pH of 4.0, a cell density of OD_{600nm} of 0.59 was observed in strain W3110 at 72 h, while a 42% increase in OD_{600nm} was observed in strain WE269. Growth rate of WE269 ($\mu_{MAX} = 0.19$) is 24.5% higher than that of W3110 ($\mu_{MAX} = 0.15$). These results suggested a moderate improvement in tolerance to mild acid stress was observed in evolved strain WE269.

In the second test, 0.2% (*v*/*v*) of 3HP was incorporated in M9 minimal medium (with a pH around 5.0). After inoculation of wild-type strain W3110 and three evolved strain WE169, WE319, and WE269 with an initial OD_{600nm} of 0.2, significant decreases of growth were observed in all test strains in the presence of 3HP, which indicated severe inhibitory effect of 3HP on *E. coli* (Fig. 3c, d). In the presence of 0.2% (*v*/*v*) of 3HP, a cell density of OD_{600nm} of 1.13 was observed in WE269 at 12 h, while an OD_{600nm} of 0.92 was observed in W3110. Growth of evolved strains was still better than that of wild-type strain ($\mu_{MAX} = 0.127$); among them, WE269 ($\mu_{MAX} = 0.144$) had shown the highest stationary cell density in the presence of 3HP. A lower degree of increases in cell density has also been observed in the other two evolved strains in the presence of 3HP as compared with the wild-type strain.

Application of the evolved strain as a platform for lactate production

In order to evaluate the merit of evolved strain as a platform for production of bulk chemicals like lactate, firstly, we determined extracellular metabolite concentrations of wild-type strain W3110 and evolved strain WE269 under growth test in 100 mL scale anaerobic bottles to reveal changes in metabolic profile (Fig. 4a, b). For both strains, lactate was the main extracellular metabolite under test conditions and reached above 3.5 g/L at 48 h. Moreover, productivity of lactate was above onefold higher in the WE269 (0.44 g/(L·h) at first 6 h) as compared with W3110 (0.17 g/(L·h) at first 6 h) before growth went into stationary phase, though no significant difference was observed in final lactate titers between these two strains. 0.72 g/L ethanol, 0.19 g/L succinate, 0.28 g/L acetate, and 0.22 g/L formate were accumulated in W3110 after 48 h of cultivation, while for WE269, 0.84 g/L ethanol, 0.20 g/L succinate, 0.28 g/L acetate, and 0.22 g/L formate were accumulated during the same time. Significant amount of residual sugar was existed in both test strains.

Secondly, we carried out batch fermentation of evolved strain WE269 in a 5-L bioreactor with M9 minimal medium as those described in methods section. pH of the fermentation broth was stabilized at 7.0 with 5 M NaOH solution; 50 g/L glucose was used as carbon source. 33.58 g/L lactate was produced by WE269 after 30 h of fermentation, while 31.00 g/L lactate was produced by W3110 after 54 h (Table 1). Productivity of lactate by WE269 was 1.12 g/(L·h) which was 96.5% higher than that of wild-type strain. Glucose was completely metabolized by W3110 after 60 h of fermentation with a

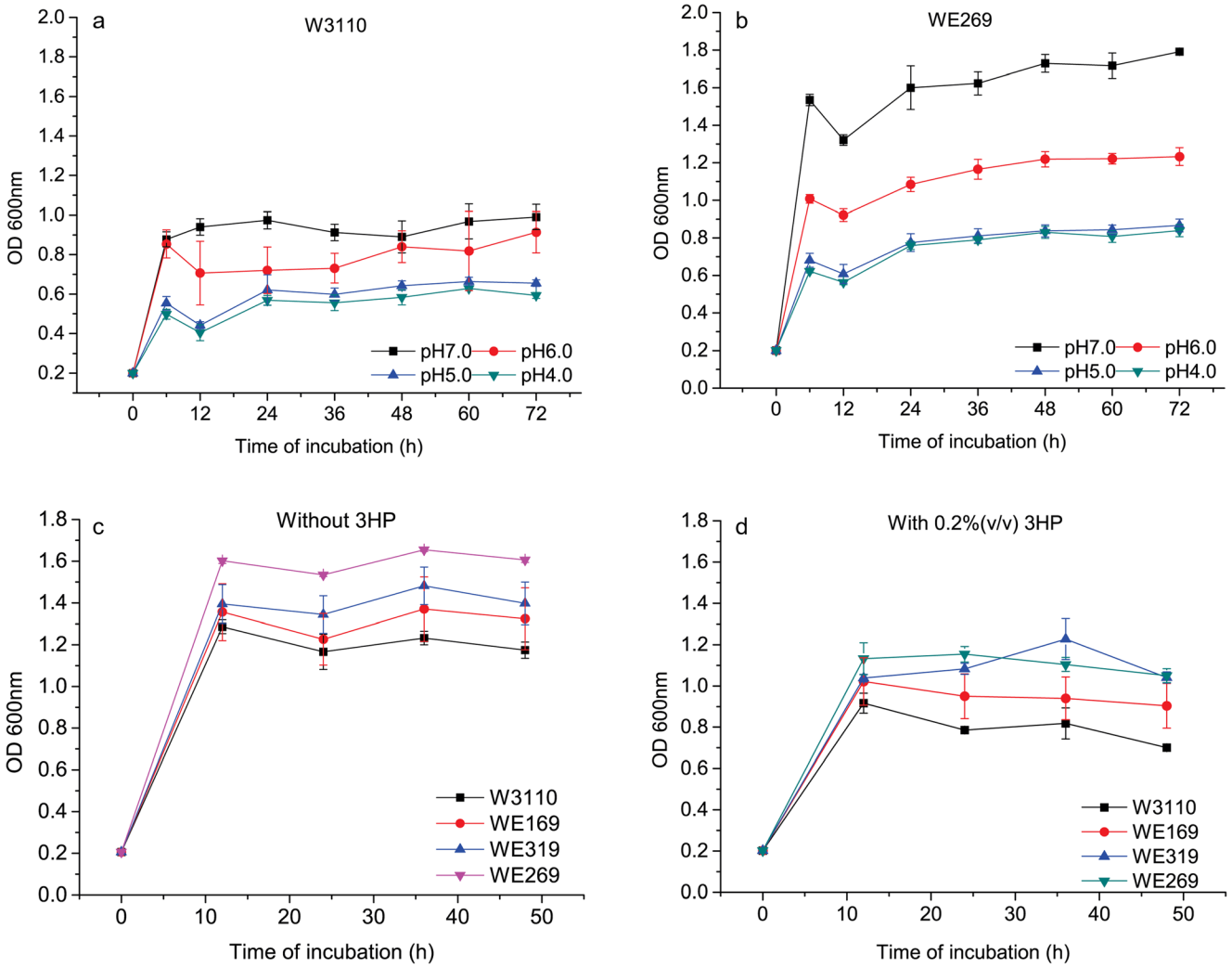

Fig. 3 Tolerance of evolved strain to acid stress and low concentration of 3HP. Test strains were anaerobically cultured in 100 mL M9 minimal medium containing 5% (*w*/*v*) glucose at 37 °C, with an initial pH of 7.0 (squares), 6.0 (circles), 5.0 (up triangles), and 4.0 (down triangles) tailored with the addition of different volume of 6 M HCl respectively (**a**, **b**). For test of evolved strains tolerance to 3HP stress, strains were anaerobically cultured in 100 mL M9 minimal medium containing 5% (*w*/*v*) glucose at 37 °C, with the absence (**c**) or presence of 0.2% (*v*/*v*) 3HP (**d**). WE169 (circles), WE319 (up triangles), and WE269 (down triangles) were evolved strains with improved growth selected from the evolution experiment; wild-type strain *E. coli* W3110 (squares) served as a control. Data are presented as the mean and standard deviation of triple replicates

metabolic rate of 0.84 g/(L·h). As for WE269, glucose was completely utilized after 30 h with a metabolic rate of 1.77 g/(L·h), 1.10 fold higher than that of wild-type strain (Fig. 4c, d). Ethanol (3.38 g/L), succinate (1.92 g/L), acetate (1.18 g/L), and formate (0.90 g/L) were detected in fermentation broth of WE269 after 30 h of fermentation. However, yield of lactate in strain WE269 was not improved (0.64 g/g) as compared with that of wild-type strain W3110 (0.65 g/g) under test conditions.

Thirdly, we deleted *pflB* gene in evolved strain WE269 via a marker-less homologous recombination method to obtain an engineered strain (BW100) for lactate production. The same gene has also been deleted in W3110 to get strain BP001. Then, lactate production via batch fermentation was carried out in both 100 mL scale anaerobic bottles and 5-L bioreactor with M9 minimal medium as described in the methods section. As for the 100 mL scale fermentation test, strain BW100 achieved a cell density of OD_{600nm} of 1.51 at the first 6 h with a μ_{MAX} of 0.275 and strain BP001 only reached a cell density of OD_{600nm} of 0.81 with a μ_{MAX} of 0.165 during the same time (Fig. 5a). However, strain WE269 and W3110 reached a higher cell density of OD_{600nm} of 1.64 and 1.0 respectively at the same time point, which indicated that deletion of *pflB* has caused growth retardation in both wild type and evolved strain. Strain BW100 produced 3.6 g/L lactate at the first 6 h of fermentation, while strain BP001 only accumulated 1.4 g/L lactate (Fig. 5b). During the same time, strain W3110 and WE269 accumulated 0.63 and 1.66 g/L lactate respectively. These results indicated that

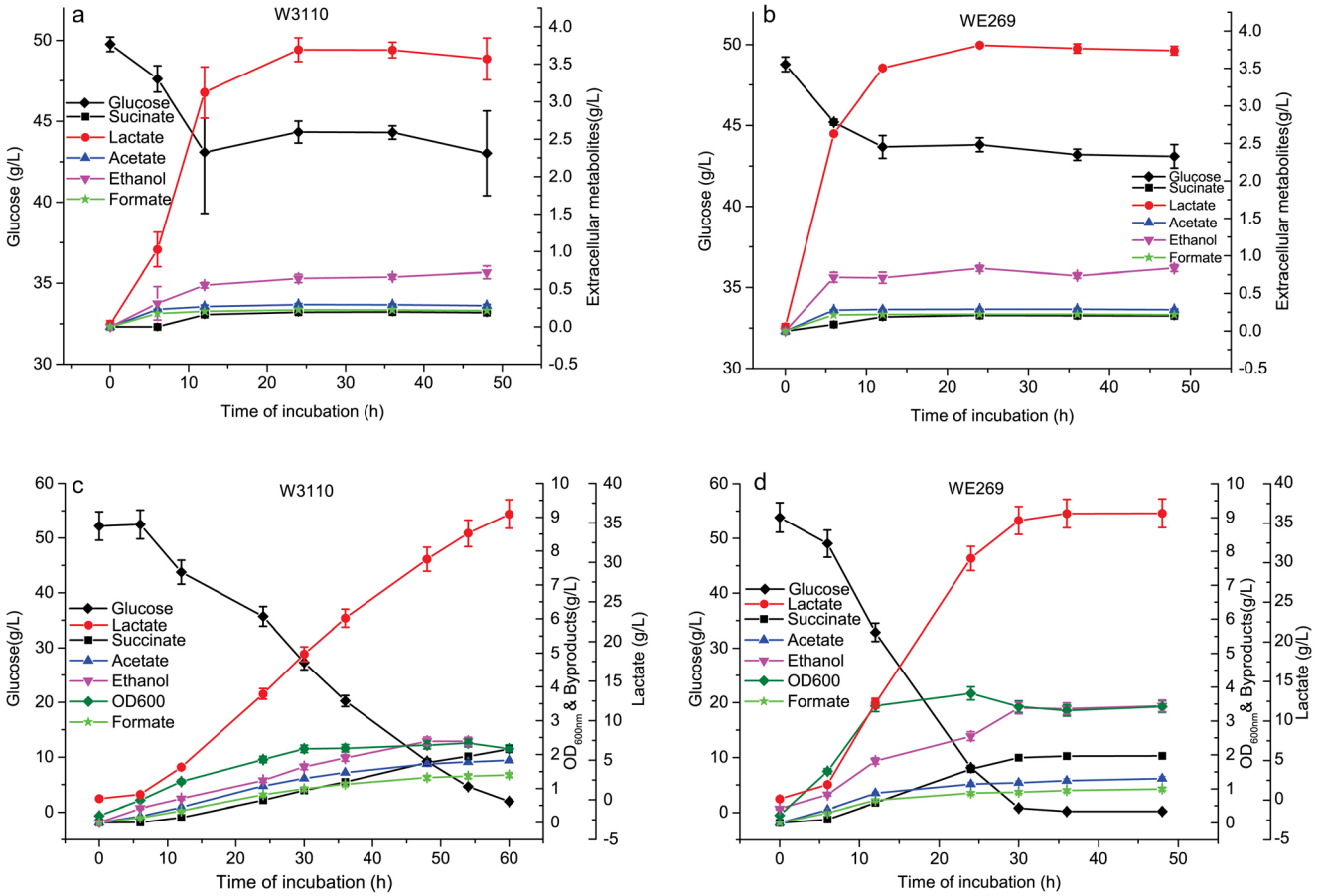

Fig. 4 Extracellular metabolites accumulation in 100 mL scale anaerobic bottles of wild-type strain W3110 (**a**) and evolved strain WE269 (**b**) and lactate production by strain W3110 (**c**), and WE269 (**d**) via batch fermentation in 5-L bioreactors with M9 minimal medium. All strains were anaerobically cultured in 100 mL M9 minimal medium containing 5% (*w*/*v*) glucose at 37 °C. The average value and standard deviation of extracellular concentrations of glucose (diamonds), succinate (squares), formate (stars), lactate (circles), acetate (up triangles), and ethanol (down triangles) from triplicate experiments are shown. Batch fermentation in 5-L bioreactors was carried out with M9 minimal medium containing 5% (*w*/*v*) glucose at 37 °C; the pH was stabilized at 7.0 with 5 M NaOH

Table 1 Lactate production by batch/fed-batch fermentation in 5-L bioreactors

Strain	Titer (g/L)	Productivity (g/(L·h))	Yield (g/g)	Biomass OD_{600nm}	Succinate (g/L)	Formate	Acetate	Ethanol
W3110[a]	31.00 ± 1.55	0.57 ± 0.03	0.65 ± 0.03	2.34 ± 0.12	1.95 ± 0.10	1.36 ± 0.07	1.78 ± 0.09	2.39 ± 0.12
WE269[b]	33.58 ± 1.68	1.12 ± 0.06	0.64 ± 0.03	3.42 ± 0.17	1.92 ± 0.10	0.90 ± 0.05	1.18 ± 0.06	3.38 ± 0.17
BP001[c]	25.30 ± 1.27	0.35 ± 0.02	0.66 ± 0.03	1.33 ± 0.07	0.67 ± 0.03	0	0.40 ± 0.02	0
BW100[d]	32.73 ± 1.64	0.45 ± 0.02	0.68 ± 0.03	1.26 ± 0.06	0.52 ± 0.03	0	0.20 ± 0.01	0
WE269[e]	88.14 ± 4.4	1.84 ± 0.09	0.85 ± 0.04	7.92 ± 0.40	4.18 ± 0.21	1.48 ± 0.07	1.93 ± 0.1	5.11 ± 0.25
BW100[f]	98.32 ± 4.9	2.05 ± 0.1	0.97 ± 0.05	4.54 ± 0.23	2.21 ± 0.11	0	0.59 ± 0.03	3.76 ± 0.19

[a] Data of W3110 presented here were determined at 54 h of batch fermentation with M9 minimal medium containing 5% (*w*/*v*) glucose

[b] Data of WE269 presented here were determined at 30 h of batch fermentation with M9 minimal medium containing 5% (*w*/*v*) glucose

[c] Data of BP001 presented here were determined at 72 h of batch fermentation with M9 minimal medium containing 5% (*w*/*v*) glucose

[d] Data of BW100 presented here were determined at 72 h of batch fermentation with M9 minimal medium containing 5% (*w*/*v*) glucose

[e] Data of WE269 presented here were determined at 48 h of fed-batch fermentation with modified NBS medium with an initial glucose concentration of 5% (*w*/*v*)

[f] Data of BW100 presented here were determined at 48 h of fed-batch fermentation with modified NBS medium with an initial glucose concentration of 5% (*w*/*v*)

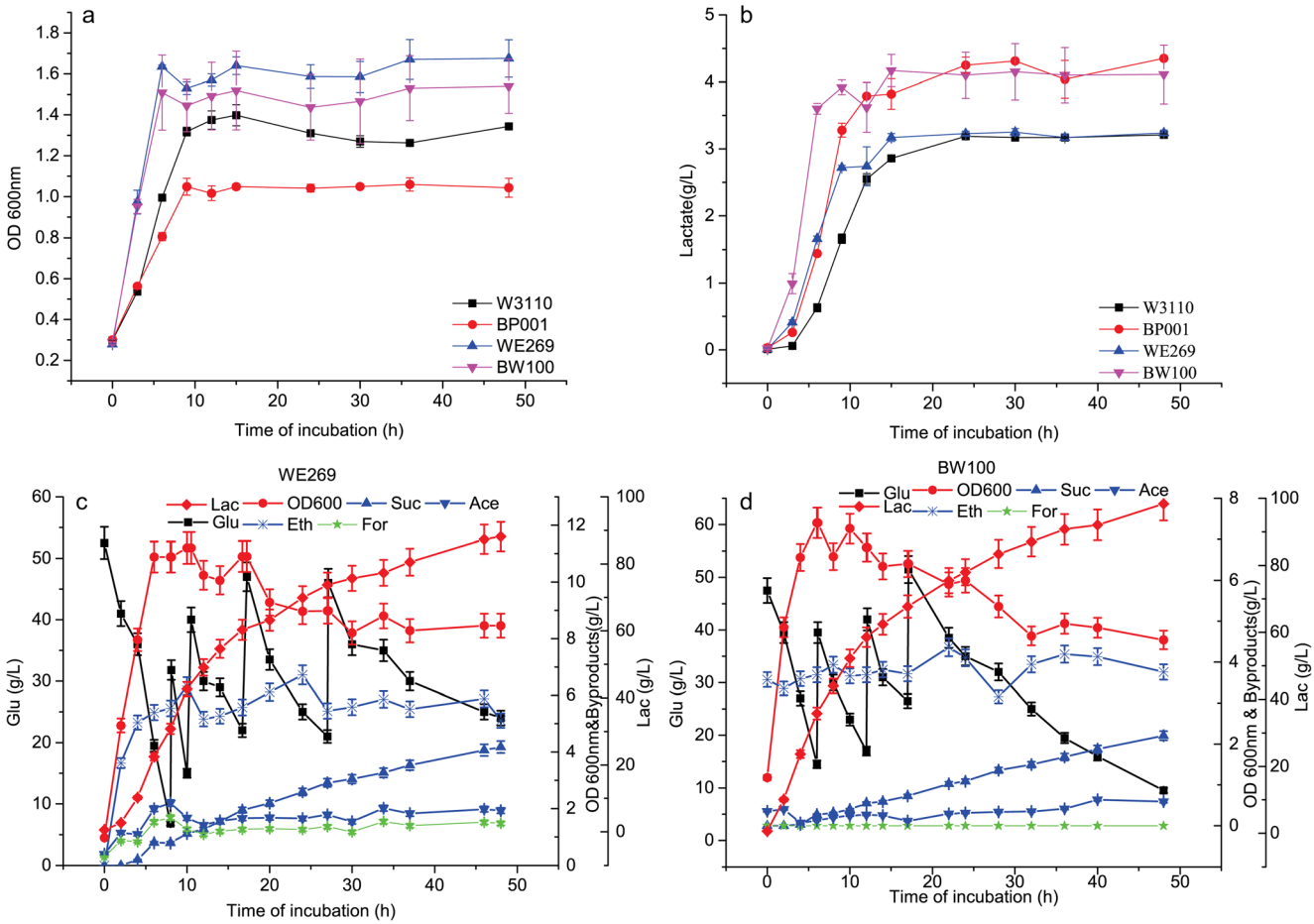

Fig. 5 Growth and lactate production by engineered strains BP001 and BW100 in 100 ml anaerobic bottles and fed-batch fermentation of strain WE269 and BW100 with modified NBS medium in 5-L bioreactors. For anaerobic bottles test, all strains were anaerobically cultured in 100 mL M9 minimal medium containing 5% (*w*/*v*) glucose at 37 °C (**a, b**). Strain BP001 (solid cycles) was constructed from wild-type strain W3110 by deletion of *pflB*, and strain BW100 (down triangles) was constructed from evolved strain WE269 by deletion of *pflB*. Wild-type strain W3110 (squares) and evolved strain WE269 (up triangles) served as controls. Data are presented as the mean and standard deviation of triple replicates. Fed-batch fermentation was carried out with modified NBS medium containing 5 g/L yeast extract with an initial glucose concentration of 5% (w/v) (**c, d**). Fermentation was inoculated with an initial cell density of OD 600 nm of 1.0. Seventy-five percent (*w*/*v*) concentrated glucose solution was fed into the bioreactors when glucose concentration was lower than 2% (*w*/*v*). pH was stabilized at 7.0 with 10 M NaOH solution

BW100 was more efficient in lactate production than the controls under test conditions. As for batch fermentation in 5-L bioreactor, 32.7 g/L lactate was produced by BW100 after 72 h of fermentation with a productivity of 0.45 g/(L·h), while strain BP001 only accumulated 25.3 g/L lactate with a maximal productivity of 0.35 g/(L·h) during the same period (Table 1). Under test conditions, lactate productivity of strain BW100 increased 28.6% as compared with that of BP001, and no obvious difference was observed in yield of lactate between these two strains. Accumulation of byproducts like succinate, acetate, and ethanol by strain BW100 and BP001 was all less than 1 g/L; this result indicated that deletion of *pflB* reduced byproduct formation in both strains.

Finally, fed-batch fermentation of lactate production by strain BW100 and WE269 was carried out in 5-L bioreactors as described in the methods section. Modified NBS medium I and II containing 5 g/L yeast extract were used for seed culture preparation and fermentation. A titer of 98.3 g/L lactate (with an optical purity of D-lactate above 95%) was produced by strain BW100 with a productivity of 2.05 g/(L·h) (Table 1). As a control, strain WE269 produced 88.14 g/L lactate under the same test condition. High yield of lactate on glucose had been achieved by strain BW100 under test conditions. Biomass formation and byproducts accumulation were much higher in strain WE269 than those of BW100, which was consistent with results of 100 mL scale anaerobic growth test and batch fermentation test in 5-L bioreactors (Fig. 5c, d). These results further supported that deletion of *pflB* hindered biomass formation and reduced byproducts formation (Zhou et al. 2003; Zhu and Shimizu 2004).

Preliminary investigation on the genetic basis of improved phenotype of evolved strain

To understand genetic bases of altered phenotypes in the evolved strain, WE169, WE319, and WE269 were sent for whole genome resequencing with W3110 served as a control. In general, an average of above 120 folds of coverage of the reference genome (*Escherichia coli* str. K-12 substr. W3110 DNA, complete genome, Genebank ID: NC_007779) was achieved by next-generation sequencing (NGS). Breseq (version 0.30.0 revision d61745a1e512) was used to analysis the NGS data along with the reference genome. Genetic changes including SNV, InDel, and evidence of structure variation were identified based on results returned from Breseq analysis. Background mutations in parent strain W3110 were subtracted when compared with the reference genome, and specific mutations in the genome of evolved strain WE169, WE319, and WE269 were identified. Mutations in protein coding sequences, promoter regions, and intergenic regions in different evolved strains were identified and grouped in a Venn Diagram (Fig. 6). Eight SNV mutations were identified in WE169 as compared with the parent strain W3110, including one readthrough mutation (*Y75_RS14300 (*33Y)*) in a hypothetic protein. Ten mutations were discovered in strain WE269, including one mutation *PdnaK(G → A)* in the possible promoter region of *dnaK*; one in the intergenic region of *purL* (*glrK/purL* intergenic (*C* → *T*)), one mutation (*nagA (Y117*)*) which has caused premature termination of *nagA* translation and one indel in a pseudogene (*ilvG* (Δ1 bp)). Fifteen mutations were discovered in strain WE319, including three mutations *PdnaK(G → A)*, *yqjH/yqjI* intergenic (*C* → *A*), and lipoprotein/*cysG* intergenic (*A* → *T*) in intergenic regions; two premature termination mutations *fhuA (E101*)* and *nagA (Y117*)*; one readthrough mutation *Y75_RS14300 (*33Y)*; and one indel mutation *ilvG* (Δ1 bp). Four identical mutations were discovered in all three evolved strains; they were *PdnaK(G → A)*, *dnaK (Q152H)*, *nadR (T233N)*, and *rpoB (T1037P)*.

To further understand how the mutations had taken place in the evolved strain, chronological order of occurrence of those common mutations between WE269 and WE319 was determined according to those described in the methods section (Fig. 7). Of the eight mutations both found in WE269 and WE319, mutations in *dnaK* (including *PdnaK(G → A)* and *dnaK (Q152H)*) took place between the 70th and 80th round of transfer. After that, mutation in *rpoB* occurred between the 90th and 110th round of transfer. Thereafter, mutation in *nadR* took place around the 169th round of transfer. After that, mutations in *ilvG*, *rpoA*, *nagA*, and *nagE* all took place between the 199th and 209th round of transfer.

To reveal effect of specific mutations on growth potential of evolved strain, we tried to construct single-gene mutants and possible multiple-gene mutants of wild-type strain W3110 in those genome loci where mutations were

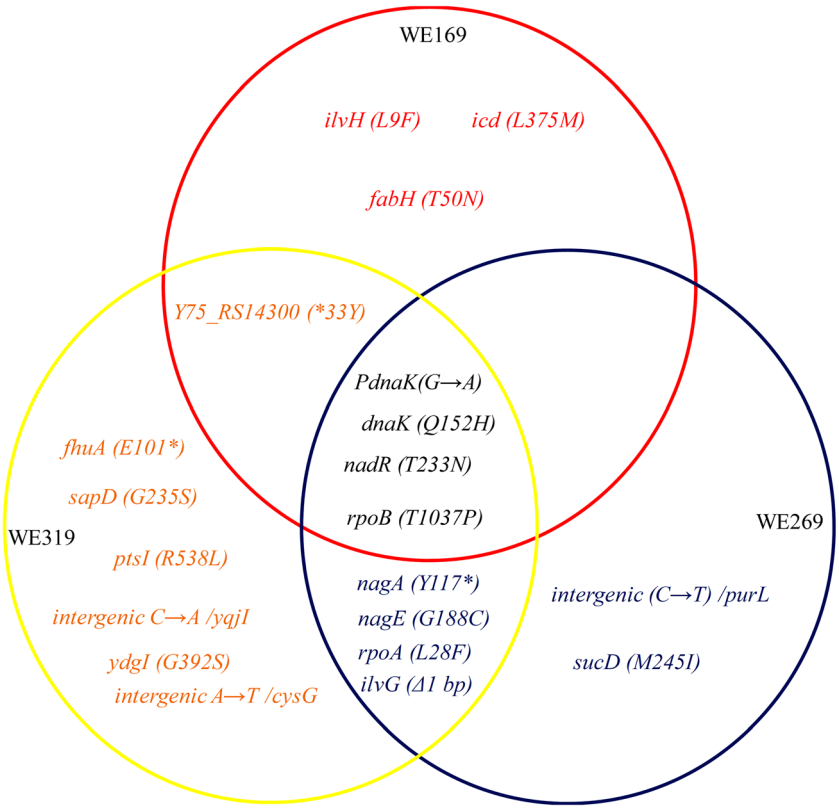

Fig. 6 Venn diagram of different mutations identified in evolved strains with improved growth selected from the evolution experiment. Genomic DNA extracted from selected evolved strains were used for whole genome resequencing. Mutations compared to the reference genome were identified with bioinformatics software Breseq; background mutations in wild-type strain *E. coli* W3110 were subtracted to get specific mutations in WE169 (red cycle), WE319 (yellow cycle), and WE269 (blue cycle)

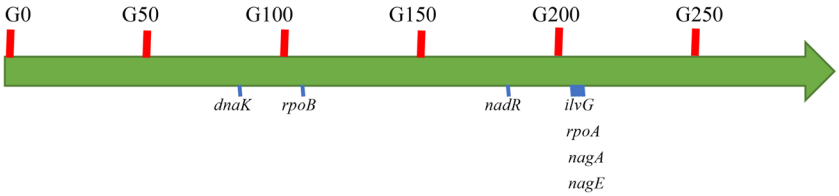

Fig. 7 Chronological order of mutations taken place in the evolutionary engineering experiments. Chronological orders of each mutation were determined by the first time of appearance of the mutation during the evolution process

found in WE269. Primers used for preparation of recombination fragments were listed in Table S2. Finally, six single-gene mutants of wild-type strain (RE02, RE03, RE04, RE06, RE07, RE08) and one double-gene mutant (RE09) were obtained (Table S1). Then, growth of these mutants of strain W3110 under anaerobic cultivation was

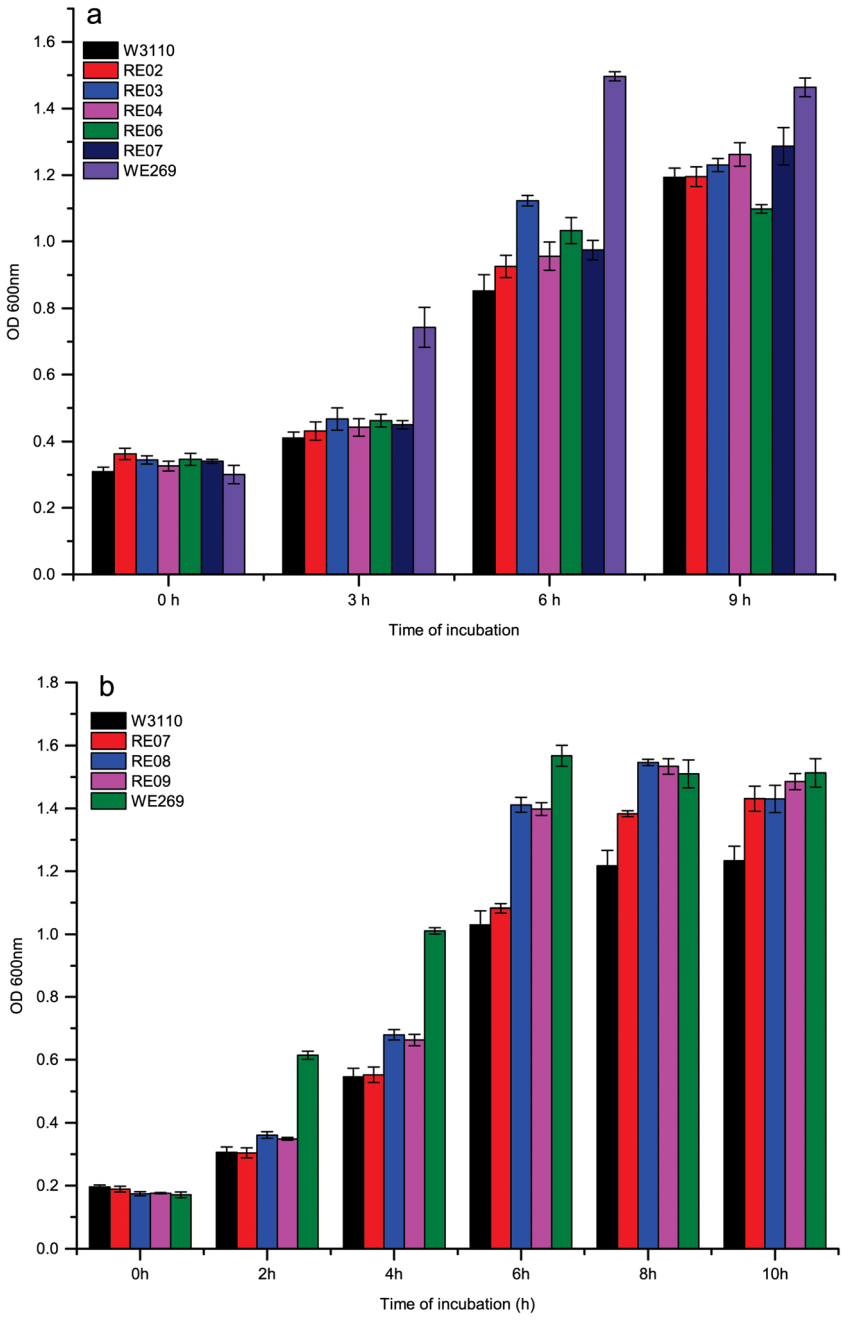

Fig. 8 Growth test of single-gene and double-gene mutants of wild-type strains *E. coli* W3110 under anaerobic cultivation. All strains were anaerobically cultured in 100 mL M9 minimal medium containing 5% (*w*/*v*) glucose at 37 °C. Single-gene and double-gene mutants were constructed by site-directed mutagenesis of wild-type strain W3110 with key mutations identified in whole genome resequencing experiments. RE02 (W3110 *nadR T233N*), RE03 (W3110 *rpoB T1037P*), RE04 (W3110 *nagA Y117**), RE06 (W3110 *rpoA L28F*); RE07 (W3110 *ilvG Δ1bp*), and RE08 (W3110 *sucD M245I:: tet*) were single-gene mutants of wild-type strain W3110, while RE09 (W3110 *ilvG Δ1bp sucD M245I:: tet*) were double-gene mutants of wild-type strain W3110. Strain W3110 (black) and WE269 (berry) served as controls. Data are presented as the mean and standard deviation of triple replicates

tested. During the exponential growth phase, all mutants have shown a better growth as compared with the wild-type strain. Among them, strain RE03 had a 32% higher cell density than that of W3110 at 6 h, while strain RE08 and RE09 have almost resumed the growth potential of evolved strain WE269 at 8~10 h (Fig. 8). This result indicated that *sucD M245I* mutation has the most significant effect on cell growth of evolved strain, while mutations in *ilvG Δ1bp*, *rpoB T1037P*, *nagA Y117**, *rpoA L28F*, and *nadR T233N* also have different degree of positive effect on cell growth under anaerobic conditions.

Discussion

Anaerobic fermentation is an attractive approach of producing bulk chemicals for its higher yield and lower energy consumption. Development of a successful microbial cell factory for anaerobic production of bulk chemicals needs to address many challenging problems and among them is the low productivity of some production host under anaerobic process (Weusthuis et al. 2011). Improving growth of typical culture like *E. coli* under anaerobic conditions is helpful to strain development for anaerobic production of bulk chemicals such as organic acids.

Evolutionary engineering of *E. coli* W3110 in M9 minimal medium under anaerobic conditions via serial transfer cultivation was carried out in this work, which finally led to an evolved strain with higher average growth rate and cell density in stationary phase. Increase in tolerance to mild acid stress (pH 4.0) and low concentration of 3HP was also observed in the evolved strain. This finding has also been supported by measuring the final pH of cultures in the growth test. The final pH of cultures of wild-type strain W3110 was 5.0, while those of evolved strain WE269 was 4.5 (data not show); this result indicated that the evolved strain accumulated more extracellular metabolites which acidified the culture to a much lower pH. This finding is helpful for the production of organic acids. Previously, researchers reported an evolved strain with increased growth under pH 4.6 obtained via adaptive evolution of *E. coli* under an external pH lower than 5 (Harden et al. 2015). In our work, no external acid stress was adopted during the evolutionary engineering process. Instead, organic acids such as lactate, acetate, and succinate accumulated via anaerobic fermentation of *E. coli* during the adaptive evolution process have served as endogenous sources of acid stress. During the adaptive evolution process, increased accumulation of acidic extracellular metabolites by the evolved strain came along with improved tolerance to acid stress. Liu reported that 1~2 g/L of 3HP slightly repressed growth of *E. coli* BL21(DE3), while 5 g/L of 3HP could acidified the medium to a pH of 4.0 (Liu et al. 2016). Thus, increase in tolerance to acid stress of the evolved strain was helpful for tolerance to low concentration of 3HP. This work has provided a new approach of developing acid tolerant strain without the introduction of external acid stress which could also be used for developing of strain tolerant to other organic acid chemicals.

We also tested feasibility of utilizing the evolved strain WE269 as a platform for construction of engineered strain for lactate production. In the growth test carried out with 100 mL anaerobic bottles, lactate productivity of evolved strain WE269 was also increased along with improved growth. When fermentation was carried out in a 5-L bioreactor with pH controlled at 7.0, 33.58 g/L lactate was accumulated by strain WE269 at 30 h with a productivity of 1.12 g/(L·h) which is 96.5% higher than that of wild-type strain. Both of these results indicated that improving anaerobic growth of *E. coli* accelerated lactate production. As no rich medium was included in the fermentation and no process optimization was conducted in this study, this value is comparable to those works reported previously (Grabar et al. 2006; Zhou et al. 2006; Zhu and Shimizu 2005). Productivity of biomass as a macromolecular by evolved strain WE269 (0.0376 g DCW/(L·h)) increased 163% as compared with that of wild-type W3110 (0.0143 g DCW/(L·h)) in this same test. It indicated that the evolved strain still favors biomass formation on product accumulation, and no obvious increase in yield has been observed during this process. This is a universal phenomenon along with microbes during the long history of evolution, while carbon metabolism and energy metabolism were interconnected during the process of cell growth and product accumulation (Wu et al. 2015). Under anaerobic fermentation conditions, lactate production regenerated NAD^+ utilized in glycolysis which produced ATP via substrate level phosphorylation. Biomass formation needs NADPH cofactor supplied by carbon flux to the PPP Pathway which is competing for total carbon flux to lactate production. Increased cell growth rate has uplifted the total lactate productivity, but may split a larger ratio of carbon flux to pathways important for biomass formation which finally sacrifice the yield of end product. However, productivity and yield of evolved strain was still very low as compared with some previous work (Niu et al. 2014). Based on this result, knockout of *pflB* in evolved strain WE269 was adopted to reduce byproduct formation; poor growth along with reduced lactate productivity was observed in anaerobic fermentation with M9 minimal medium. This phenomenon is consistent with an early work which reported that deletion of *pflB* decreased cell growth of the production host (Zhu and Shimizu 2005). Though similar effect on cell growth by deleting *pflB* has been observed in both wild type and evolved strain, the evolved strain still accumulated more lactate. This result further supported that improving growth of production host accelerated anaerobic production of lactate. In a fed-batch fermentation carried out with modified NBS medium in a 5-L bioreactor, 98.3 g/L lactate was produced by engineered strain BW100 after 48 h with a productivity of

Table 2 D-Lactate production by engineered *E. coli* and other microbes from representative works

Strain	Substrate	Medium	Scale (L)	Titer (g/L)	Yield (g/g)	Productivity (g/L·h)	Reference
Anaerobic fermentation by engineered *E. coli*							
SZ194	glucose	NBS	0.5	115	0.95	0.96	(Zhou et al. 2006)
TG108/TG113	glucose	NBS	0.5	115	0.95	2.29/1.92	(Grabar et al. 2006)
HBUT-D15	glucose	Modified NBS	30m^3	108	0.91	4.29	(Fu et al. 2017)
HBUT-D	sucrose	NBS	15	85	0.85	1	(Wang et al. 2012)
BLac-2106	Crude glycerol	NBS	1	105	0.87	2.63	(Wang et al. 2015)
JH15	Glucose +Xylose	Modified NBS	7	83	0.83	0.86	(Lu et al. 2016)
BW100	glucose	Modified NBS	5	98.3	0.97	2.08	This study
Aerobic-anaerobic bi-stage fermentation by engineered *E. coli*							
B0013-080A	Glycerol	Modified M9	7	119.8	0.87	4.11	(Tian et al. 2016)
	Glucose	Modified M9	7	129.5	0.977	3.66	
Lactate production by other microbes							
Corynebacterium glutamicum	Glucose		0.1	120\	0.87	4.0	(Okino et al. 2008)
Klebsiella pneumoniae	Glycerol		5	142.1	0.82	2.96	(Feng et al. 2014)
Saccharomyces cerevisiae	Glucose		0.1	112.0	0.80	2.2	(Baek et al. 2016)
Alkaliphilic Bacillus sp.	peanut meal, non-steril			143.99	0.96	1.67	(Assavasirijinda et al. 2016)
Sporolactobacillus inulinus Y2–8	corn flour hydrolyzate		7.5	218.8	0.98	1.62	(Zhao et al. 2014)

2.05 g/(L·h). Yield of lactate to glucose was also higher than that of batch fermentation carried out with M9 minimal medium, the main reason maybe rich medium component and NBS medium was more suitable for anaerobic growth of *E. coli* which finally led to higher productivity and yield. To our knowledge, it is one of the best lactate producers of *E. coli* which has been achieved via single stage anaerobic fermentation with glucose as carbon sources (Table 2).

System biology methods are powerful tools for investigation on complex phenotypes-genotype relationships of microbes. Whole genome resequencing combined with site-directed mutagenesis of the parent strain genome was conducted to identify key mutations which may responsible for improved growth in evolved strain. At the beginning, single-gene mutants of strain W3110 with specific loci identified in evolved strain were constructed; finally, six mutants were obtained while those mutations in *PdnaK(G → A)*, *dnaK (Q152H), nagE(G188C) and intergenic (C-T)/ purl* were not successful. One possible reason of the unsuccessful construction of these mutants maybe those single-gene mutations could lead to cell death. Among them, two mutations were related to the gene encoding DnaK, also known as HSP70. DnaK participates in response to hyperosmotic shock, oxidative stress, and heat shock response as a molecular chaperon, which is important under stress conditions (Thompson et al. 2012; Zhang et al. 2016). *nagE* encodes a protein involved in N-acetylglucosamine transport which is important for cell membrane biosynthesis; *purL* encodes a synthase involved in the purines biosynthetic pathway; effect of these genes on improving anaerobic growth of *E. coli* could not be evaluated via the approach used here. Among the six single-gene mutants, mutation in *sucD M245I* has the most important effect on improving anaerobic growth of *E.coli*; *ilvG(Δ1b)* and *rpoB (T1037P)* mutation also have shown significant positive effect (Fig. 8). *sucD* encoding a succinyl-CoA synthetase functions in the citric acid cycle (TCA) which is active under aerobic conditions, mutation in *sucD M245I* has caused a change of Met to Ile close to the active center (H247) in the conserved domains of SucD which may led to disfunction of this enzyme (Fig. S2, S3). Silencing genes encoding enzymes functioning in aerobic metabolism may provide great advantage for anaerobic growth. *ilvG* encoding an acetolactate synthase isozyme 2 large subunit which catalysis the transformation of 2 molecules of pyruvate to one molecule of 2-acetolactate and CO_2. *ilvG(Δ1b)* mutation caused frameshift mutation in the coding sequence which may led to the disfunction of this enzyme; thus, more pyruvate has been save for growth and lactate production. Beside *rpoB (T1037P)*, another mutation in gene encoding RNA polymerase, *rpoA (L28F)* was also found in evolved strain WE319 and WE269. Similar finding has also been reported in a previous work; mutations in *rpoB* and *rpoC* which have diverse effect on RNA transcription and reprogrammed *E. coli* for optimal growth in evolved strains were identified (Conrad et al. 2010). These results revealed that modification of the RNA transcription machinery seems to be a versatile approach of adaption to new environment by evolutionary engineering. Mutations

in *nagA (Y117*)* which caused a premature stop of translation in the coding sequence and *nadR* encoding a protein related to NAD synthesis and regulation have shown a limited effect on improving anaerobic growth of *E. coli*. In previous work, an IS150 insertion in *nadR* was also reported during experimental adaptation of *E. coli* to an anaerobic environment; this mutation was suggested to have blocked the negative control on NAD synthesis and finally enhanced anaerobic fermentation metabolism (Finn et al. 2017). However, effect of single-gene mutation *nadR(T233N)* on cell growth of W3110 was not obvious in this study; it may need to work together with other important mutations.

In order to further find out possible synergetic effect between different mutations, then we tried to construct multiple-gene mutation in wild-type strain. As the combination space is too large for engineering work by random combination of all mutations identified, we grouped these mutations to several categories based on their functions. The first category was tried to build one strain containing mutations in *rpoA*, *rpoB*, *PdnaK*, and *dnaK*. The second category was tried to build one strain containing mutations in *ilvG*, *sucD,* and *nadR*. The third category was tried to build one strain containing mutations in *nagA* and *nagE*. However, only one strain RE09 containing mutations in *ilvG* and *sucD* was successfully constructed after several rounds of failing and trying, and it has almost recovered the full growth potential of evolved strain WE269. The marker-less homologous recombination methods used in constructing these multiple-genes mutants have faced difficulties along with the complex physiological context of the engineered host strain. In our future work, CRISPR-Cas9 based recombination methods may facilitate the multi-loci engineering of target strain (Li et al. 2015). After determining the chronological order of those common mutations taken place during the evolution process, we found that mutations related to DNA replication and transcription machinery were taken place at a very early stage during the evolution which may provide a huge selection space for improving anaerobic growth of *E. coli*. To find out whether gene expression profiles have changed in the evolved strain, we also used Q-PCR to quantitate transcription level of genes in key carbon metabolic knots, DNA replication and transcription, efflux pumps, fatty acid synthesis, ATP, and cofactor synthesis (Fig. S4). Several genes related to PPP pathway (*ybhE* and *zwf*) and DNA replication (*dnaE* and *dnaK*) have been upregulated above 10 folds; several efflux pumps genes have also been upregulated 4~10 fold. Upregulation in PPP pathway could provide more NADPH which is important for biomass formation. Upregulation of DNA replication genes involved in stress tolerance and related cationic ions efflux pumps also have positive effect on anaerobic growth of *E. coli* in minimal mineral medium with high concentration of glucose. Two transcriptase genes *rpoA* and *rpoB* have been down regulated above twofolds along with two genes involved in fatty acid synthesis. Downregulation of transcriptase genes and several fatty acid synthetase genes maybe an approach of saving energy under anaerobic growth. Changes in transcription of genes involved in ATP synthesis (*atpA* and *atpB*) behave differently; this may be due to measurement error. ATP levels in evolved strain were a little lower as compared with the wild-type strain, which may indicate less feedback inhibition of the carbon assimilation in physiological state under test conditions (Fig. S5).

In conclusion, improving anaerobic growth of production host via evolutionary engineering is an effective approach for accelerating lactate production. Successful construction of an efficient lactate producer suggested advantages of the evolved strain as a platform for strain development of anaerobic production of organic acids. Systematic biology combined with classic molecular biology investigation demonstrated that mutation in *sucD* and *ilvG* has the most important impact on anaerobic growth of W3110, while modification of RNA transcription and DNA replication machinery genes occurred at an early stage of the evolution process also has positive impact on improving anaerobic growth of *E. coli*.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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