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ORIGINAL PAPER

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Engineering lactic acid bacteria with pyruvate decarboxylase and alcohol dehydrogenase genes for ethanol production from *Zymomonas mobilis*

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Abstract Lactic acid bacteria are candidates for engineered production of ethanol from biomass because they are food-grade microorganisms that can, in many cases, metabolize a variety of sugars and grow under harsh conditions. In an effort to divert fermentation from production of lactic acid to ethanol, plasmids were constructed to express pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH), encoded by the *pdc* and *adhB* genes of *Zymomonas mobilis*, in lactic acid bacteria. Several strains were transformed with the plasmids, and transcription of *pdc* and *adhB* was confirmed by northern hybridization analysis of transformants. PDC and ADH enzyme activities were at least 5- to 10-fold lower in these bacteria compared to *Escherichia coli* transformed with the same plasmid. Glucose fermentations were carried out, and some, but not all, of the transformed strains produced more ethanol than the untransformed parent strains. However, lactic acid was the primary fermentation product formed by all of the transformants, indicating that ADH and PDC activities were insufficient to divert significant carbon flow towards ethanol.

Keywords Ethanol · Pyruvate decarboxylase · Alcohol dehydrogenase · Lactic acid bacteria · Molecular biology

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Introduction

Agricultural biomass has the potential to supplement starch as a feedstock for fuel ethanol production. Hydrolysis of the lignocellulose in biomass yields a mixture of hexose and pentose sugars, including glucose, galactose, xylose, and arabinose. However, the current starch-based technology has not yet been extended to a biomass-to-ethanol process, in part because the yeast *Saccharomyces cerevisiae* does not metabolize pentoses. Naturally occurring yeasts that ferment xylose have low ethanol yields and production rates [3]. Consequently, ethanol fermentation from biomass-derived sugars must employ new fermentative microorganisms with an expanded substrate range.

Recombinant organisms have been constructed that ferment sugar mixtures selectively to ethanol. These include *S. cerevisiae* [20] and the Gram-negative bacteria *Zymomonas mobilis* [31], *Escherichia coli* [10, 21], and *Klebsiella oxytoca* [24]. Gram-positive bacteria are another group with the potential for ethanol production. Many of these microorganisms have the ability to metabolize a variety of sugars, including pentose sugars. Gram-positive bacteria are widely used in the food industry, and thus are generally recognized as safe. Some grow at low pH and at 50°C or higher; these traits would allow adaptation to current fermentation systems, which would otherwise be vulnerable to contamination. In addition, many strains are relatively tolerant to ethanol. Some lactobacilli have been identified that are capable of withstanding 16% (v/v) ethanol [16]. Thus, Gram-positive ethanologens would offer a unique combination of desirable traits.

This study focused on the lactic acid group of Gram-positive bacteria. Homofermentative lactic acid bacteria ferment glucose to lactate via reduction of pyruvate by lactate dehydrogenase (LDH). Heterofermentative strains also produce acetate, ethanol, and CO_2 via phosphoketolase pathway activity. In engineered strains, expression of pyruvate decarboxylase (PDC) and alcohol

dehydrogenase (ADH) could divert carbon flow to ethanol rather than to lactate. The central metabolite pyruvate is converted to acetaldehyde by PDC and subsequently to ethanol by ADH. This strategy has been applied successfully to *E. coli* [10, 21] using the *pet* operon, which carries the *pdc* and *adhB* genes from *Z. mobilis*. The *pet* operon has also been placed in the Gram-positive bacteria *Bacillus* [2] and *Lactobacillus casei* [17], but selective production of ethanol has not been achieved. In work described here, two variations of the *pet* operon were constructed that have expression signals appropriate for lactic acid bacteria, and several strains were transformed with each plasmid. Gene expression, PDC and ADH enzyme activities, and ethanol yields were compared for the transformed and parent strains.

Materials and methods

Bacterial strains, culture media, and growth conditions

Bacterial strains used in this study are described in Table 1. *E. coli* was grown at 37°C aerobically in BHI medium (Becton Dickinson, Sparks, Md.) and anaerobically as described by Dien et al. [10]. Where appropriate, media were supplemented with ampicillin, chloramphenicol, erythromycin, or kanamycin at 100, 20, 150, and 50 mg l^{-1}, respectively. Lactic acid bacteria were grown in MRS medium (Becton Dickinson) supplemented with 10 mg l^{-1} erythromycin for transformed strains. *Lactococcus lactis* was grown at 30°C. Other strains were grown at 37°C. Cells used for enzyme assays were grown anaerobically for 18–24 h in 100 ml MRS medium flushed with nitrogen gas and containing 500 mg l^{-1} cysteine HCl, in glass bottles sealed with butyl rubber stoppers. Fermentation experiments were conducted in triplicate in 125 ml Erlenmeyer flasks, unless stated otherwise. MRS medium (50 ml) was supplemented with 40 g l^{-1} glucose (resulting in 60 g l^{-1} total glucose, because MRS contains 20 g l^{-1}), and buffered with 9 g $CaCO_3$ (marble chips 6210, Mallinckrodt, St. Louis, Mo.) per flask. The flasks were capped with rubber stoppers vented with 22 gauge needles. Cultures were gently agitated to promote mixing of the carbonate, and sampled after 72 h. *Lactobacillus plantarum* TF103 (an *ldh* mutant that grows best under anaerobic culture conditions [12]) and its transformants were grown for fermentation experiments anaerobically as described above.

Plasmid construction

PCR, ligations, and *E. coli* transformations were performed by standard techniques [27]. PCR products were cloned using the TA Cloning Kit (Invitrogen, Carlsbad, Calif.). Competent strains of lactic acid bacteria were prepared and transformed by electroporation [4]. Transformation was verified by PCR screening of erythromycin-resistant colonies, using oligonucleotide primers from the *Z. mobilis pdc* gene sequence [6] to detect the presence of the recombinant plasmids. Plasmid DNA was prepared with the Qiaprep Spin Kit (Qiagen, Valencia, Calif.). For Gram-positive bacteria, cells from 10-ml overnight cultures were incubated with 2 mg ml^{-1} lysozyme for 5 min at 37°C, and plasmid DNA was isolated according to the manufacturer's protocol. Cloned DNA was sequenced with the Big Dye Terminator Ready Reaction Kit (Applied Biosystems, Foster City, Calif.).

Two plasmids (pNET125 and pNET131; Fig. 1) were constructed to express PDC and ADH in lactic acid bacteria. First, the fragment of DNA containing the *pdc* and *adhB* genes was cloned by PCR amplification from pLOI297 [1] so that the *pdc* start codon is present within an engineered *Nde*I site, on a construct named pNET101. The cloned PCR product contained the *pdc* and *adhB* genes, beginning with the *pdc* start codon and ending four bases downstream of the *adhB* stop codon; this included the intergenic region that was created when the unlinked *pdc* and *adhB* genes were combined to form the *pet* operon [21]. The primers used for amplification were 5′-GCATCATATGAGTTATACTGTCGG-TACC-3′ (spanning the 5′ end of *pdc*; start codon underlined) and 5′-GAAGCCCGGGAAATTAGAAAGCGCTCAGG-3′ (spanning the 3′ end of *adhB*; stop codon on opposite strand underlined).

Two promoters (Fig. 1) were cloned separately, then joined to the *Z. mobilis pdc* start codon at the *Nde*I site. One promoter sequence (in pNET125; Fig. 1A) was PCR-amplified from the *Streptococcus bovis ldh* gene on pUCLDH1 [30] using primers 5′-ACTAGTTTATTCGTGACATTTCTCACTTAACG-3′ and 5′-CATATGTTTCCTCCTTGGTTTACAAGTGCG-3′ (*Nde*I recognition sequence underlined). The amplified region encompassed the 5′ end of the *ldh* gene in order to couple translation of the *pet* genes to a fragment of the *ldh* open reading frame. A consensus ribosome-binding site (RBS) was added [8], and the *ldh* gene was truncated after 30 residues by the addition of a stop codon, designed to overlap the *pdc* start codon. The modified *S. bovis* promoter region was fused to the *pdc* start codon, forming the final expression construct pNET125. This plasmid has the *S. bovis* promoter plus the *pet* genes, in the low copy shuttle vector pTRKL2 [25].

The second promoter sequence (in pNET131; Fig. 1B) has a consensus *L. lactis* promoter (CP40), with optimized sequences flanking the consensus −35 and −10 regions [23], and an added RBS sequence. The promoter sequence represented in Fig. 1B was constructed from complementary single-stranded oligonucleotides (20 μM each), which were annealed in 25 mM Tris, pH 8 and 10 mM $MgCl_2$, and cooled from 95°C to room temperature over 2 h. The annealed double-stranded fragment was used for blunt-end ligation without further purification. The cloned promoter region was fused to the *pet* genes at the introduced *Nde*I site (Fig. 1B), and the entire region spanning the promoter plus *pet* genes was cloned into the high-copy shuttle vector pTRKH2 [25], to form pNET131.

RNA analyses

Bacterial RNA was isolated from actively growing cultures. Cell pellets were suspended in buffer containing 10 mM MOPS, pH 7.3

Table 1 Bacterial strains

Strain	Description	Source or Reference
Escherichia coli INVαF′	Cloning strain	Invitrogen (Carlsbad, Calif.)
E. coli NZN111	*ldhA*::Kn Δ*pfl*::Cm	[5]
Lactococcus lactis LM0230	Plasmid-cured; *lac*$^-$ *prot*$^-$	[11]
Lactobacillus plantarum NCIMB8826	Wild-type; parent to TF103	[12]
L. plantarum TF103	NCIMB8826 *ldhD*$^-$ *ldhL*$^-$	[12]
Lactobacillus paracasei paracasei 3	Isolate from yeast ethanol fermentation	[23]
L. plantarum 18a	Isolate from yeast ethanol fermentation	[23]
L. plantarum 18c	Isolate from yeast ethanol fermentation	[23]
Lactobacillus casei 686	Ethanol tolerant	[17]

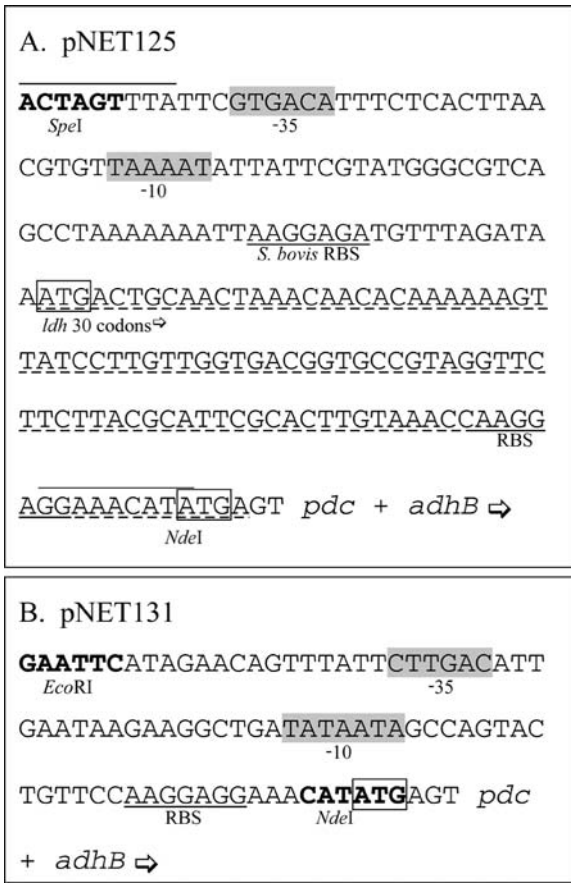

Fig. 1 Sequence of promoters used for *pet* gene expression in pNET125 (**A**) and pNET131 (**B**). Promoter consensus −35 and −10 regions are *shaded*, ribosome binding site (RBS) sequences are *underlined*, and start codons are *boxed*. Restriction sites introduced for cloning are indicated in *bold*. The *Streptococcus bovis ldh* gene fragment is shown in **A** with a dotted underline. The *overlined* bases were added to the sequence by PCR

and 10 mM KCl, incubated with 2 mg ml^{-1} lysozyme for 5 min at 37°C, and processed essentially as described by Gosink et al. [18]. Briefly, the suspensions were diluted with an equal volume of a solution containing 1% SDS, 20 mM MOPS pH 7.5, 40 mM EDTA, and 200 mM NaCl, boiled for 2 min, and extracted with phenol-chloroform. RNA was precipitated in ethanol, suspended in sterile water, and treated with DNase (Ambion, Austin, Tex.). Hybridizations were performed according to standard protocols [27]. Chemiluminescent DNA probes were generated by PCR amplification (Roche, Indianapolis, Ind.) from pLOI297, using oligonucleotide primers derived from the published gene sequences for *pdc* [6] and *adhB* [7]. Northern blots were performed on 10 μg RNA per sample.

Enzyme assays

Cell extracts for enzyme assays were prepared from 10 ml of *E. coli* cultures and 50 ml of other cultures. Pellets were suspended in 1.5 ml sodium phosphate (25 mM, pH 7), and disrupted using a Savant FastPrep 120 (Bio101, Vista, Calif.) with 0.1 mm diameter zirconia/silica beads (Biospec, Bartlesville, Okla.). *E. coli* cells were disrupted with one pulse at speed setting 4 for 20 s. Lactic acid bacteria were incubated with 2 mg ml^{-1} lysozyme for 30 min at 37°C, and then subjected to three pulses at speed setting 6.5 for 15 s, with incubation on ice for 2 min between pulses. Protein in crude cell extracts was measured using the Bio-Rad (Hercules, Calif,) dye-binding reagent with bovine serum albumin as a standard.

PDC activity was measured using 3-fluoropyruvic acid (14 mM) as the substrate in an assay mixture containing 20 mM KMES buffer pH 6.5, 1 mM thiamine pyrophosphate, 2 mM $MgCl_2$, and 10% (v/v) crude extract. PDC-catalyzed conversion of fluoropyruvate [15, 28] to acetate, carbon dioxide, and fluoride ion was measured at 35°C with a fluoride-selective electrode (Analytical Sensors, Sugar Land, Tex.) connected to an ion meter (Accumet AR25, Fisher Scientific, Springfield, N.J.) and calibrated with sodium fluoride. Activity measured in the fluoropyruvate assay was determined to be linear with respect to time and protein concentration. ADH activity was determined at 30°C by measuring the acetaldehyde-dependent oxidation of NADH to NAD+ at 340 nm [7]. LDH activity was measured at 30°C as the pyruvate-dependent oxidation of NADH to NAD^+ at 340 nm [26]. Assays were performed in duplicate on at least two separate extracts.

Determination of fermentation products

Concentrations of ethanol, glucose, and organic acids were determined by high-pressure liquid chromatography (Thermo Finnigan, San Jose, Calif.) using an Aminex HPX-87H column (Bio-Rad, Richmond, Calif.) and a Thermo Finnigan Spectrasystem RI-150 refractive index detector. Samples were run at 65°C and eluted at 0.6 ml min^{-1} with 5 mM sulfuric acid.

Results and discussion

Construction of broad-host-range plasmids containing the *pet* genes

The *pet* operon, for expression of PDC and ADH from *Z. mobilis*, was modified for expression in Gram-positive lactic acid bacteria. Two plasmids, each with a promoter and RBS fused to the fragment containing the *Z. mobilis pdc* and *adhB* genes, were constructed (Fig. 1). Plasmid pNET125 contained a promoter from the *ldh* gene in *S. bovis* [30], and a consensus *L. lactis* RBS [8]. This plasmid also contained the sequence corresponding to the first 30 codons of the *ldh* gene, prior to the start of the *pdc* gene, with a stop codon introduced to overlap the *pdc* start codon. This type of translational coupling is a strategy for increasing the efficiency of translation of heterologous genes. Plasmid pNET131 had the same RBS as pNET125, but used a moderately strong synthetic promoter characterized for use in *L. lactis* [22].

Expression of the *pet* operon in *E. coli*

To confirm that the *pet* genes were cloned intact on pNET125 and pNET131, expression of the *pet* genes was initially examined in *E. coli*. *E. coli* INVαF′, transformed with each plasmid, was tested for ADH and PDC activities. ADH was conveniently detected in both transformants (Table 2) by measuring the reduction of NAD+ in the presence of ethanol. Measurement of PDC in this strain by standard methods was problematic because of LDH background activity. The standard

Table 2 Pyruvate decarboxylase (*PDC*) and alcohol dehydrogenase (*ADH*) activity in *E. coli*INVαF′

Plasmid	PDC (U mg protein^{-1})[a]	ADH (U mg protein^{-1})[a]
–	0.09 ± 0.01	0.03 ± 0.01
pNET125	2.04 ± 0.01	1.21 ± 0.06
pNET131	1.57 ± 0.16	1.21 ± 0.21

[a]One unit of activity is 1 μmol product formed per minute

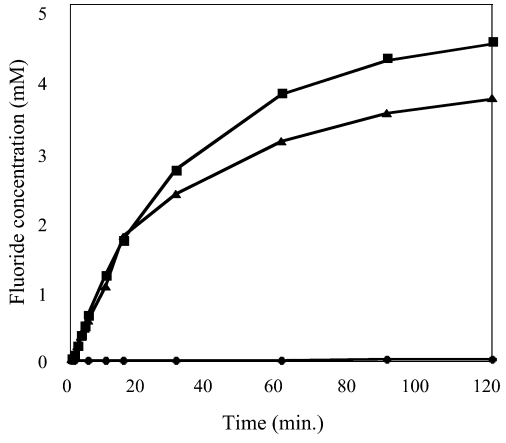

Fig. 2 Detection of pyruvate decarboxylase (PDC) activity in *Escherichia coli* cell extracts using a fluoride release assay, with fluoropyruvate as substrate. *E. coli* INVαF′ was transformed with pNET131 (■), pNET125 (▲), or the cloning vector pTRKH2 (●)

assay for measuring PDC is to follow NADH,H oxidation following addition of pyruvate and the ADH enzyme. However, LDH also oxidizes NADH,H in the presence of pyruvate, and interferes with measurement of PDC activity. Therefore, an alternative assay method was employed to determine PDC activity, using the artificial substrate fluoropyruvate, which is converted by PDC to acetate, CO_2, and F^-. This method has the advantage, compared to the PDC/ADH coupled assay, of being free from interference by lactate dehydrogenase. Fluoropyruvate has been used to study the mechanism of substrate activation of PDC [15, 28], and the method was adapted here to measure PDC enzyme activity in cell extracts. As shown in Fig. 2, fluoride was released by *E. coli* transformed with either pNET125 or pNET131, but not by the untransformed cloning strain, despite the presence of background LDH activity. The PDC and ADH activities associated with pNET125 and pNET131 indicate that both enzymes were expressed and are functional in *E. coli* (Table 2).

The plasmids were also transformed into an *E. coli* fermentative mutant, NZN111. This strain cannot grow fermentatively, due to mutations in the *ldhA* and *pfl* genes, which encode LDH and pyruvate-formate lyase [5]. The *pdc* and *adhB* genes, which provide an alternate pathway for cells to oxidize NADH, restored the ability of the mutant to grow fermentatively when supplied in trans in an *ldhA*, *pfl* mutant [19]. Similarly, NZN111 transformed with pNET125 or pNET131 grew anaerobically in glucose-containing medium, while the untransformed parent strain did not grow (results not shown). Thus, complementation of the growth defect in NZN111 further confirmed the presence of functional *pet* genes on pNET125 and pNET131.

The *pet* genes in lactic acid bacteria

After the presence of ADH and PDC activities was confirmed for the two plasmids in *E. coli*, each was used to transform *L. lactis* LM0230. To determine the utility of the two promoter-RBS systems used to drive gene expression in pNET125 and pNET131, northern hybridizations were performed to confirm the presence of the *pet* transcript in the transformed strains. The *pdc* and *adhB* probes each yielded a 3.3 kb band, detected in RNA from *L. lactis* LM0230 transformed with pNET125 and pNET131 (not shown). This result was consistent with the predicted size of the *pdc* and *adhB* cotranscript, which is approximately 3.1 kb. No hybridization was observed to RNA from a control strain, *L. lactis* transformed with the cloning vector pTRKH2. Higher levels (approximately 10-fold stronger hybridization signal) of *pdc* and *adhB* message were detected in *L. lactis* transformed with pNET131, compared to pNET125. The increased level of transcript detected for pNET131 can likely be attributed to two factors: the higher copy number replicon present in this plasmid, and the use of a promoter designed specifically for *L. lactis*.

The two plasmids were next introduced into six other strains of lactic acid bacteria (Table 1). The strains were chosen to encompass a variety of strains among the lactic acid group of bacteria, including homolactic and facultative heterolactic acid fermenters. Emphasis was placed on strains that can grow on xylose and have high ethanol tolerance. Three of the strains used were isolated as contaminants from fuel ethanol fermentations. Finally, one of the strains chosen for this study (*L. plantarum* TF103) is an *ldh* mutant, which may be useful in attempting to divert cellular metabolism of pyruvate toward ethanol. The presence of the *pet* transcript was verified in each of these strains transformed with pNET131 (not shown). The promoters constructed and used here to drive transcription of the *pet* genes may therefore prove generally useful for gene expression in the lactic acid group of bacteria.

PDC and ADH activities in lactic acid bacteria

PDC and ADH enzyme activities from pNET125 and pNET131 were measured in the transformants (Table 3). Native LDH was also measured. As expected, native LDH activity was high in all strains (Table 3) except the *ldh* mutant *L. plantarum* TF103 [12]. To circumvent the LDH background activity, fluoride released from flu-

Table 3 PDC and ADH activity in lactic acid bacteria. *LDH* Lactate dehydrogenase

Strain	Plasmid	PDC (U mg protein^{-1})[a]	ADH (U mg protein^{-1})[a]	LDH (U mg protein^{-1})[a]
L. lactis LM0230	pTRKH2	0.003 ± 0.002	0.031 ± 0.024	20.7 ± 1.7
	pNET125	0.004 ± 0.007	0.007 + 0.014	18.0 ± 3.1
	pNET131	0.033 ± 0.018	0.028 ± 0.025	17.3 ± 1.3
L. casei 686	–	0.009 + 0.001	1.16 ± 0.21	72.5 ± 18.0
	pNET125	0.009 + 0.001	1.28 ± 0.08	70.5 ± 8.1
	pNET131	0.006 + 0.001	1.68 ± 0.13	86.7 ± 11.7
L. plantarum 18a	–	0.006 ± 0.007	0.006 ± 0.007	5.22 ± 0.61
	pNET131	0.008 ± 0.006	0.034 ± 0.024	8.53 ± 2.66
L. plantarum 18c	–	0.006 ± 0.003	0.000 ± 0.000	6.9 ± 0.7
	pNET125	0.091 ± 0.020	0.245 ± 0.028	9.0 ± 1.5
	pNET131	0.013 ± 0.008	0.296 ± 0.043	5.6 ± 1.2
L. paracasei 3	–	0.010 ± 0.011	0.002 ± 0.002	3.59 ± 0.61
	pNET131	0.017 ± 0.016	0.010 ± 0.011	3.71 ± 0.44
L. plantarum NCIMB8826	–	0.002 ± 0.002	0.005 ± 0.006	63.1 ± 1.6
	pNET131	0.002 ± 0.002	0.090 ± 0.081	56.2 ± 2.5
L. plantarum TF103	–	0.003 ± 0.004	0.293 ± 0.128	0.007 ± 0.001
	pNET131	0.004 ± 0.005	0.292 ± 0.052	0.083 ± 0.070

[a]One unit of activity is one μmol product formed per minute

oropyruvate was measured as an indicator of PDC activity. The assay detected PDC activity in some of the transformed strains of lactic acid bacteria, compared to virtually no background in untransformed strains and in controls transformed with the cloning vector. This assay therefore proved to be sensitive for detection of PDC activity, and may be generally useful for systems where endogenous NADH oxidation occurs, and/or PDC activity is low. PDC activities were two orders of magnitude lower than those measured for the *E. coli* transformants, and only three strains (*L. lactis* LM0230, *L. plantarum* 18c, and *L. paracasei* 3) showed increased PDC activity. Results for the fluoropyruvate assay of PDC activity are shown in Fig. 3 for *L. lactis* LM0230. Strains with detectable PDC activity also had increased ADH activity (Table 3). In addition, *L. casei* 686 and *L. plantarum* NCIMB8826 showed increased ADH activity. *L. casei* 686 had native ADH activity comparable to that of the transformed *E. coli* strains. Otherwise, ADH activity in the Gram-positive transformants was significantly lower than that measured for *E. coli* transformants.

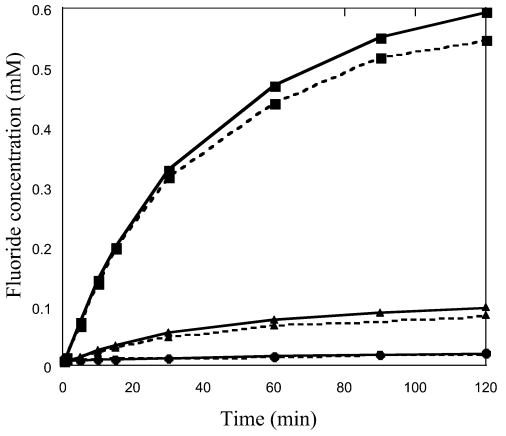

Fig. 3 Recombinant PDC activity in cell extracts of *Lactococcus lactis* transformed with pNET131 (■), pNET125 (▲), or the cloning vector pTRKH2 (●). PDC activity was detected using a fluoropyruvate assay. Cell extracts were assayed in duplicate (*dashed lines*)

Fermentation of glucose by the transformed strains

The strains were next tested for the production of ethanol from glucose (Table 4). All of the control strains, except for *L. plantarum* 18c, produced some ethanol. This result was not surprising because lactic acid bacteria are known to produce ethanol as a minor fermentation product. A few strains transformed with either pNET125 and/or pNET131 showed increased ethanol production compared to the untransformed parent strains (Table 4). The most ethanol was produced by *L. casei* containing pNET131. However, the background ethanol concentration was nearly as high (268 mM). This strain also had the highest ADH activity, comparable to that of the *E. coli* transformed strains (Table 3). *L. plantarum* strain 18c, in which no ethanol was produced by the parent strain, did produce a small amount of ethanol (2.9–3.0 mM) when transformed with the *pet* plasmids. Therefore, it can be concluded from glucose fermentations experiments that although the genes encoding ADH and PDC were introduced into these strains, activity in most transformants was insufficient to produce ethanol that exceeded background levels. No acetaldehyde was detected in fermentations with any of the strains, and lactate was the major fermentation product for all strains except *L. plantarum* TF103, an *ldh* mutant that produces mainly acetoin from glucose [12, 13].

Initial work with *Bacillus* species [2] led to the conclusion that there is no intrinsic barrier to ethanol production in Gram-positive bacteria, and Ingram et al. [21] further observed that the *Z. mobilis* PDC, with a K_m of 0.4 mM, should be able to compete for pyruvate with

Table 4 Ethanol produced by transformants of lactic acid bacteria

Strain	Plasmid	Glucose used (mM)	Final lactic acid (mM)	Final ethanol (mM)
L. lactis LM0230	pTRKH2	79.9 ± 5.0	163.4 ± 9.9	2.5 ± 0.5
	pNET125	85.7 ± 3.5	153.4 ± 4.7	1.7 ± 0.3
	pNET131	77.6 ± 4.3	145.2 ± 2.0	1.8 ± 1.4
L. casei 686	–	324.6 ± 3.9	262.5 ± 13.7	267.7 ± 9.6
	pNET125	282.8 ± 60.6	223.5 ± 43.4	241.6 ± 56.0
	pNET131	329.1 ± 0.1	272.5 ± 3.1	278.3 ± 3.6
L. plantarum 18a	–	209.8 ± 1.8	390.8 ± 14.5	1.4 ± 2.5
	pNET131	226.8 ± 40.0	322.4 ± 33.8	2.0 ± 1.3
L. plantarum 18c	–	240.5 ± 6.0	476.4 ± 14.3	0.0 ± 0.0
	pNET125	154.3 ± 0.7	462.1 ± 27.4	2.9 ± 0.1
	pNET131	333.3 ± 0.0	373.0 ± 13.2	3.0 ± 0.3
L. paracasei 3	–	228.1 ± 5.7	560.7 ± 11.7	6.9 ± 7.3
	pNET125	188.7 ± 14.7	395.1 ± 21.5	31.4 ± 4.9
	pNET131	216.5 ± 14.9	498.0 ± 40.5	3.6 ± 0.7
L. plantarum NCIMB8826	–	215.4 ± 5.2	498.4 ± 15.4	3.3 ± 0.1
	pNET125	144.7 ± 7.5	314.9 ± 21.1	4.9 ± 0.5
	pNET131	193.8 ± 4.1	463.4 ± 9.7	4.5 ± 3.7
L. plantarum TF103	–	141.3 ± 10.9	9.4 ± 2.3	133.4 ± 0.1
	pNET131	129.1 ± 4.6	12.2 ± 1.3	121.1 ± 8.1

LDH enzymes. Subsequently, Gold et al. [16] engineered a *pet* operon in *L. casei* 686, a facultatively heterofermentative strain with high native ADH activity, and obtained 6.5–8.7 g l^{-1} ethanol from glucose. Transformation of our plasmids into this strain yielded no increase in ethanol production above the 12 g l^{-1} produced by the untransformed parent (Table 4). The difference between our results and the previous work by Gold et al. may be related to the ability of many lactic acid bacteria to shift from homofermentative growth to mixed acid fermentation, depending on growth conditions [9, 14].

As observed for *Bacillus* [2], gene transcription is unlikely to be the only factor important for high-level ethanol production. Here, the *pet* transcript was detected in all of the transformants tested, yet most had low PDC and ADH enzyme activities and produced no ethanol above control levels. Since the lactic acid bacteria comprise a diverse group, the impact of factors such as translational efficiency, protein folding, and metabolic flux are likely to differ among strains. In some cases, low enzyme activities and low ethanol production may be due to poor translation of the *pet* genes because of inefficient translation initiation, or to differences in codon usage in lactic acid bacteria compared to the *Z. mobilis pet* genes. One of the plasmids, pNET125, was designed to couple translation of the *pet* genes to a truncated *ldh* gene, but this did not result in increased enzyme activities compared to pNET131 (Table 2). Possibly, a *pdc* gene obtained from an alternate source would be better expressed in the lactic acid bacteria. The *pdc* gene from *Sarcina ventriculi* [29] is a possible candidate, because *S. ventriculi*, like the lactic acid group of bacteria, is Gram-positive. It is clear that enhancement of gene expression in a suitable strain, along with optimized growth conditions, will be needed to obtain higher enzyme activities and increased ethanol production.

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