J Ind Microbiol Biotechnol (2006) 33: 1–7
DOI 10.1007/s10295-005-0001-3

ORIGINAL PAPER

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Metabolic engineering of a *Lactobacillus plantarum* double *ldh* knockout strain for enhanced ethanol production

Received: 11 April 2005 / Accepted: 13 June 2005 / Published online: 29 September 2005

Abstract *Lactobacillus plantarum* ferments glucose through the Embden–Meyerhof–Parnas pathway: the central metabolite pyruvate is converted into lactate via lactate dehydrogenase (LDH). By substituting LDH with pyruvate decarboxylase (PDC) activity, pyruvate may be redirected toward ethanol production instead of lactic acid fermentation. A PDC gene from the Gram-positive bacterium *Sarcina ventriculi* (*Spdc*) was introduced into an LDH-deficient strain, *L. plantarum* TF103, in which both the *ldhL* and *ldhD* genes were inactivated. Four different fusion genes between *Spdc* and either the *S. ventriculi* promoter or three *Lactococcus lactis* promoters in pTRKH2 were introduced into TF103. PDC activity was detected in all four recombinant strains. The engineered strains were examined for production of ethanol and other metabolites in flask fermentations. The recombinant strains grew slightly faster than the parent TF103 and produced 90–130 mM ethanol. Although slightly more ethanol was observed, carbon flow was not significantly improved toward ethanol, suggesting that a further understanding of this organism's metabolism is necessary.

Keywords Ethanol fermentation · Metabolic engineering · Lactic acid bacteria · Pyruvate decarboxylase · *Sarcina ventriculi*

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Introduction

Lactic acid bacteria (LAB) are a group of facultatively anaerobic, Gram-positive bacteria. LAB typically lack a respiratory chain, and they use fermentations of various sugars to produce the energy required for cellular maintenance and growth. For homofermentive LAB, lactate is the sole end product of fermentation, whereas in heterofermentive LAB, a mixture of ethanol, CO_2, acetate, and lactate is usually produced via the pentose phosphoketolase pathway.

LAB have GRAS (generally recognized as safe) status, and naturally occurring LAB have been successfully used in the fermentation industry. In recent years, genetically modified LAB have been explored for production of lactic acid, B-vitamins, and low-calorie sugar alcohols like sorbitol and mannitol [11, 12]. LAB can grow at lower pH and many strains are ethanol-tolerant. These traits are useful for developing new microorganisms for fermentative production of ethanol from lignocellulosic biomass [3]. Conversion of lignocellulose to ethanol requires microorganisms capable of fermenting both glucose and xylose sugars released from hydrolysis of lignocellulosic biomass [3]. Many of the LAB can metabolize multiple sugars, including pentoses. Therefore, they appeared to be ideal hosts for metabolic engineering toward ethanol production from biomass. However, LAB do not produce large amounts of ethanol, as they ferment sugars to lactate. The objective of this study is to use *Lactobacillus plantarum* as a model strain to explore the possibilities of re-channeling the lactate fermentation capacity toward ethanol production.

The enzymes responsible for ethanol production from pyruvate are pyruvate decarboxylase (PDC; EC 4.1.1.1) and alcohol dehydrogenase (ADH; EC 1.1.1.1). PDC catalyzes the decarboxylation of pyruvate to acetaldehyde and CO_2, and acetaldehyde is then reduced to ethanol by ADH. The ethanol-producing genes *pdc* and *adhB* from *Zymomonas mobilis* were assembled into a

portable *pet* operon [13]. The introduction of the *pet* operon in *Escherichia coli* resulted in ethanol yield of 86–92% of the theoretically possible yield [4, 13].

The *pet* operon was also introduced in Gram-positive *Bacillus* and LAB strains [2, 9, 14, 17]. Little or no ethanol was produced by these recombinant strains, suggesting that this *pdc* gene of Gram-negative origin may not function appropriately in Gram-positive bacteria. This could be due to codon bias [7] and/or significant differences in gene expression between Gram-negative and Gram-positive species.

Unlike ADHs, which are common in many organisms including bacteria, PDCs can only be found in a few bacterial species, although they are widely distributed in plants. The only *pdc* gene from a Gram-positive bacterium described recently was from *Sarcina ventriculi* (*Spdc*) [21]. Under acidic conditions (pH 3.0), *S. ventriculi* uses PDC to alter carbon and electron flow from acetate, formate, and ethanol to predominantly ethanol [15]. *S. ventriculi* PDC is activated by pyruvate and exhibits sigmoidal kinetics similar to the enzyme from fungi and higher plants instead of Michaelis–Menten kinetics [21]. The low G + C codon usage of this Gram-positive *Spdc* gene offered potential in engineering Gram-positive hosts for high-level PDC expression and possibilities for development of a second generation of ethanologenic microorganisms that could use biomass-derived sugars. The objectives of the present paper are to determine if the Gram-positive *Spdc* can be functionally expressed in *L. plantarum*, and to test whether a lactate-deficient *L. plantarum* strain can be engineered to make ethanol as its primary fermentation product.

Materials and methods

Bacterial strains and growth conditions

S. ventriculi JK was cultivated under anaerobic conditions as described by Goodwin and Zeikus [10]. *L. plantarum* NCIMB8826 derivative strain TF103 was kindly provided by Dr. Ferain [5]. The TF103 strain, which is defective for both D- and L-LDH (lactate dehydrogenase) activities, was grown at 37°C, 100 rpm in an MRS (BD Diagnostic Systems, Sparks, MD) broth with chloramphenicol (10 μg ml^{-1}) or erythromycin (5 μg ml^{-1}). *E. coli* strain DH5α, BL21(DE3)pLysS (Invitrogen, Carlsbad, CA), and BL21-CodonPlus-RIL (Stratagene, La Jolla, CA) cells were grown at 37°C in BHI (BD Diagnostic Systems) or Luria–Bertani [20] media supplemented with erythromycin (150 μg ml^{-1}), or ampicillin (50 μg ml^{-1}), or chloramphenicol (35 μg ml^{-1}) when required.

Cloning of *Spdc* in pBluescript and pTRKH2

Chromosomal DNA was isolated from *S. ventriculi* using the Bactozol Kit (Molecular Research Center, Cincinnati, OH), as described in the manufacturer's protocol, with additional phenol–chloroform extractions. PCR was performed using *S. ventriculi* genomic DNA as template and Spdc5′XbaI and Spdc3′XhoI primers (Table 1), derived from the GenBank-deposited sequence AF354297, with *Xba*I and *Xho*I sites at the ends of the primers. The 1.7 kb full length *Spdc* gene amplified from genomic DNA was purified (Qiagen PCR purification kit), digested, and cloned into pBluescript SK as well as shuttle vector pTRKH2 [18] at *Xba*I and *Xho*I sites to obtain pTRKH2 *Spdc*. Standard molecular biology techniques were used as described by Sambrook et al. [20]. Plasmid DNA purification from *E. coli* was prepared using the Qiaprep Spin Kit (Qiagen, Valencia, CA). Plasmid was isolated from *L. plantarum* strains according to O'Sullivan and Klaenhammer [19]. DNA sequencing and data analyses were carried out as described [14].

Expression of *Spdc* gene in *E. coli*

The PCR primers Spdc5′ATG BamHI and Spdc3′KpnI (Table 1) were used to amplify the *Spdc* gene and clone this into pRSETa (*Bam*HI/*Kpn*I). The resultant pRSETaSpdc was transformed into *E. coli* BL21 (DE3), pLysS, and BL21-CodonPlus-RIL cells. The in vivo IPTG (isopropyl–*β*–D-thio-galactoside) induction and expression were performed according to the vendor's instructions. The cell pellets were lysed using the CelLytic B plus kit (Sigma, St. Louis, MO). About 10 μl of protein extracts of soluble as well as insoluble material (resuspended in 50 μl of 1× phosphate-buffered saline) were subjected to electrophoresis on a 12.5% SDS polyacrylamide gel [20]. To enhance the solubility of overexpressed protein, the overnight cultures were diluted tenfold into 10 ml Terrific Broth medium [20]

Table 1 Oligonucleotides used in this study

M13 Reverse	CAGGAAACAGCTATGAC
T7 Promoter	TAATACGACTCACTATAGGG
Spdc5′XbaI	GGCT**TCTAGA**TAAAAAATGAATTGGAGG
Spdc3′XhoI	GCGGG**CTCGAG**ATTAGTAGTTATTTTG
Spdc5′ATG BamHI	GCC**GGATCC**ATGAAAATAACAATTGCAG
Spdc 3′KpnI	GCC**GGTACC**ATTAGTAGTTATTTTG
Spdc 3′1431	TCTGCTGCATATCCTGCA
Amj5′EcoRV	ACC**GATATC**ATTTTTGGTTGCCATTTGTT
Amj3′XbaI	CC**TCTAGA**CTAGACAACAAAATAG

containing different concentrations (0, 200, and 500 mM) of glycylglycine (Sigma) and grown at 37°C to an $OD_{600} = 1$. Protein expression was then induced with 0.5 mM IPTG for 14–16 h at 27°C [8].

Expression of *Spdc* in *L. plantarum* TF103

Gram-positive promoter- *Spdc* fusions were constructed as follows. Plasmid clones AMJ772, AMJ769, and AMJ692 containing three *Lactococcus* promoters in pAK80 [16] were used as templates to amplify the 118 bp promoter sequences (Fig. 1), using primers Amj5′EcoRV and Amj3′XbaI (Table 1). These promoter sequences were cloned into pTRKH2 Spdc at *Eco*RV/*Xba*I sites to generate pTRKH2 772Spdc, pTRKH2 769Spdc, and pTRKH2 692Spdc, respectively. These constructs were transformed into *L. plantarum* strain TF103 by electroporation as described [1]; recombinant strains were obtained with erythromycin selection.

Pyruvate decarboxalase assay

PDC enzymatic activities were assayed as previously described [14]. Briefly, cultures of the recombinant *E. coli* and *L. plantarum* TF103 cells containing *Spdc* fusions were lysed using a beadbeater (Biospec products, Bartlesville, OK) in 50 mM sodium phosphate buffer (pH 6.5), with 2 mM TPP and 20 mM $MgSO_4$. The protein concentration was determined using the BIO-RAD protein assay kit (BIO-RAD Laboratories, Hercules, CA). The protein extract (150–250 μg ml^{-1}) was added to a 50 mM sodium phosphate buffer (pH 6.5) containing 30 mM acetaldehyde, 40 mM benzaldehyde, 2 mM TPP, and 20 mM $MgSO_4$ in 500 μl and incubated for 30 min at 25°C. The supernatant of the reaction mixture was then assayed for (R)–PAC by adding 20 μl tetrazolium red containing 0.2% 2,3,5-triphenyltetrazolium chloride (dissolved in methanol) and 10 μl 3 M NaOH (130 mM final). The amount of formazane formed was measured spectrophotometrically at 510 nm. A unit of PDC-specific activity was defined as 1 μmol of formazane formed per minute per milligram of protein.

Fermentation of engineered TF103 strains

Flask fermentations were used for assessment of ethanol production by recombinant *L. plantarum* TF103 strains. Duplicate fermentations were carried out in MRS media with 4% of glucose plus erythromycin as described [17]. Samples were taken out at time points from 72 to 240 h of fermentation. The concentrations of residual glucose and fermentation products, including lactate, acetate, and ethanol were measured by HPLC [4]. The metabolic yield (g ethanol/g glucose) was calculated from data obtained in two experiments, and analyses of other fermentation products were made from the fermentation duplicates.

Results

Overexpression of the *Spdc* gene in *E. coli*

To assess whether a functional PDC could be produced, the cloned *Spdc* ORF was subcloned in frame with His-tag sequences into an *E. coli* expression vector, pRSETa. SDS-PAGE analysis of cell extract from IPTG induced *E. coli* BL21Codon plus RIPL cells with pRSETaSpdc showed a strong band, corresponding to a polypeptide about 61 kDa, present in the insoluble fraction (Fig. 2). The size of the overexpressed SPDC agreed with the reported native 58 kDa plus about 3 kDa from the vector [21]. The result from SDS-PAGE suggested that the SPDC is expressed as insoluble aggregates of folding intermediates known as inclusion bodies. In order to induce correct folding of overexpressed SPDC protein, a dipeptide, glycylglycine, was used in a culture medium with or without IPTG induction. The PDC enzymatic activities were measured spectrophotometrically. As shown in Fig. 3, the highest PDC activity was observed in the soluble fraction of cell lysates treated with 200 mM glycylglycine without IPTG induction. It appeared that IPTG treatment was not necessary for SPDC expression using the pRSETa vector. The enzymatic activities of insoluble fraction were not determined.

pTRKH2Spdc
Spdc5'XbaI--ATAAAAAATGAATT**GGAGG**AAATACATA**ATG**----------Spdc3'XhoI
RBS

pTRKH2692Spdc -35 -10 +1
AmjEcoRV-ATTTTTGGTTGCCATTTGTTA**ACGCTGC**CTCCTCTCCCTAG**TGCTATAAT**AAAAATG-----
----------AmjXbaI\Spdc5'XbaI -ATAAAAAATGAATT**GGAGG**AAATACATA**ATG**----Spdc3'XhoI
RBS

pTRKH2769Spdc -35 -10 +1
AmjEcoRV-ATTTTTGGTTGCCATTTGTTA**TTGACA**CCTCCTCTCCCTAG**TGCTATAAT**AAAAATG----
----------AmjXbaI\Spdc5'XbaI -ATAAAAAATGAATT**GGAGG**AAATACATA**ATG**----Spdc3'XhoI
RBS

pTRKH2772Spdc -35 -10 +1
AmjEcoRV-ATTATGCTTTGGCAGTTTATTC**TTGACA**TGTAGTGAGGGGGC**TGGTATAAT**AAAATAG—
----------AmjXbaI\Spdc5'XbaI -ATAAAAAATGAATT**GGAGG**AAATACATA**ATG**----Spdc3'XhoI
RBS

Fig. 1 Gram-positive promoter sequences from p692, p769, and p772, as described by Madison et al. [16], were used to construct *Spdc* fusions in pTRKH2 vector. Sequences of −35, −10, and +1 transcription initiation site were indicated and ribosome binding site were underlined

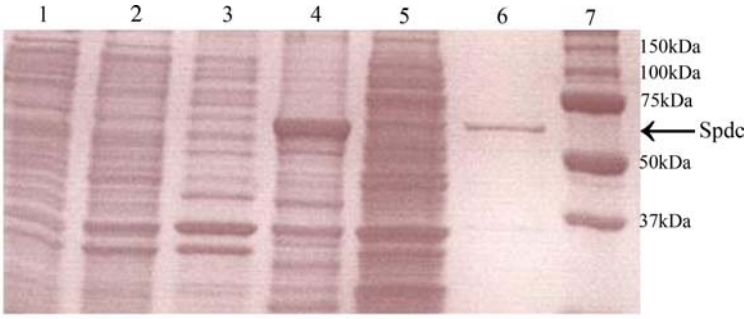

Fig. 2 SDS-PAGE analysis of *S. ventriculi pdc* gene expressed in *E. coli* BL21Codon Plus RIPL cells. *Lane 1*: soluble fraction of IPTG induced pRSETa cell lysate, *lane 2*: insoluble fraction of IPTG induced pRSETa cell lysate, *lane 3*: soluble fraction of IPTG induced pRSETaSpdc cell lysate, *lane 4*: insoluble fraction of IPTG induced pRSETaSpdc cell lysate, *lane 5*: soluble fraction of IPTG induced and 200 mM glycylglycine treated pRSETaSpdc cell lysate, *lane 6*: insoluble fraction of IPTG induced and 200 mM glycylglycine treated pRSETaSpdc cell lysate, *lane 7*: BioRad precision plus protein standards, sizes are indicated on the right. The *arrow* indicates overexpressed PDC about 61 kDa in the insoluble fraction

Introduction of Gram-positive promoter- *Spdc* fusions into TF103

Three Gram-positive promoters, including the acid-inducible promoter p692, the highly expressed promoter p769, and the relatively moderate-strength promoter p772, as well as the native *Spdc* 5′ flanking sequences, were fused with the *Spdc* gene in the broad-host-range vector pTRKH2 (Fig. 1). These constructs were introduced in *L. plantarum* TF103, in which *ldhL* and *ldhD* genes were inactivated [5]. There were no observed DNA rearrangements of the fusion genes or pTRKH2 in TF103, as revealed by restriction enzyme analyses of the plasmids isolated from the recombinant strains (data not shown). Subsequently, PDC enzymatic activities were measured in the recombinant TF103 strains, and higher-than-background activities were observed in all four strains (Fig. 4). Differences in PDC activity in the strains might be due to the differences in the promoter sequences, with the strain containing *Spdc* fused to an acid-inducible promoter, pTRKH2 692Spdc, showing the highest PDC activities (Fig. 4). As reported previously, the acid-inducible promoter is more active at pH 5.5 than at pH 7.0 [16], and the pH value of cultures of these strains ranged from 4.20 to 5.80 when harvested for activity assays.

Fermentation analysis of the recombinant strains

The ethanologenic capacities of the engineered strains were examined in flask fermentations. HPLC analyses of the fermentation products indicated that all four strains carrying the *Spdc* gene produced greater amounts of ethanol than the control strain that was transformed with the pTRKH2 vector only. Strains 692Spdc and 769Spdc showed slightly higher ethanol production than the other tested strains (Fig. 5). However, the recombinant strains also produced significant amounts of lactate. The control strain carrying the pTRKH2 vector grew very slowly (Fig. 6a) and produced mainly acetate (76 mM), some lactate (60 mM), and ethanol (17 mM) (Fig. 5). Analyses of HPLC data from two separate fermentations indicated that the metabolic yield for ethanol (grams of ethanol produced per gram of sugar consumed) ranged from 0.15 to 0.28 g of ethanol produced per gram of glucose used (Fig. 6b).

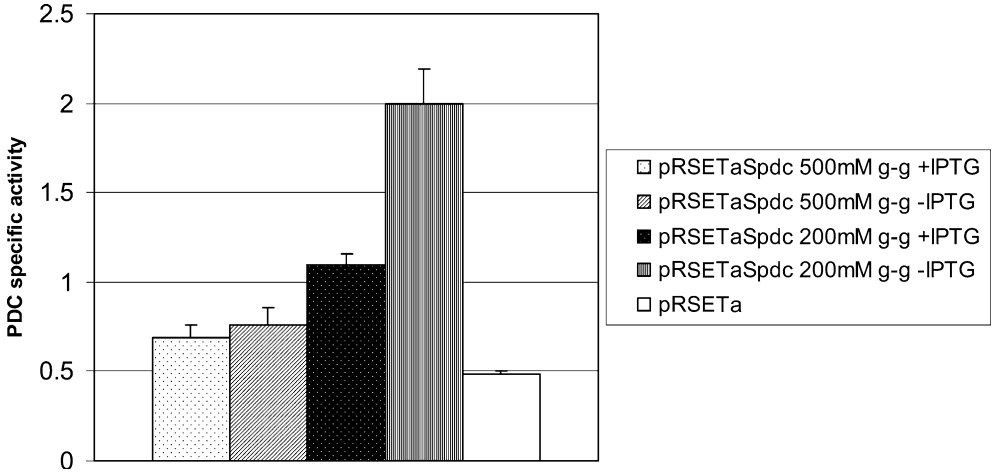

Fig. 3 PDC enzymatic activities in recombinant *E. coli* cells carrying pRSETa or pRSETaSpdc, treated with 500, 200 mM glycylglycine (g–g), with (+) or without (−) IPTG induction

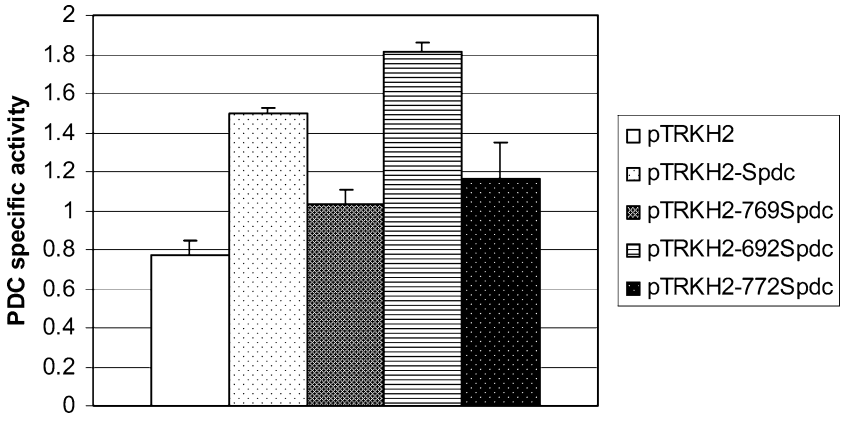

Fig. 4 PDC activities in recombinant *L. plantarum* TF103 cells, where *Spdc* gene was driven by different promoters. *L. plantarum* TF103 carrying pTRKH2 vector was used as a negative control. The data are from three assays, each done in duplicate

Discussion

A primary goal of metabolic engineering is to manipulate biological systems to perform in a desired fashion using an appropriate host. In this study, a Gram-positive *L. plantarum* strain was used as host to introduce a Gram-positive *pdc* gene toward increased ethanol production. Previous studies showed that PDC is the key enzyme for ethanol production from pyruvate, and the low G + C contents of the *S. ventriculi pdc* gene made it a reasonable candidate to be introduced into LAB through genetic engineering.

For most LAB, sugars (hexoses and in some cases pentoses) are the primary substrates for fermentation, and pyruvate is the central metabolite formed from sugar substrates via different pathways. Pyruvate is mainly converted into lactate through the catalytic action of LDH. *L. plantarum* strain TF103 contains the genetic knockouts of both *ldh* (lactate dehydrogenase) genes [5]. Metabolic analysis of *L. plantarum* TF103 indicated that pyruvate accumulated, and lactate was either very low [18] or undetectable [6], depending on growth and fermentation conditions. Therefore, *L. plantarum* TF103 was chosen as a host strain for genetic manipulation in an effort to channel pyruvate into the ethanol-fermentation pathway by introducing the Gram-positive *Spdc* gene. It was anticipated that by overexpressing the *S. ventriculi pdc* gene, the central metabolite pyruvate would be converted to acetaldehyde in TF103, and acetaldehyde would subsequently be converted to ethanol by several endogenous ADH activities.

The differing PDC enzymatic activities observed in the recombinant strains suggested that the strength of each promoter for driving *Spdc* expression in TF103 varied. It appeared that, based on SPDC enzymatic assays, the acid-inducible promoter p692 was the most

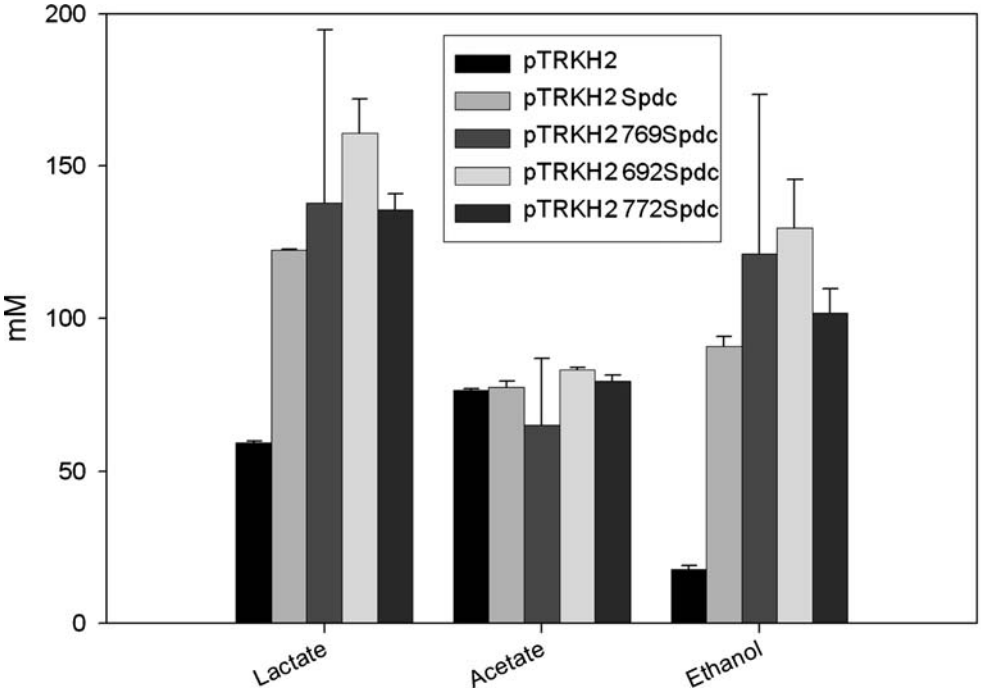

Fig. 5 HPLC analyses of glucose fermentation using the recombinant *L. plantarum* TF103 strains. The concentration (mM) of lactate, acetate, and ethanol produced are shown

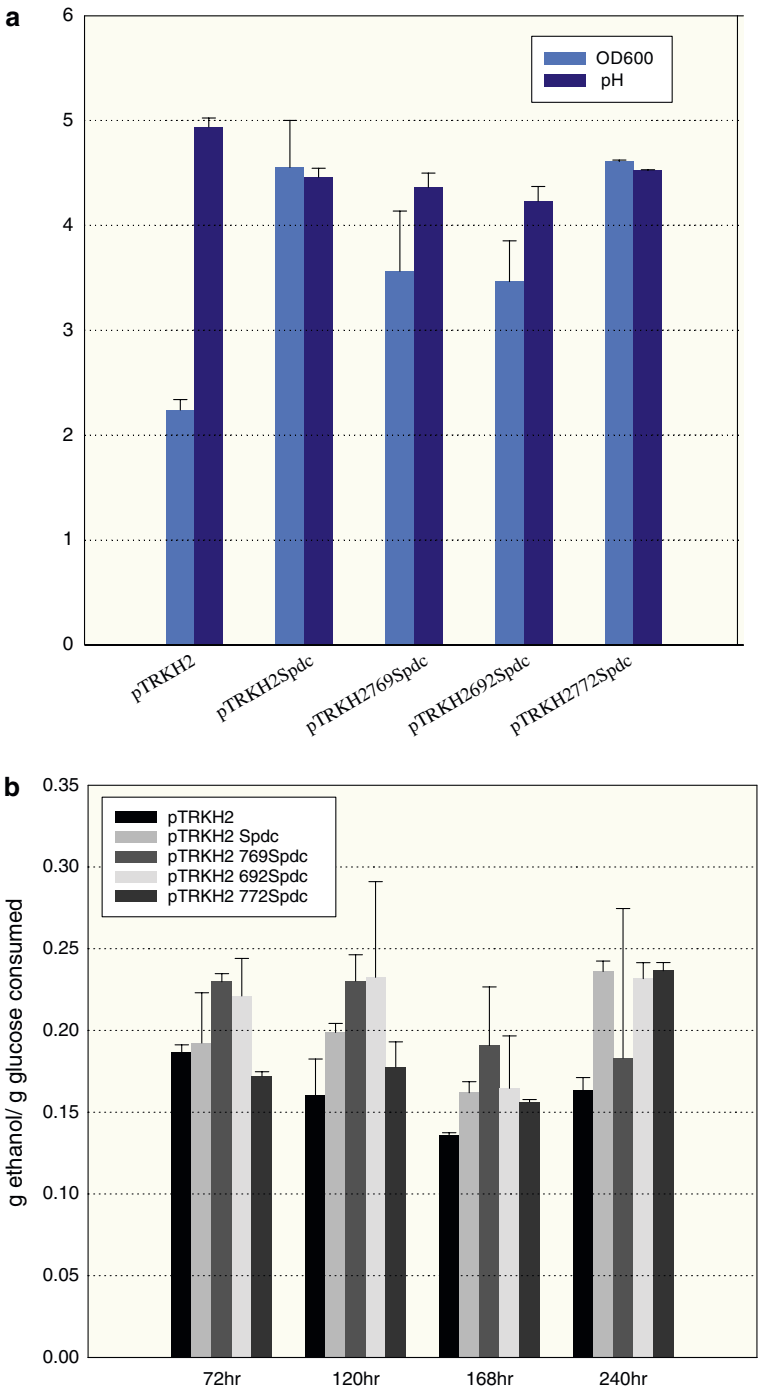

Fig. 6 **a** Final readings of the optical density OD_{600} and pH values from 240 h fermentation flasks obtained by using each recombinant strain carrying pTRKH2, pTRKH2 Spdc, pTRKH2 769Spdc, pTRKH2 692Spdc, and pTRKH2 772Spdc. **b** The metabolic yield for ethanol, grams of ethanol produced per gram of glucose consumed, is shown as the average of two fermentation experiments over a 240-h time period

efficient for conferring higher *Spdc* expression in TF103. Further fermentation analyses indicated that the recombinant strains showed higher ethanol (90–130 mM) and increased lactate (120–170 mM) production than control strain TF103 transformed with pTRKH2 (17 mM ethanol and 60 mM lactate) (Fig. 5). Strains 692Spdc and 769Spdc showed slightly higher ethanol production than the other tested strains (Fig. 5).

About 60 mM lactate was produced in TF103 transformed with the control vector pTRKH2 (Fig. 5), although the TF103 parent strain is reported to produce 12–14 mM lactate [6, 18]. Previous fermentation analyses of *L. plantarum* NCIMB8826, which is the parent strain of the pTRKH2 recombinant TF103 strain, showed production of about 498 mM lactate and 4 mM ethanol [17]. Therefore, the lactate (60 mM) produced by the TF103 (lacking *ldhD-ldhL*) carrying pTRKH2 (derived from but not the same as TF103) is unlikely, due to reversion of the strain. The increased lactate production is unexpected, remains to be understood, and could be due to added erythromycin selection pressure of the pTRKH2 in TF103. Antibiotic stress

might force the activation of chromosomal genes for other dehydrogenases that are usually not active, attributable to the lower affinities to lactate when both *ldhL* and *ldhD* genes are functional. Further studies are necessary to prove the proposed mechanism. Interestingly, the production of lactate was also observed in a separate study with the *L. fermentum* double *ldhD-ldhL* mutant strain GRL1032, although lactate was reduced to one-tenth the amounts produced by the parent strain [1].

In summary, as the deletion of both *ldhL* and *ldhD* genes in TF103 led to an imbalance of cellular redox potential, it also resulted in slower growth than parent strain *L. plantarum* NCIMB8826 [6]. The *Spdc* engineered strains carrying *Spdc* gene grow faster than control TF103 carrying pTRKH2, but the growth is still slower than the wild type NCIMB8826 strain. The engineered strains on the TF103 background produced slightly more ethanol; however, they also produced more lactate. Although SPDC was expressed in the engineered TF103 strains, the carbon flow was not significantly improved toward ethanol, suggesting a further understanding of this organism's metabolism is necessary.

Acknowledgements We thank Theresa Holly, Patricia O'Bryan, and Rachel Furman for excellent technical assistance. The valuable review of this manuscript by Dr. Timothy D. Leathers is gratefully acknowledged.

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