Enzyme and Microbial Technology 53 (2013) 97–103

Contents lists available at SciVerse ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

Metabolic engineering of *Lactobacillus plantarum* for succinic acid production through activation of the reductive branch of the tricarboxylic acid cycle

Akira Tsuji[a], Sanae Okada[a], Pascal Hols[b], Eiichi Satoh[a,*]

[a] Department of Applied Biology and Chemistry, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya, 156-8052 Tokyo, Japan
[b] Institut des Sciences de la Vie, Université catholique de Louvain, Place Croix du Sud 5, B-1348 Louvain-La-Neuve, Belgium

ARTICLE INFO

Article history:
Received 14 November 2012
Received in revised form 19 April 2013
Accepted 22 April 2013

Keywords:
Succinic acid
Lactobacillus plantarum
Metabolic engineering
Pyruvate carboxylase
Phosphoenolpyruvate carboxykinase
Malic enzyme

ABSTRACT

Biosynthesis of succinic acid is an alternative method from conventional chemical synthesis. For this application, several bacteria and fungi have been employed and genetically modified. Lactic acid bacteria (LAB) are gaining recognition as novel producers of useful compounds by metabolic engineering. Among LAB, *Lactobacillus plantarum* NCIMB 8826 is an interesting candidate for succinic acid production by metabolic engineering since it has an incomplete tricarboxylic acid (TCA) cycle and naturally produces small amounts of succinic acid. In this study, we constructed recombinant LAB and evaluated them as hosts of succinic acid production. We examined the enzymes pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PEPCK), and malic enzyme for their potential to improve metabolic flux from glycolysis to the reductive TCA cycle in a lactate dehydrogenase-deficient strain of *L. plantarum* NCIMB 8826 (VL103). We investigated the effects of overexpression or coexpression of each enzyme on succinic acid production. Our results suggested that PC is the key enzyme for succinic acid production by *L. plantarum* VL103, whereas PEPCK is critical for increasing biomass. The highest yield of succinic acid was obtained through coexpression of PC and PEPCK in *L. plantarum* VL103. This recombinant strain produced a 22-fold higher amount of succinic acid than the wild-type and converted 25.3% of glucose to succinic acid.

1. Introduction

Lactic acid bacteria (LAB) are favorable microorganisms for metabolism modification, since they harbor small genomes and simple metabolic pathways. Furthermore, a range of methods for genetic manipulation have been developed to facilitate metabolic engineering [1–3]. LAB are gaining recognition as alternative producers of valuable metabolites by metabolic engineering. For example, the modification of pyruvate metabolism has enabled efficient production of diacetyl, L-alanine or sorbitol [4–6]. In addition, modifications of more complex biosynthetic pathways have improved production of exopolysaccharides, vitamin B_2, and vitamin B_6 [7,8].

Succinic acid is used as a raw material for the production of the biodegradable plastic polybutylene succinate and as a food additive. Because succinic acid is synthesized mainly by chemical methods from petroleum, alternative production methods, such as microbial fermentative production, are desirable [9]. There are three routes that form succinic acid: the reductive branch of the tricarboxylic acid (TCA) cycle, which is primarily active under fully anaerobic conditions, the oxidative branch of the TCA cycle, which is primarily active under aerobic conditions, and the glyoxylate shunt, which is essentially active under aerobic conditions upon adaptation to growth on acetate. The aerobic routes synthesize 1 mol of succinic acid from 1 mol of glucose, while the anaerobic pathway outputs 2 mol of succinic acid in combination with fixation of CO_2. Thus, succinic acid is efficiently produced under anaerobic conditions using the reductive branch of the TCA cycle [9]. Fixation of carbon dioxide is essential in this reaction.

Succinic acid production with genetic modification was demonstrated in various microorganisms [10]. The highest succinic acid productivity, 88.1 mM h^{-1}, was achieved in a continuous culture of *Anaerobiospirillum succiniciproducens* with integrated membrane for cell recycling [11]. The concentration up to 1.2 M was shown in a cell recycling fed-batch culture of *Corynebacterium glutamicum* [12]. A mutant *Escherichia coli* strain deficient in the phosphotransferase system produced 99.2 g succinic acid l^{-1} by anaerobic fed-batch fermentation [13]. Although these microorganisms produce succinic acid efficiently, they have some disadvantages, as some of them are obligate anaerobic, potentially pathogenic, or

* Corresponding author. Tel.: +81 3 5477 2328; fax: +81 3 5477 2619.
E-mail address: satoh@nodai.ac.jp (E. Satoh).

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http://dx.doi.org/10.1016/j.enzmictec.2013.04.008

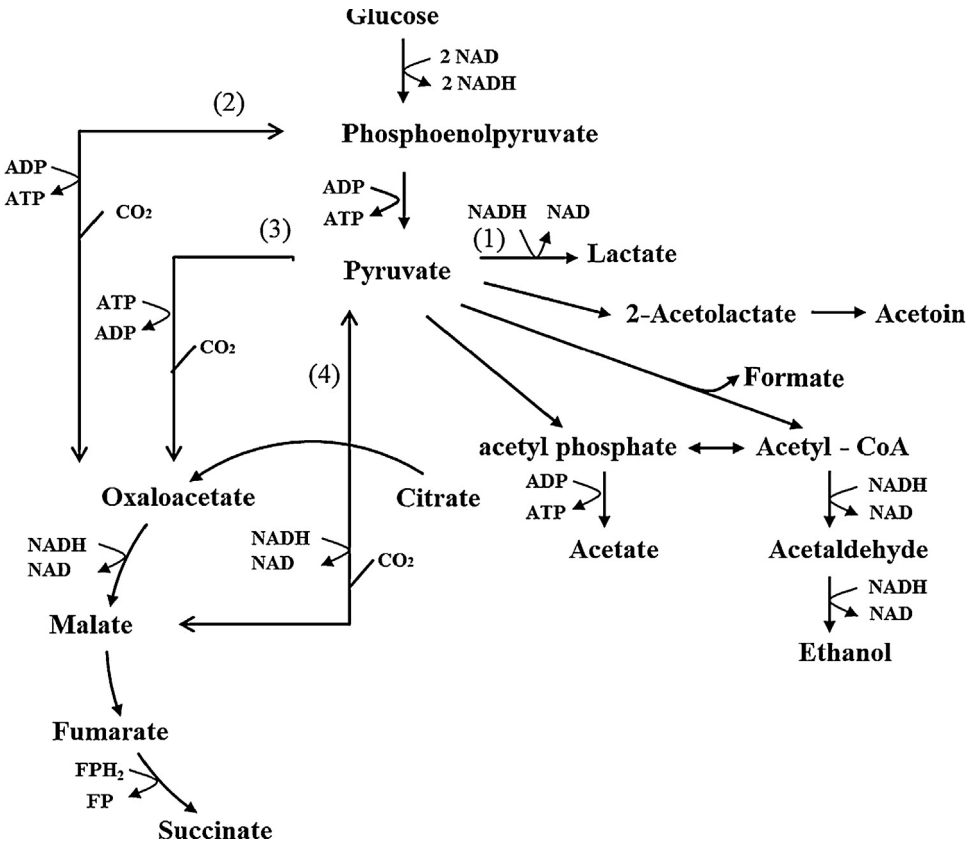

Fig. 1. The metabolic pathways related to succinic acid formation in *Lactobacillus plantarum* NCIMB 8826. (1) Lactate dehydrogenase; (2) pyruvate carboxylase; (3) phosphoenolpyruvate carboxykinase; (4) malic enzyme.

show poor growth, low tolerance to acid and osmotic stress [10]. Many LAB are generally recognized as safe (GRAS) and have been extensively used for industrial production of fermented food; they exhibits decent growth rate with various carbohydrates. In addition, as highly acid- and osmo-tolerant facultative anaerobes, LAB constituted an adequate model for succinic acid production by fermentation.

Lactobacillus plantarum NCIMB 8826 (identical to strain WCFS1) was isolated from human saliva [14]. This strain has been used as a model in metabolic engineering studies because its complete genome has been sequenced and deeply annotated [15,16]. This strain has an incomplete TCA cycle and produces small amounts of succinic acid (Fig. 1) [17]. *L. plantarum* VL103, the L- and D-lactate dehydrogenase (LDH)-deficient strain of *L. plantarum* NCIMB 8826 [18], was engineered to produce sorbitol by efficient rerouting of carbon metabolism [6]. As previously reported, productivity of succinic acid in *L. plantarum* VL103 was 2.44 mM and it could be increased up to 17 mM by supplementation of sodium bicarbonate to the culture medium [19]. This study indicated that *L. plantarum* VL103 could produce more succinic acid in a modified culture condition; however, no additional recombination which potentially increases the productivity has been tested.

To improve of succinic acid biosynthesis by *L. plantarum* VL103, metabolic flux from glycolysis to the reductive TCA cycle needs to be increased. In *E. coli* and *C. glutamicum*, the modification of metabolic pathway was reportedly achieved via overexpression of phosphoenolpyruvate and pyruvate-carboxylating enzymes [12]. The overexpression of *Lactococcus lactis* pyruvate carboxylase (PC) in *E. coli* increased the pyruvate carboxylating flux and improved succinate production [20]. Similarly, the homologous overexpression of the malic enzyme (ME) in *E. coli* resulted in pyruvate carboxylation and high succinate production, which was the major fermentation end-product [21,22]. Phosphoenolpyruvate could also be carboxylated to oxaloacetate by the phosphoenolpyruvate carboxykinase (PEPCK); oxaloacetate is then converted to succinate by the reductive TCA cycle. The heterologous overexpression of the *Actinobacillus succinogenes* PEPCK in the same host resulted in a significant improvement of succinate production [23].

To our knowledge, there is no report on succinate production via overexpression or coexpression of carbon fixation enzymes in LAB. Therefore, this study aims to use LAB as an alternative model to construct improved succinic acid producing strains. We investigated succinic acid yield and biomass production resulting from overexpression or coexpression of carbon fixation enzymes (PC, ME, PEPCK) in small-scale static cultures of *L. plantarum* VL103 recombinant strains.

2. Materials and methods

2.1. Bacterial strains and growth conditions

L. plantarum VL103 and derivatives were grown in modified de Man, Rogosa and Sharpe (MRS) medium (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) (2% glucose, 1.0% tryptone, 1.0% beef extract, 1.0% yeast extract, 0.2% K_2HPO_4, 0.1% Tween 80, 0.02% $MgSO_4$ $7H_2O$, 0.005% $MnSO_4$ $4H_2O$, 0.2% ammonium citrate, 0.5% sodium acetate) supplemented with 50 mM $NaHCO_3$ and erythromycin (5 μg/ml). Fermentation experiments were performed under tube-scale static conditions in 10 ml of modified MRS medium at 37 °C.

2.2. Plasmid and strain construction

In *L. plantarum*, *pycA* (lp_2136), *pckA* (lp_3418), and *mae* (lp_1105) encodes PC, PEPCK and ME, respectively. These genes were amplified by PCR from chromosomal DNA of *L. plantarum* VL103 using the following primer pairs: *pyc*AF (5′-TACGGATCCATTGGTGAAGAAAGTATTATTT-3′) and *pyc*AR (5′-TGCACTGCAGTCAACTAAGTTCTTAACTAC-3′) for *pycA*; *pck*AF (5′-CGCGGATCCTATGAGCACTAAAAATTCTTA-3′) and *pck*AR (5′-TTTTTGCACTGCAGCTATTTTGTAATTGGTTGTT-3′) for *pckA*; and *mae*F (5′-CGGGATCCAATGGTAGAACAAGATGAGAT-3′) and