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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

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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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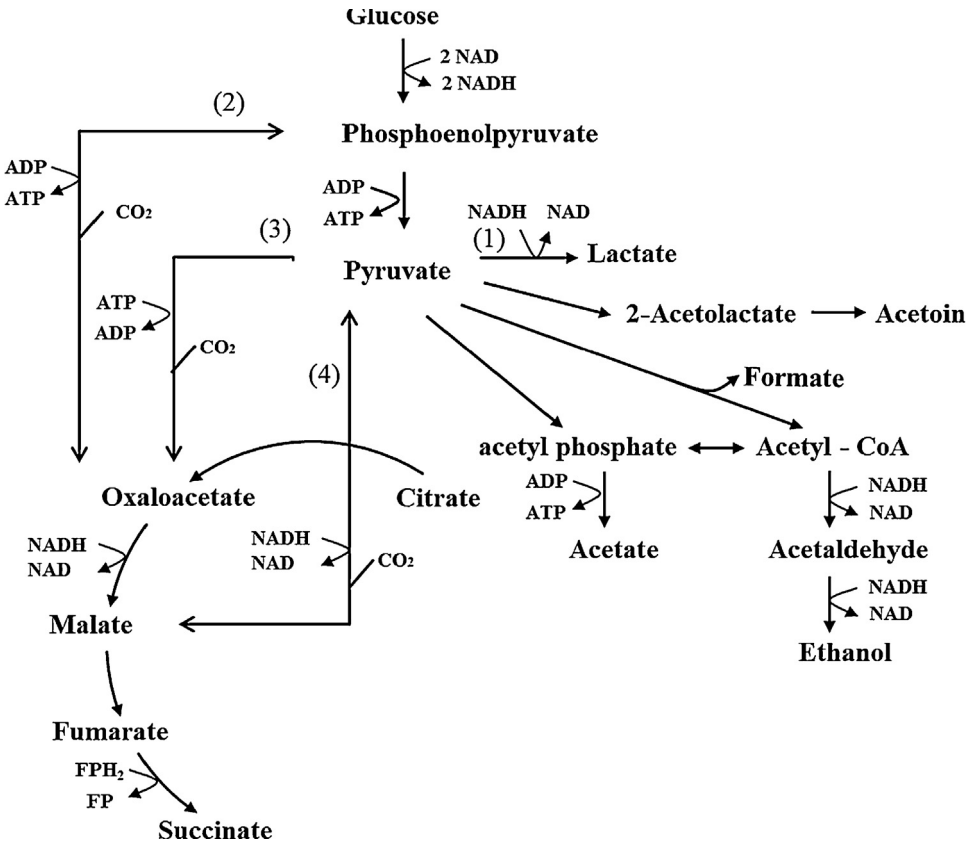

Fig. 1. The metabolic pathways related to succinic acid formation in *Lactobacillus plantarum* NCIMB 8826. (1) Lactate dehydrogenase; (2) pyruvate carboxylase; (3) phosphoenolpyruvate carboxykinase; (4) malic enzyme.

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

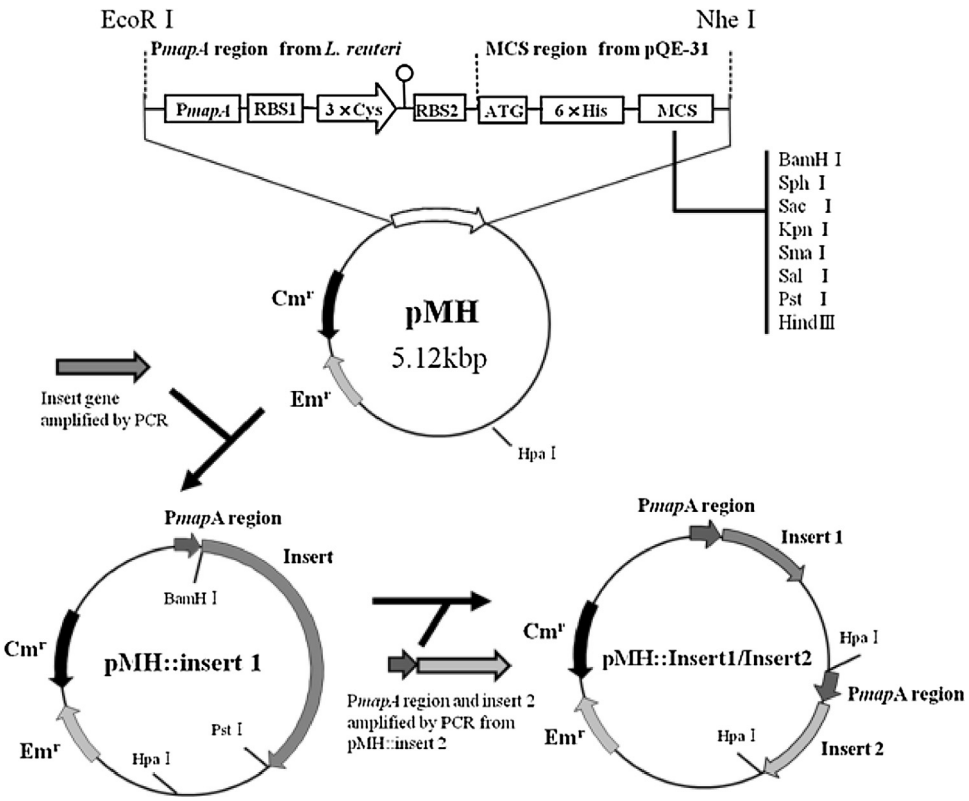

Fig. 2. Construction of overexpression plasmid and coexpression plasmid.

*mae*R (5′-AAAACTGCAGCTCCTATGATTAATGCTGTG-3′) for *mae*. BamHI and PstI restriction sites underlined in the primer sequences were attached upstream and downstream of the coding region. BamHI/PstI-digested PCR products were cloned into the corresponding sites of pMH, digested with the same enzymes, yielding the plasmids pMH::*pycA*, pMH::*pckA*, and pMH::*mae*. The plasmid pMH is a pGK13 [24] derivative which was constructed in a previous study [19]. This plasmid contains the expression signals of the *mapA* (Mucus Adhesion Promoting protein) operon from *Lactobacillus reuteri* DSM 20016^T, a multiple cloning site, and the translational start codon from pQE-31 (Qiagen, Hilden, Germany). The coexpression plasmid of PC with PEPCK (pMH::*pycA*/*pckA*), PC with ME (pMH::*pycA*/*mae*), and PEPCK with ME (pMH::*pckA*/*mae*) were constructed as shown in Fig. 2. The PEPCK expression cassette, consisting of *mapA* operon promoter fused to *pckA* gene of pMH::*pckA*, were cloned into pMH::*pycA* and pMH::*mae*, yielding plasmids pMH::*pycA*/*pckA* and pMH::*pckA*/*mae*, respectively. The ME expression cassette of pMH::*mae* were cloned into pMH::*pycA*, yielding plasmid pMH::*pycA*/*mae*. *L. plantarum* VL103 was transformed with the resulting plasmids by electroporation as described previously [25].

2.3. Enzymatic assays

The bacteria were grown in MRS medium until the mid-exponential phase (optical density [OD] at 660 nm of 2.0), harvested by centrifugation at 13,000 × *g* for 10 min, and mechanically disrupted with glass beads. Enzyme activity was assayed spectrophotometrically at 25 °C in 1 ml of reaction solution, which consists of 50 mM potassium phosphate buffer (pH 7.0), 9.7 mM Na_2CO_3, and 9.7 mM $MgSO_4$, and 0.14 mM nicotinamide adenine dinucleotide (NADH). Sodium pyruvate (3.1 mM), 9.7 U/ml malate dehydrogenase, and 3.1 mM adenosine triphosphate (ATP) was added to the reaction solution in which PC activity was measured. Phosphoenolpyruvate (3.1 mM) (potassium salt), 9.7 U/ml malate dehydrogenase, and 3.1 mM adenosine diphosphate (ADP) was added to the reaction solution in which PEPCK activity was measured. Sodium pyruvate (3.1 mM) was added to the reaction solution in which ME activity was measured. The reaction was initiated by the addition of 0.1 ml of cell extract (10 μg of total protein extract), and monitored by the decrease in OD at 340 nm. One unit of enzyme was defined as the amount that catalyzed the oxidation of 1 mmol NADH to nicotinamide adenine dinucleotide (NAD^+) per minute at 25 °C. Protein concentrations were determined according to the Bradford method, using bovine serum albumin as a standard [26].

2.4. Analytical methods

Samples of culture were filtered (pore size: 0.22 μm) after centrifugation at 13,000 × *g* for 10 min. The supernatant was analyzed for glucose consumption and production of fermentation end-products. Concentrations of organic acids were determined by high-performance liquid chromatography (HPLC) using a Waters® 2695 Separations Module, 2996 Photodiode Array Detector, and Reagent Manager (Waters Corporation, Milford, MA, USA) with columns RSpak KC-811 (Shodex, Tokyo, Japan) and RSpak KC-G (Shodex). The column was eluted isocratically at 60 °C at a flow rate of 0.8 ml/min with 0.08% $HClO_4$, Ethanol and acetoin concentrations were determined by HPLC, as for the concentrations of organic acids. The column was eluted isocratically at 60 °C at a rate of 1 ml/min using surface plasmon waves, which facilitate detection of refractive index changes. Glucose was measured with a commercial glucose CII-test kit (Wako Pure Chemical, Osaka, Japan). Analysis of intracellular metabolic products was performed according to the method described previously [27].

Cell growth was determined by measuring the increase in OD at 660 nm spectrophotometrically (UV-2000; Shimadzu Scientific Instruments, Inc., Columbia, MD, USA). One unit of OD corresponded to 2.5×10^6 CFU/ml.

2.5. Statistical analysis

Statistical evaluation of the fermentation product, enzymatic activity, and cell growth were performed by Student's *t*-test with the level of significance set at $p < 0.05$.

3. Results and discussion

3.1. Metabolic characteristics of L. plantarum VL103 overexpressing pyruvate carboxylase, phosphoenolpyruvate carboxykinase, and malic enzyme

From analysis of the genome sequence, *L. plantarum* NCIMB8826 contains gene candidates coding for PC, PEPCK, and ME. Each gene candidate was individually cloned on a multicopy plasmid for its overexpression in *L. plantarum* VL103. To confirm their functionality, PC, PEPCK, and ME enzyme activity was measured. Table 1 shows the enzyme activities of *L. plantarum* VL103(pMH) used as control (empty vector) and its derivatives. Each overexpression strain exhibited an increased activity of its corresponding enzyme. The activities of PC, PEPCK, and ME in *L. plantarum* VL103 were 2.4-, 1.7-, and 1.3-fold higher than those of the control strain,

Table 1
Enzymatic activities detected in cell extracts[a] from the *Lactobacillus plantarum* VL103 and derivatives.

L. plantarum VL103strains	Enzymatic activity[b] (U/mg of protein) of strain		
	PC	PEPCK	ME
pMH	0.78 ± 0.018	0.39 ± 0.033	0.088 ± 0.011
pMH::*pycA*	1.89 ± 0.078	–	–
pMH::*pckA*	–	0.68 ± 0.063	–
pMH::*mae*	–	–	0.119 ± 0.006

Values represent the mean and standard error derived from three independent experiments, with each experiment performed in triplicate. PC, pyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; ME, malic enzyme.
[a] Cell extracts were prepared according to the method described in Section 2.
[b] One unit of activity corresponds to the conversion of 1 mmol substrate per min.

respectively. These differences were statistical significant between control and the derivatives. These results suggested that these three enzymes are active and potentially capable of increasing metabolic flux from glycolysis to the reductive TCA cycle.

Presence of metabolites in the culture supernatants of *L. plantarum* VL103(pMH) and derivatives were determined every 24 h by HPLC (Table 2). Succinic acid concentration reached its peak after 48 h (Table 2), and remained constant until the end of fermentation (data not shown). All the derivatives yielded more succinic acid than the control strain VL103(pMH) after 48 h of fermentation (1.5 to 3-fold increase). *L. plantarum* VL103(pMH::*pycA*) yielded the highest succinic acid concentration (52.5 mM), which is 3- and 22-fold higher than the LDH-deficient strain VL103(pMH) (16.9 mM) and the wild-type (2.44 mM), respectively. Notably, *L. plantarum* VL103(pMH::*pycA*) converted 23.8% of glucose to succinic acid. This strain exhibited the highest increase in enzyme activity and succinic acid yield among the recombinant strains overexpressing each enzyme. These results demonstrated that PC overexpression is effective for constructing improved succinic acid-producing *L. plantarum* strains and suggest that PC is a key enzyme for succinic acid production in this model microorganism.

An intracellular accumulation of succinic acid would limit the production capacity from the change intracellular pH conditions. However, *L. plantarum* VL103(pMH) and derivatives did not accumulate succinic acid intracellularly (data not shown). Succinic acid is generally transported by diffusion or by a dicarboxylate transport protein located in the plasma membrane [28,29]. Although the transport mechanism responsible for the import and export of succinic acid in *L. plantarum* is unknown, *L. plantarum* VL103(pMH) and derivatives are capable of exporting succinic acid into the culture medium at high concentration without major intracellular accumulation.

L. plantarum VL103(pMH) and derivatives generate additional fermentation by-products, including acetoin, ethanol, and formate. Acetoin production by the enzyme overexpressing strains was lower than that by *L. plantarum* VL103(pMH) after 48 h of fermentation. The LDH-deficient strain of *L. plantarum* exhibited a reduced rate of NADH reoxidation, causing intracellular pyruvate accumulation as well as extracellular production (Table 2) [1]. Conversion of accumulated pyruvate to acetoin is also an important mechanism for pH homeostasis in *L. plantarum* [30]. The *L. plantarum* VL103(pMH) and derivatives produce increased amount of acetoin compared to the wild type. However, the production of acetoin does not lead to the conversion of NADH to NAD^+ while succinic acid biosynthesis results in the consumption of 2 mol of NADH. Our results suggest that reduced acetoin production in strains overexpressing each enzyme reflects increased conversion of NADH to NAD^+ due to elevated succinic acid production, resulting in an improvement in NADH reoxidation of the LDH-deficient strain.

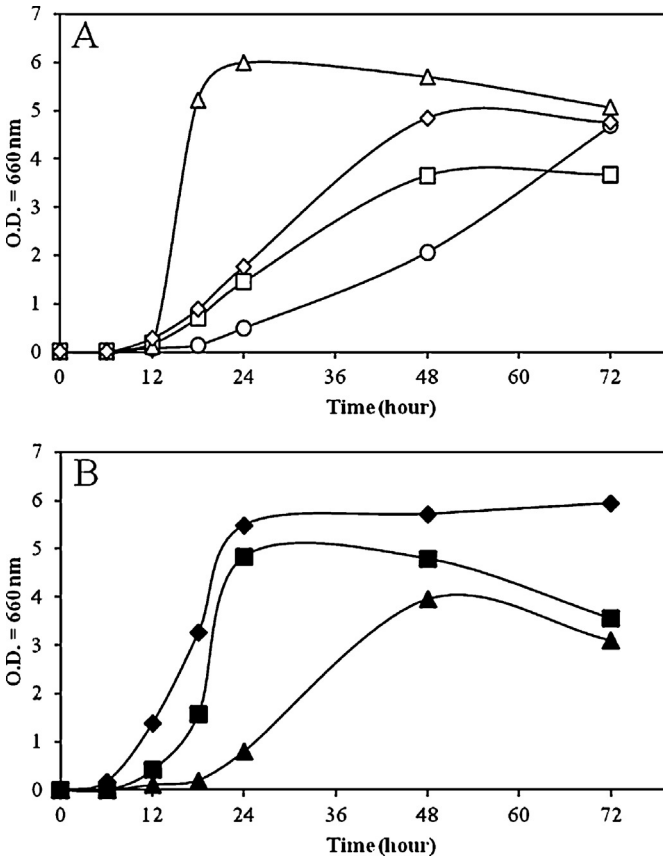

Fig. 3. Cell growth of the *Lactobacillus plantarum* VL103(pMH) and derivatives in modified MRS medium, ascertained by measuring the increase in optical density at 660 nm. Data are mean of three independent experiments and those in parentheses are standard deviation. Symbols: ○, *L. plantarum* VL103(pMH); □, *L. plantarum* VL103(pMH::pycA); △, *L. plantarum* VL103(pMH::pckA); ◊, *L. plantarum* VL103(pMH::mae); ■, *L. plantarum* VL103(pMH::pycA/pckA); ▲, *L. plantarum* VL103(pMH::pycA/mae); ♦, *L. plantarum* VL103(pMH::pckA/mae).

L. plantarum VL103 and derivatives consume acetate which is present in the MRS medium (initial concentration 61.0 mM). It has been previously reported that LDH-deficient LAB produce ethanol by consuming acetate to improve intracellular redox balance [1,6]. Consumption of acetate by *L. plantarum* VL103(pMH) and derivatives (up to 36 mM) could play a role in the maintenance of the redox balance. However, our results suggested that acetate is converted in other compounds than ethanol because the concentration of ethanol in the medium does not increase.

3.2. Cell proliferation of *L. plantarum* VL103 overexpressing pyruvate carboxylase, phosphoenolpyruvate carboxykinase, and malic enzyme

Growth curves were established for *L. plantarum* VL103(pMH) and derivatives (Fig. 3A). The three derivatives showed a higher specific growth rate than *L. plantarum* VL103(pMH). Similarly, the specific growth rate of metabolically engineered *E. coli* with PC or PEPCK overproduction was shown to be increased compared to the wild-type strain [21,23]. The overexpression of each enzyme in *L. plantarum* VL103 reduced intracellular pyruvate accumulation and improved glucose consumption rate, probably resulting from an improved redox balance linked to succinate production. However, *L. plantarum* VL103(pMH::*pycA*) showed a lower total cell number than *L. plantarum* VL103(pMH) after 72 h (Fig. 2A). This lower biomass could be explained by the consumption of adenosine triphosphate (ATP) during the conversion of pyruvate

Table 2
Fermentation product concentrations in modified MRS medium produced by *Lactobacillus plantarum* NCIMB 8826 and derivatives.

L. plantarum strains	Time (h)	Glucose consumed (mM)	Fermentation product (mM)						
			Succinate	Lactate	Pyruvate	Formate	Acetate	Ethanol	Acetoin
NCIMB 8826	24	90.31 ± 0.74	1.48 ± 0.06	160.62 ± 0.77	ND	ND	61.34 ± 2.42	ND	0.28 ± 0.03
	48	109.69 ± 0.01	2.44 ± 0.14	189.50 ± 2.61	ND	ND	63.96 ± 0.45	ND	0.28 ± 0.03
VL103									
pMH	24	26.28 ± 0.55	11.00 ± 0.23	ND	6.01 ± 0.82	12.58 ± 0.76	53.66 ± 2.39	0.01 ± 0.00	15.18 ± 0.74
pMH::*pycA*		36.61 ± 0.75	33.28 ± 2.07	ND	ND	ND	36.71 ± 3.79	0.01 ± 0.00	30.96 ± 1.14
pMH::*pckA*		108.80 ± 0.31	19.14 ± 0.74	ND	ND	37.58 ± 1.23	48.78 ± 2.15	0.01 ± 0.00	54.75 ± 1.69
pMH::*mae*		61.28 ± 0.38	20.14 ± 2.01	ND	ND	14.18 ± 1.10	50.83 ± 3.06	0.01 ± 0.00	44.79 ± 1.32
pMH	48	109.00 ± 0.30	16.87 ± 0.88	ND	ND	16.33 ± 0.52	58.95 ± 2.52	0.02 ± 0.00	75.12 ± 1.17
pMH::*pycA*		107.20 ± 0.10	52.46 ± 0.35	ND	ND	13.03 ± 0.61	25.35 ± 1.48	0.02 ± 0.00	62.64 ± 3.04
pMH::*pckA*		108.30 ± 0.05	25.32 ± 0.71	ND	ND	45.65 ± 0.56	43.32 ± 0.90	0.02 ± 0.00	64.44 ± 2.55
pMH::*mae*		108.40 ± 0.05	33.24 ± 0.50	ND	ND	31.69 ± 0.27	43.60 ± 2.69	0.02 ± 0.00	60.65 ± 2.27

ND, not detected. Values represent the mean and standard error derived from three independent experiments, with each experiment performed in triplicate.

to oxaloacetate by PC. Unlike PC, the reaction catalyzed by ME does not consume ATP. Hence, the change in specific growth rate of *L. plantarum* VL103(pMH::*mae*) might be due to the depletion of accumulated pyruvate in the cell. The *L. plantarum* VL103(pMH::*pckA*) exhibited a higher specific growth rate and higher total cell number than *L. plantarum* VL103(pMH::*mae*) and *L. plantarum* VL103(pMH::*pycA*). This increased biomass could be due to avoidance of the rate-limiting step in glycolysis, i.e., the reaction between phosphoenolpyruvate and pyruvate [31]. Instead, *L. plantarum* VL103(pMH::*pckA*) produced ATP by converting phosphoenolpyruvate into oxaloacetate. Thus, *L. plantarum* VL103(pMH::*pckA*) improves its succinic acid production by increasing total biomass. Since reduced cell growth is an obstacle for the construction of metabolically engineered bacteria, PEPCK overexpression is an interesting approach for the engineering of succinic acid-producing bacteria since it increases succinic acid concentration by enhancing biomass formation.

3.3. The metabolic characteristics and cell proliferation of *L. plantarum* VL103 coexpressing pyruvate carboxylase or phosphoenolpyruvate carboxykinase and other enzymes

PC, PEPCK, and ME influence succinic acid yield in different ways. PC overexpression yielded the highest amount of succinic acid, but reduced total cell number. Conversely, PEPCK overexpression increased total cell number, but resulted in a lower succinic acid yield per biomass. In addition, ME overexpression showed an intermediate situation.

We hypothesized that a combined high succinic acid yield and cell proliferation could be generated by coexpression of PC, PEPCK, and ME. The *L. plantarum* VL103(pMH::*pycA*/*mae*), *L. plantarum* VL103(pMH::*pckA*/*mae*) and *L. plantarum* VL103(pMH::*pycA*/*pckA*) were constructed, and their metabolites were quantified after 24 and 48 h of culture by HPLC (Table 3), and their growth curves were drawn (Fig. 3B). Succinic acid concentration reached its peak after 48 h and remained constant until the end of fermentation (data not shown). The coexpression strains also exhibited the properties of both enzyme overexpressing strains. All these strains showed a higher specific growth rate than *L. plantarum* VL103. The coexpressing strains exhibited the combined properties of overexpression of each enzyme with respect to by-product generation. Acetic acid consumption and decreased acetoin production were confirmed, as well as the improved glucose consumption rate.

The total cell number of *L. plantarum* VL103(pMH::*pycA*/*pckA*) was higher than *L. plantarum* VL103(pMH::*pycA*), but lower than *L. plantarum* VL103(pMH::*pckA*). The *L. plantarum* VL103(pMH::*pycA*/*mae*) showed a lower total cell number than *L. plantarum* VL103(pMH::*mae*) but similar to *L. plantarum* VL103(pMH::*pycA*). On the other hand, *L. plantarum* VL103(pMH::*pckA*/*mae*) exhibited the highest total cell number than *L. plantarum* VL103(pMH) and all derivatives after 72 h. The coexpression of PC with PEPCK or PC with ME resulted in defective cell proliferation than overexpression ME or PEPCK, respectively. This might be because PC consumed ATP which was required for cell growth.

The *L. plantarum* VL103(pMH::*pycA*/*mae*) yielded slightly more succinic acid than *L. plantarum* VL103(pMH::*pycA*): an increase from 52.5 to 53.5 mM, corresponding to 24.3% of glucose conversion to succinic acid. These results demonstrate that increased succinic acid yield in *L. plantarum* VL103(pMH::*pycA*/*mae*) relative to *L. plantarum* VL103(pMH::*pycA*) was also achieved through

Table 3
Fermentation product concentrations in modified MRS medium produced by *Lactobacillus plantarum* strains coexpressing enzymes.

L. plantarum VL103 strains	Time (h)	Glucose consumed (mM)	Fermentation product (mM)					
			Succinate	Pyruvate	Formate	Acetate	Ethanol	Acetoin
pMH::*pycA*/*pckA*	24	76.39 ± 0.34	33.26 ± 0.78	ND	5.40 ± 0.30	38.44 ± 0.79	0.01 ± 0.00	48.15 ± 1.58
pMH::*pycA*/*mae*		74.89 ± 0.61	35.56 ± 0.41	ND	4.51 ± 0.28	41.81 ± 0.73	0.01 ± 0.00	51.56 ± 2.04
pMH::*pckA*/*mae*		100.61 ± 1.36	20.71 ± 0.20	ND	7.51 ± 0.65	51.69 ± 1.11	0.01 ± 0.00	50.05 ± 1.70
pMH::*pycA*/*pckA*	48	107.90 ± 0.31	55.69 ± 0.28	ND	30.10 ± 1.92	30.96 ± 1.45	0.02 ± 0.00	60.19 ± 1.97
pMH::*pycA*/*mae*		109.00 ± 0.23	53.45 ± 0.92	ND	20.62 ± 0.95	38.56 ± 1.73	0.01 ± 0.00	64.44 ± 2.55
pMH::*pckA*/*mae*		108.00 ± 0.42	26.81 ± 0.28	ND	35.35 ± 1.69	36.97 ± 0.58	0.02 ± 0.00	62.56 ± 2.12

ND, not detected. Values represent the mean and standard error derived from three independent experiments, with each experiment performed in triplicate.

elevated specific growth rate. However, these results showed a no significant difference between *L. plantarum* VL103(pMH::*pycA*) and *L. plantarum* VL103(pMH::*pycA*/*mae*). Moreover, the succinic acid yield of *L. plantarum* VL103(pMH::*pckA*/*mae*) was lower than *L. plantarum* VL103(pMH::*mae*) (26.8 vs. 33.2 mM). The ME catalyze the reversible conversion of malate and NAD^+ to pyruvate and NADH. In general, malic enzymes have a higher affinity toward malate than toward pyruvate [31]. Thus, the *L. plantarum* VL103(pMH::*pckA*/*mae*) and *L. plantarum* VL103(pMH::*pycA*/*mae*) were suggested that malate might be converted to pyruvate via ME. Furthermore, *L. plantarum* VL103(pMH::*pycA*/*pckA*) yielded more succinic acid than *L. plantarum* VL103(pMH::*pycA*): an increase from 52.5 to 55.7 mM, corresponding to 25.3% of glucose conversion to succinic acid. From these results, it can be concluded that increased succinic acid yield in *L. plantarum* VL103(pMH::*pycA*/*pckA*) relative to *L. plantarum* VL103(pMH::*pycA*) was achieved through increased biomass formation, although the mechanism of biomass increase by PEPCK remain unclear.

The strategies of metabolic engineering in *E. coli* for succinic acid production are classified into three main methods: improvement of substrate transportation, enhancement of pathways directly involved in the succinic acid production, deletion of pathways involved in by-product accumulation. These methods and their combinations are used for succinic acid production in several bacteria and fungi. In this study, combination of deletion of lactate pathways and enhancement of the carbon fixation enzymes was performed. The results showed that PC-overexpression was effective in high succinic acid production, but slowed the growth rate down. The coexpression of PC and PEPCK was shown to exhibit increase of succinic acid yield and biomass. Furthermore, our experimental set up is simple and exhibits high usability.

Since the productivity of succinic acid by the currently constructed *L. plantarum* is not comparable to the other producer microbes, this *L. plantarum* may not be suitable for industrial production of the organic acid. However, LAB can be applied to food fermentation or used as food additives, while most other succinic acid-producing microorganisms are not applicable for those purposes. Moreover, the genetic modifications done in this study are gene deletion and self-cloning, which are food-grade techniques. In this respect, this metabolically engineered LAB may be appealing to food industry.

The future developments of this study will consist of knockout of the production pathway of byproducts such as acetoin and formic acid which consume approximately 70% of glucose, regulation of succinic acid and substrate transporters in *L. plantarum*, and examination of a range of culture conditions in order to go a step further in the optimization of succinic acid production by *L. plantarum*.

4. Conclusion

In this study, succinic acid production by *L. plantarum* VL103 was significantly enhanced through genetic engineering. Overexpression or coexpression of carbon fixation enzymes has been reported to improve metabolic flux from glycolysis to the reductive TCA cycle, resulting in an increase of succinic acid production. Here, we show that individual overexpression or coexpression of the carbon fixation enzymes, PC, PEPCK, and ME, in *L. plantarum* VL103 resulted in all cases in an improvement of succinic acid production. We demonstrated that this improvement by PEPCK overexpression relies on an enhanced biomass formation while PC overexpression showed the higher succinate yield. By combining both properties through the coexpression of PEPCK and PC, 25.3% of sugar could be converted to succinic acid, which is the most efficient rate reported to date in the context of metabolic engineering of LAB for succinic acid production.

References

[1] Kleerebezemab M, Hols P, Hugenholtz J. Lactic acid bacteria as a cell factory: rerouting of carbon metabolism in *Lactococcus lactis* by metabolic engineering. Enzyme and Microbial Technology 2000;26:840–8.

[2] Papagianni M. Recent advances in engineering the central carbon metabolism of industrially important bacteria. Microbial Cell Factories 2012;11:50.

[3] de Vos WM, Hugenholtz J. Engineering metabolic highways in *Lactococci* and other lactic acid bacteria. Trends in Biotechnology 2004;22:72–9.

[4] Nadal I, Rico J, Pérez-Martínez G, Yebra MJ, Monedero V. Diacetyl and acetoin production from whey permeate using engineered *Lactobacillus casei*. Journal of Industrial Microbiology and Biotechnology 2009;36:1233–7.

[5] Hols P, Kleerebezem M, Schanck AN, Ferain T, Hugenholtz J, Delcour J, et al. Conversion of *Lactococcus lactis* from homolactic to homoalanine fermentation through metabolic engineering. Nature Biotechnology 1999;17:588–92.

[6] Ladero V, Ramos A, Wiersma A, Goffin P, Schanck A, Kleerebezem M, et al. High-level production of the low-calorie sugar sorbitol by *Lactobacillus plantarum* through metabolic engineering. Applied and Environment Microbiology 2007;73:1864–72.

[7] Kleerebezem M, Hugenholtz J. Metabolic pathway engineering in lactic acid bacteria. Current Opinion in Biotechnology 2003;14:232–7.

[8] Sybesma W, Burgess C, Starrenburg M, van Sinderen D, Hugenholtz J. Multivitamin production in *Lactococcus lactis* using metabolic engineering. Metabolic Engineering 2004;6:109–15.

[9] McKinlay JB, Vieille C, Zeikus JG. Prospects for a bio-based succinate industry. Applied Microbiology and Biotechnology 2007;76:727–40.

[10] Song H, Lee SY. Production of succinic acid by bacterial fermentation. Enzyme and Microbial Technology 2006;39:352–61.

[11] McKinlay JB, Vieille C. ^{13}C-metabolic flux analysis of *Actinobacillus succinogenes* fermentative metabolism at different $NaHCO_3$ and H_2 concentrations. Metabolic Engineering 2008;10:55–68.

[12] Okino S, Noburyu R, Suda M, Jojima T, Inui M, Yukawa H. An efficient succinic acid production process in a metabolically engineered *Corynebacterium glutamicum* strain. Applied Microbiology and Biotechnology 2008;81:459–64.

[13] Chatterjee R, Millard CS, Champion K, Clark DP, Donnelly MI. Mutation of the ptsG gene results in increases production of succinate in fermentation of glucose by *Escherichia coli*. Applied and Environment Microbiology 2001;67:148–54.

[14] Kleerebezem M, Boekhorst J, van Kranenburg R, Molenaar D, Kuipers OP, Leer R, et al. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proceedings of the National Academy of Sciences of the United States of America 2003;100:1990–5.

[15] Liu S, Nichols NN, Dien BS, Cotta MA. Metabolic engineering of a *Lactobacillus plantarum* double ldh knocked-out strain for enhanced ethanol production. Journal of Industrial Microbiology and Biotechnology 2006;33:1–7.

[16] Siezen RJ, Francke C, Renckens B, Boekhorst J, Wels M, Kleerebezem M, et al. Complete resequencing and reannotation of the *Lactobacillus plantarum* WCFS1 genome. Journal of Bacteriology 2012;194:195–6.

[17] Ferain T, Schanck AN, Delcour J. ^{13}C nuclear magnetic resonance analysis of glucose and citrate end products in an ldhL–ldhD double-knockout strain of *Lactobacillus plantarum*. Journal of Bacteriology 1996;178:7311–5.

[18] Ferain T, Hobbs Jr JN, Richardson J, Bernard N, Garmyn D, Hols P, et al. Knockout of the two ldh genes has a major impact on peptidoglycan precursor synthesis in *Lactobacillus plantarum*. Journal of Bacteriology 1996;178:5431–7.

[19] Satoh E, Endo M, Okada S, Uchimura T, Hols P. Production of succinic acid by *Lactobacillus plantarum* using metabolic rerouting. Food Preservation Science 2008;34:59–64 [in Japanese].

[20] Wang J, Zhu J, Bennett GN, San KY. Succinate production from different carbon sources under anaerobic conditions by metabolic engineered *Escherichia coli* strains. Metabolic Engineering 2011;13:328–35.

[21] Hong SH, Lee SY. Metabolic flux analysis for succinic acid production by recombinant *Escherichia coli* with amplified malic enzyme activity. Biotechnology and Bioengineering 2001;74:89–95.

[22] Lin H, San KY, Bennett GN. Effect of Sorghum vulgare phosphoenolpyruvate carboxylase and *Lactococcus lactis* pyruvate carboxylase coexpression on succinate production in mutant strains of *Escherichia coli*. Applied Microbiology and Biotechnology 2005;67:515–23.

[23] Kim P, Laivenieks M, Vieille C, Zeikus JG. Effect of overexpression of *Actinobacillus succinogenes* phosphoenolpyruvate carboxykinase on succinate production in *Escherichia coli*. Applied and Environment Microbiology 2004;70:1238–41.

[24] Kok J, Van der Vossen JM, Venema G. Construction of plasmid cloning vectors for lactic streptococci which also replicate in *Bacillus subtilis* and *Escherichia coli*. Applied and Environment Microbiology 1984;48:726–31.

[25] Aukrust T, Blom H. Transformation of *Lactobacillus* strains used in meat and vegetable fermentations. Food Research International 1992;25:253–61.

[26] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Analytical Biochemistry 1976;72:248–54.
[27] Raab AM, Gebhardt G, Bolotina N, Weuster-Botz D, Lang C. Metabolic engineering of *Saccharomyces cerevisiae* for the biotechnological production of succinic acid. Metabolic Engineering 2010;12:518–25.
[28] Olsen EB, Russell JB, Henick-Kling T. Electrogenic L-malate transport by *Lactobacillus plantarum*: a basis for energy derivation from malolactic fermentation. Journal of Bacteriology 1991;173:6199–206.
[29] Janausch IG, Zientz E, Tran QH, Kroger A, Unden G. C4-dicarboxylate carriers and sensors in bacteria. Biochimica et Biophysica Acta 2002;1553: 39–56.
[30] Tsau JL, Guffanti AA, Montville TJ. Conversion of pyruvate to acetoin helps to maintain pH homeostasis in *Lactobacillus plantarum*. Applied and Environment Microbiology 1992;58:891–4.
[31] Sauer U, Eikmanns BJ. The PEP-pyruvate-oxaloacetate node as the switch point for carbon flux distribution in bacteria. FEMS Microbiology Review 2005;29:765–94.