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Production of Lactate in *Escherichia coli* by Redox Regulation Genetically and Physiologically

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Abstract *Escherichia coli* grows fermentatively in glucose-containing medium under anaerobic condition with formation of a mixture of organic acids (lactate, acetate, formate, and succinate) and ethanol to accommodate reducing equivalents generated during glycolysis. In this paper, we tried to improve the lactate accumulation in *E. coli* by redox regulation genetically and physiologically. Our results indicated that genetic regulation of the host by reducing the reductive by-product may improve the lactate production. In addition, lactate accumulation was also improved under reduced and anaerobic cultivation conditions. Engineered *E. coli* SDU4 was able to accumulate lactate under strictly anaerobic conditions to 100 g/L with a yield of 1.97 mol/mol glucose.

Keywords *Escherichia coli* · Metabolic engineering · Redox · Lactate

Introduction

Lactic acid (2-hydroxypropionic acid) is the most widely occurring hydroxycarboxylic acid, having versatile applications in food, cosmetic, pharmaceutical, textile, leather, and chemical industries [1–4]. Meanwhile, the great potential market for the biodegradable polymer has increased the global interest in the production of lactic acid [1, 5, 6]. Many bacteria, such as the *Lactobacillus* species, were found to produce lactic acid in a large quantity by fermentation of glucose and other renewable resources [7]. *Escherichia coli*, a well characterized bacterium, has many advantages as a host for production of lactic acid, including the ability to produce optically pure lactate, rapid growth under both aerobic and anaerobic conditions, and its simple nutritional requirements. Most important, *E. coli* was

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able to utilize xylose as carbon sources for lactate production. The ease of genetic manipulation of *E. coli* makes possible metabolic engineering strategies for improving lactate accumulation in *E. coli* [8, 9].

Fermentation of sugars through native metabolic pathways in *E. coli* under anaerobic conditions produces a mixture of products consisting primarily of lactate, formate, acetate, and ethanol with smaller amounts of succinate (Fig. 1). The relative proportions of these products varied with the relative in vivo enzyme activities such as lactate dehydrogenase (*ldhA*), pyruvate formate lyase (*pflB*), and phosphoenolpyruvate carboxylase (*ppc*). Meanwhile, this product ratio also changed with the redox potential influenced by environmental factors, such as the availability of oxygen, the oxidation state of substrate as well as the presence of redox agents [10] in order to balance the reducing equivalents generated from glycolysis [11].

Fermentative production of lactic acid from glucose or other renewable sugars by metabolically engineered *E. coli* has been reported in several papers. For example, a *pfl ldhA* mutant (FBR11) containing a plasmid with the *ldh* gene from *Streptococcus bovis* (L-lactate dehydrogenase) produced L-lactic acid anaerobically on complex media [4]. The lactate production of this strain reached 73 g/L with a small amount of succinate (0.9–2.2 g/L). A *pta ppc* mutant (JP203) was able to generated 62 g/L of D-lactate in a two-state cultivation [8]. Recent results indicate that some characteristics of *E. coli* compare favorably with those of lactic acid bacteria, which often attain yields surpassing 90% [7]. Lactate is a reduced compound; redox regulation inside the cell or in the medium should be beneficial to lactate accumulation. To investigate this, we tried to regulate the redox potential intracellularly and extracellularly. Genetic reduction of reductive by-product can clearly redivert the metabolic pathway to lactate. Meanwhile,

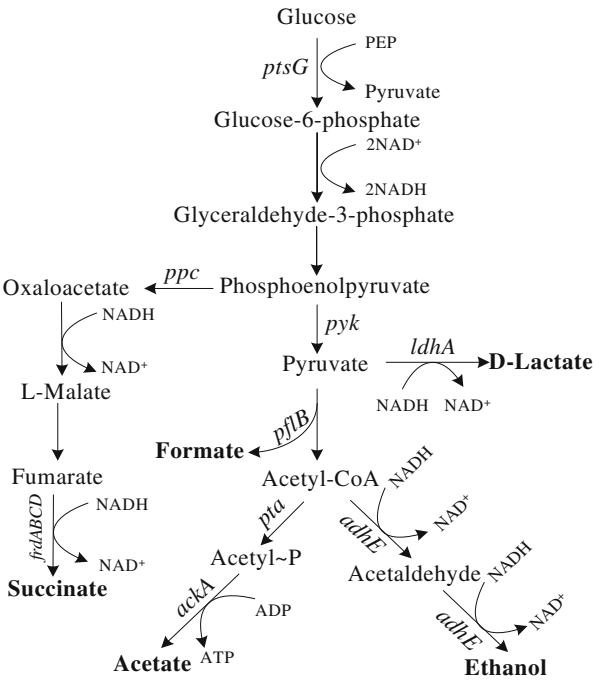

Fig. 1 Scheme of glucose fermentation by *E. coli*. Primary fermentation products are indicated by *boldface type*. Genes encoding important enzymes are indicated by *italics*

the lactate accumulation of the engineered *E. coli* was also improved under more reducing environmental and cultivation conditions.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions E. coli W3110, which is usually suitable for anaerobic growth, [12] and its constructed mutants, along with plasmids used in this study, are listed in Table 1. During the mutant construction, cultures were grown at either 30, 37, or 42 °C in Luria–Bertani (LB) broth or agar [13]. Antibiotics were included as appropriate at the following concentrations: kanamycin, 25 μg/ml; chlortetracycline, 34 μg/ml; and ampicillin, 100 μg/ml. For fermentation, all *E. coli* strains were inoculated from fresh plates into 5 ml of LB medium with appropriate antibiotics and grown for 6 h at 37 °C prior to inoculation in defined media.

Genetic Methods The general method to construct each mutant (*pflB*: GI89107753; *adhE*: GI89108085) followed the method of Datsenko and Wanner [14]. Polymerase chain reaction (PCR) was performed with appropriate hybrid primers (Table 2) complementary to *E. coli* W3110 chromosomal target genes and to the antibiotic cassette in pKD3 or pKD4 [14]. With a 40 bp homologous region at each end, the PCR product was introduced into the cell by electroporation and recombined into the chromosome of *E. coli* W3110. PCR verification was carried out to screen mutant having the correct structure using appropriate combinations of primers. The pCP20, a temperature-sensitive helper plasmid containing the thermally induced FLP recombinase gene, was transformed into the appropriate constructs to remove the antibiotic gene. All resistant transformants were selected by growth at 30 °C, after which colonies were verified for loss of all antibiotic-resistant markers at 42 °C.

Fermentation Cultivation was performed in LB medium or minimal salt medium (M9 medium) at 37 °C and 100 rpm supplied with glucose and/or glycerol or sorbitol. L-cysteine HCl or Na_2S of different concentrations was added if necessary. One hundred-milliliter serum bottles containing 50 ml of culture medium were used for normal anaerobic cultivation. N_2 was introduced at the beginning of cultivation when necessary. Strictly anaerobic fermentation was performed in a working volume of 3 L using a 5-L fermentor with nitrogen bubbled through the medium. The pH was controlled at 6.5–7.0 by addition of NH_4OH (25%). Samples were removed at intervals for the analysis of substrate, organic acids, ethanol, and cell mass.

Analyses Optical density at 600 nm was measured with Heλios UV-Visible Spectrophotometers (Thermo Electron Corporation, Waltham, MA, USA). Concentrations of products as well as residual substrates were quantified using high-performance liquid chromatography (Shimadzu LC-VP system; Shimadzu Corporation, Kyoto, Japan) equipped with

Table 1 *E. coli* strains and plasmids used in this study

Strains	Relevant characteristics	Source or reference
E. coli 3110	Wild type	Lab collection
SDU2	W3110, Δ*focA-pflB*::FRT	This study
SDU4	W3110, Δ*focA-pflB*::FRT Δ*adhE*::FRT	This study

Table 2 Oligonucleotides used for mutant construction

Name	Sequences of hybrid primers
pflB_F	5′-tcggcaacattatcggtggtggtttgttggttgggttgacGTGTAGGCTGGAGCTGCTT-3′
adhE_F	5′-attcgagcagatgatttactaaaaaagtttaacattatcGTGTAGGCTGGAGCTGCTTC-3′
pflB_R	5′-atagattgagtgaaggtacgagtaataacgtcctgctgctATGGGAATTAGCCATGGTC-3′
adhE_R	5′-atcggcattgcccagaaggggccgtttatgttgccagacATGGGAATTAGCCATGGTCC-3′

UV (210 nm) and refractive index detectors. Products were separated by using a cation exchange column (Aminex HPX-87H; Bio-Rad Laboratories, Hercules, CA, USA) with 5 mM H_2SO_4 as the mobile phase (0.6 ml/min, 65 °C). For analyzing, 1 ml of culture was centrifuged at 12,000 g for 5 min and the supernatant was then filtered through a 0.22-mm syringe filter. Standards were prepared for glucose and succinate, and calibration curves were created.

Results and Discussion

Intracellular Redox Regulation of *E. coli*

E. coli produced extracellularly a mixture of lactate, succinate, formate, acetate, and ethanol under anaerobic conditions. Metabolite interconversion at the phosphoenolpyruvate–pyruvate–oxaloacetate node involves a structurally entangled set of reactions that interconnects the major pathways of carbon metabolism and thus, is responsible for the distribution of the carbon flux among catabolism, anabolism, and energy supply of the cell [15]. Both succinate and lactate are direct metabolic products of this node. Many researchers have reported that the redox potential in vivo is important for lactate and succinate accumulation [16]. To accumulate lactate in *E. coli*, *pflB*, which encodes an anaerobic pyruvate formate lyase, should be knocked out. Knockout of *pflB* not only decreased the carbon flow to acetyl-CoA under anaerobic condition but also reduced the anaerobic consumption of NADH through reductive TCA. Our result indicated that deletion of gene *pflB* (*E. coli* SDU2) increased the lactate production to 1.67 mol/mol glucose with a sharp decline in formate generation (Table 3). To further reduce the ethanol formation under aerobic condition, which also consume 1 mol of NADH per mole of pyruvate, *adhE* should also be knocked out. Deletion of *adhE* (*E. coli* SDU4) led to additional improvement of lactate by 30% to 1.73 mol/mol of glucose at the expense of ethanol generation (Table 3). For sustained anaerobic fermentation, an organism must regenerate the reducing equivalent (NAD^+) consumed during glycolysis. *E. coli* accomplishes this task by formation of ethanol, lactate, or succinate [17]. When one of these pathways was blocked, *E. coli* will conceivably alter the distribution of these products to overcome the imbalanced reducing equivalents caused by the pathway knockout. Therefore, inactivation of AdhE not only rediverted the carbon flow but also rediverted the reducing equivalents—NADH for lactate and/or succinate accumulation. In our experiment, the lactate accumulation in *E. coli* SDU4 was slightly increased, indicating that the surplus NADH by inactivating AdhE gave priority to lactate accumulation over succinate formation. Metabolic engineering of microorganism should consider both carbon flow and redox balance.

Table 3 Analysis of the fermentation products of genetically modified *E. coli*

Strain	Genotype	Amount of products formed (mol/mol glucose consumed)[a]				
		Lactate	Succinate	Formate	Acetate	Ethanol
W3110	Wild type	1.34±0.02[b]	0.09±0.01	0.43±0.03	0.4±0.02	0.18±0.01
SDU2	W3110, Δ*pflB*	1.67±0.02	0.08±0.01	0.03	0.02	0.09±0.01
SDU4	W3110, Δ*pflB* Δ*adh*	1.73±0.01	0.07±0.01	ND	0.18±0.01	ND

ND not detected

[a] Fermentation tests were conducted in M9 medium supplemented with 20 g/L of glucose for 72 h of cultivation

[b] The mean values±SD of three independent experiments are shown

Extracellular Redox Regulation of *E. coli*

The redox potential of fermentation system was influenced by environmental factors such as the oxidation state of the substrate, the availability and nature of electron acceptors, as well as the presence of redox agents. Substrates, such as glycerol and sorbitol, were supposed to possess a highly reduced state [16, 18]. Addition of these compounds may greatly reduce the redox potential of the substrate and significantly increase the product yield of lactate by providing more reducing equivalents. In our study, lactate was found to be the main fermentation product of *E. coli* SDU4 with a molecular ratio of 18.3:1 to succinate when glucose was used as carbon source (Table 4). The production ratio of lactate to succinate dramatically increased to 40.5:1 with 10 g/L of glucose plus 10 g/L of glycerol as carbon source, and 149.8:1 when 20 g/L of glycerol was used exclusively. This result indicated that glycerol is an ideal feed stock for the fermentative production of fuels and chemicals with reduced states [19]. Based on this consideration, a homofermentative

Table 4 Lactate accumulation of *E. coli* SDU4 under different fermentation conditions

Fermentation conditions	Succinate production		Lactate production		Lactate/succinate (mol/mol)
	g/L	mM	g/L	mM	
20 g/L glucose	1.16±0.02[a]	9.79±0.05	16.14±0.04	179.15±0.17	18.3
10 g/L glucose+10 g/L glycerol	0.66±0.01	5.55±0.05	20.27±0.11	225.01±0.42	40.53
20 g/L glycerol	0.21±0.01	1.79±0.02	24.11±0.2	267.66±0.58	149.75
10 g/L glucose+10 g/L sorbitol	0.46±0.02	3.88±0.04	16.41±0.15	182.21±0.33	46.96
20 g/L sorbitol	ND	ND	4.42±0.04	49.07±0.05	–
20 g/L glucose+0.5 mM L-cysteine HCl	1.33±0.01	11.22±0.05	18.59±0.08	206.36±0.19	18.39
20 g/L glucose+4 mM L-cysteine HCl	0.97±0.02	8.19±0.07	19.04±0.05	211.42±0.2	25.8
20 g/L glucose+1 mM Na_2S	1.3±0.02	11.05±0.05	18.77±0.1	208.42±0.2	18.86
20 g/L glucose+8 mM Na_2S	1.2±0.01	10.16±0.03	17.81±0.04	197.74±0.16	19.47

ND not detected

[a] The mean values±SD of three independent experiments are shown

metabolic pathway from glycerol to D-lactate was recently constructed in *E. coli* [20]. In our study, the same effect was also obtained when sorbitol, another substrate with a reduced state, was supplemented as carbon source. Since only little sobitol was utilized, no succinate was found in the medium. All these confirmed that the substrate redox state is important for lactate production.

To see if additional reducing agents in the medium also have similar results to substrates with different redox states, reducing agents L-cysteine–HCl or Na_2S at different concentrations were added in the fermentation medium. A positive correlation was found between the reducing potential and lactate production (Table 4). Adding the reducing agent improved the lactate production. This result suggested that application of substrates or media with different redox states affected the product distribution and accumulation. Physiological regulation of the redox state in lactate fermentation should include both application of the reduced substrate and fine regulation of the anaerobic condition and/or redox potential in the medium.

Production of Lactate Under Anaerobic Condition

To produce lactate from glucose with *E. coli* SDU4, the strain was cultivated in M9 medium under anaerobic condition in 5 L fermentor. In our experiment, secretion of lactate in *E. coli* SDU4 expanded from 1.17 mol/mol glucose under normal anaerobic condition (no N_2 was provided, data not shown) to 1.97 mol/mol glucose under strict anaerobic condition (Fig. 2, N_2 was provided during the fermentation). The final lactate production reached 100 g/L with trace amount of other by-product. This result confirmed that decreasing the dissolved oxygen tension resulted in an increased NADH/NAD^+ ratio, and improvement of lactate production was achieved consequently. Many succinate fermentations were preceded with complicated processes, including sparging with gas (CO_2, H_2, O_2, or air) and dual aerobic and anaerobic process steps. Malate dehydrogenase (*Mdh* gene) activity is depressed during anaerobic conditions [21]. There is a twofold difference between expressions of Mdh under aerobic versus anaerobic conditions. Considering the response of lactate flux to the genetic perturbations, lactate dehydrogenase might be a means to afford the cell some metabolic flexibility in adapting readily to redox demands [22].

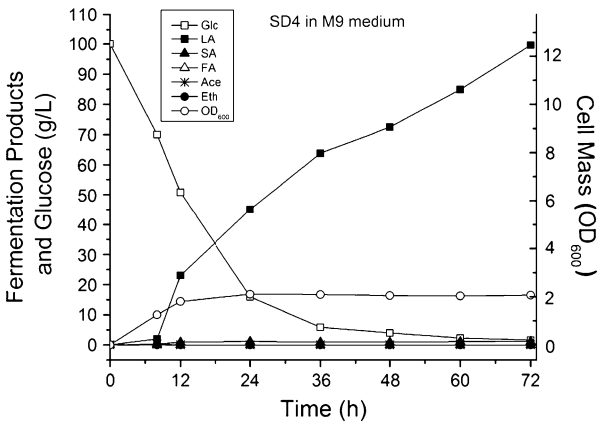

Fig. 2 Strict anaerobic fermentation of *E. coli* SDU4 in M9 medium supplemented with 100 g/L of glucose

Conclusion

Lactate fermentation can theoretically be achieved in *E. coli* with a lactate yield of 2 mol/mol glucose if both carbon and redox balance in the conversion of glucose exclusively to lactate. We obtained a lactate yield of 1.97 mol/mol glucose under low cell density fermentation and strictly anaerobic conditions. Our results confirmed that genetic engineering to reduce the reductive by-product is important. Lactate accumulation can be improved under reduced in vivo and environmental conditions.

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