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ORIGINAL RESEARCH PAPER

Heterologous expression of pyruvate decarboxylase in *Geobacillus thermoglucosidasius*

Ann H. Thompson · David J. Studholme · Edward M. Green · David J. Leak

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Abstract Expression of a pyruvate decarboxylase (Pdc) pathway in metabolically versatile thermophilic bacteria could create novel ethanologenic organisms, but no suitable thermostable Pdc is available. We have demonstrated that Pdc from *Zymomonas mobilis* can be expressed in an active form in *Geobacillus thermoglucosidasius* at up to 52°C, while expression of Pdc polypeptides up to 54°C was evident from Western blotting. By using an unstable lactate dehydrogenase (*ldh*) mutant of *G. thermoglucosidasius*, indirect evidence of Pdc activity in vivo was also obtained.

Keywords Decarboxylase · Ethanol · *Geobacillus* · Pyruvate · Thermophile

A. H. Thompson · D. J. Studholme · D. J. Leak (✉)
Division of Biology, Faculty of Natural Sciences, Imperial College London, South Kensington, London SW7 2AZ, UK
e-mail: d.leak@imperial.ac.uk

Present Address:
D. J. Studholme
Sainsbury Laboratory, John Innes Centre, Norwich NR4 7UH, UK

Present Address:
E. M. Green
Green Biologics Ltd, 45A, Milton Park, Abingdon, Oxfordshire OX14 4RU, UK

E. M. Green
Agrol Ltd, The White House, 2 Meadrow, Godalming, Surrey GU7 3HN, UK

Introduction

Thermophiles are potentially useful organisms for ethanol production, generally offering a catabolic versatility (Lynd 1989; Sommer et al. 2004) with the possibility of combining ethanol production with removal by gas stripping, or mild vacuum, in a continuous process.

Despite the evident advantages of using thermophiles, one major disadvantage is that there is no thermophilic equivalent of *Saccharomyces cerevisiae* or *Zymomonas mobilis* which produce ethanol as their sole fermentation product. Although some near theoretical carbohydrate to ethanol production efficiencies have been cited in the literature, these have a strong dependence on physiological and nutritional conditions (e.g., Hild et al. 2003) and there is no good example of an obligately homo-ethanologenic thermophile. A potential solution to this problem is to redirect the fermentation pathways in a suitable thermophile towards production of ethanol. This strategy has been very effective in producing ethanologenic *Escherichia coli* (Ingram et al. 1987; Alterthum and Ingram 1989) and *Bacillus megaterium* (Talarico et al. 2005) in which the pyruvate decarboxylase (Pdc) from *Z. mobilis* or *Sarcina ventriculi* has been expressed, together with an alcohol dehydrogenase. However, to date, pyruvate decarboxylase seems only to have been found in mesophiles. Therefore, as a precursor to selecting more thermostable mutants of Pdc we have investigated whether Pdc from *Z. mobilis*

can be functionally expressed in thermophilic facultative *Geobacillus* spp.

The host for expression of Pdc was *G. thermoglucosidasius* TN, an unstable L-lactate dehydrogenase (Ldh) mutant, isolated after di-epoxybutane mutagenesis (Cohn 1961) of strain LLD-R. Although cultures of TN seem to revert less readily than LLD-15 (San Martin et al. 1992), lactate-producing revertants ultimately predominate in extended continuous cultures maintained under metabolic stress which, as outlined below, was advantageous for demonstrating a beneficial effect of Pdc expression. San Martin et al. (1992, 1993) demonstrated that selection of revertants could be avoided by maintaining the specific sucrose consumption rate below 4.2 g sucrose h^{-1} (g cells)$^{-1}$ at pH 7, but this maximum flux rate was diminished at lower pH.

This suggests that lactate production is only a selective advantage once flux through the other available fermentation pathways is saturated, consistent with lactate being a fermentative overflow metabolite. Acetate, ethanol and formate production via the pyruvate formate lyase pathway (PFL) is the main fermentation pathway, while evidence has been presented for induction of pyruvate dehydrogenase in *ldh* mutants under anaerobic conditions, once flux through PFL becomes limiting (San Martin et al. 1993). This complicates the demonstration of increased ethanol production via a new Pdc pathway, because flux through Pdh can also increase ethanol production which is impossible to quantify independently. However, pyruvate accumulation always precedes "takeover" by lactate producing revertants. As expression of an active Pdc should provide an alternative pathway for pyruvate metabolism, a decrease in pyruvate accumulation and selection pressure for revertants should be a good indicator of Pdc expression, even in the absence of quantitative differences in flux to ethanol.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown at 37°C in Luria–Bertani broth (LB) or agar (LA). *Z. mobilis* NCIMB8938 was grown in a medium containing 50 g glucose l^{-1}, 2 g yeast extract l^{-1}, 2 g NH_4Cl l^{-1}, 1 g KH_2PO_4 l^{-1}, 0.49 g $MgSO_4 \cdot 7H_2O$ l^{-1} , 50 mg NaCl l^{-1}, 50 mg KCl l^{-1}, 7.2 mg $ZnSO_4 \cdot 7H_2O$ l^{-1}, 5.54 mg $MnCl_2 \cdot 4H_2O$ l^{-1}, 5 mg $FeSO_4 \cdot 7H_2O$ l^{-1}, 2 mg $CuSO_4 \cdot 5H_2O$ l^{-1}, 1.1 mg $CaCl_2$ l^{-1}, 0.74 mg

Table 1 Strains and plasmids used in this study

Strain	Description/genotype	Source/reference
E. coli TOP10	F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*)Φ80*lacZ*ΔM15 Δ*lacX74 recA1 ara*D139 Δ(*ara-leu*)7697 *galU galK rpsL* (Str^R) *endA1 nupG*	Invitrogen
G. stearothermophilus NCA1503	Wild type strain	NCIMB 8924
G. thermoglucosidasius TN	*ldh* non-sporulating	Agrol Ltd
Z. mobilis	Wild type strain	NCIMB 8938
Plasmids		
pLOI275	*pdc* gene from *Z. mobilis* in pUC18	Ingram and Conway (1988)
pBST22	*E. coli-Geobacillus* shuttle vector	Liao et al. (1986)
pMETH	Expression vector for *Hae*III methylase	Green et al. (2002)
		Agrol Ltd
pBST22:ZYM	*Pdc* from *Z. mobilis* under the control of the *lct* promoter from *G. stearothermophilus* NCA1503, in pBST22	This work

$CoSO_4 \cdot 5H_2O$ l^{-1}. *G. thermoglucosidasius* TN was routinely grown on LA plates and in LB or Welker's modified LB (Chen et al. 1986). For continuous culture the medium contained 10 g yeast extract l^{-1}, 5 g tryptone l^{-1}, 1 g NaH_2PO_4 l^{-1}, 2 g NH_4Cl l^{-1}, 0.4 g KCl l^{-1}, 0.4 g $MgCl_2 \cdot 6H_2O$ l^{-1}, 0.4 g $MnCl_2 \cdot 4H_2O$ l^{-1}, 6 mg $CaCl_2$ l^{-1} and 10 ml trace elements l^{-1} (San Martin et al. 1992). Glucose was heat-sterilised separately and added to give 10 or 100 g l^{-1}.

For chemostat culture, cells were grown in a 1.5 l (water jacketed) vessel (LSL-Biolafitte) with temperature, agitation, dissolved O_2 and pH measurement and control. The pH was maintained using 100 g NaOH l^{-1}. For anaerobic growth, cells were initially grown aerobically in batch and continuous culture and then switched to N_2 sparging (50 ml min^{-1}) with agitation at 400 rpm to achieve anaerobic conditions. Fermentation substrates and products were measured by separation on a BioRad Aminex HPX-87H HPLC column with 10%(v/v) H_2SO_4 as the mobile phase with UV and RI detection. Ethanol was also analysed by GC-FID with separation on a Porapak Q column at 180°C and N_2 carrier gas at 30 ml min^{-1}.

Molecular biology

All *E. coli* manipulations used standard protocols (Sambrook et al. 1989). Plasmid DNA was routinely purified from *E. coli* using Wizard miniprep kits (Promega). Chromosomal DNA was isolated from thermophilic bacilli by an adaptation of the method of Cutting and van der Horn (1990). Cultures were grown to an OD_{600} of 1 in Welker's modified LB at 55°C, harvested, washed in warm (55°C) lysis buffer and then re-suspended in 0.1 vol (based on the original culture) lysis buffer containing 2.5 mg lysozyme ml^{-1}. This suspension was then incubated without shaking at 48°C for 10 min, sarkosyl added to give 15 g l^{-1} and incubation continued at room temperature for 5 min. DNA was extracted from the lysed cells with equal volumes of phenol, then 1:1 (v/v) phenol/chloroform, then twice with 24:1 (v/v) chloroform/isoamyl alcohol and finally ethanol precipitated.

All PCR amplifications for cloning purposes used Vent polymerase (New England Biolabs), while confirmatory PCR used BioTaq DNA polymerase (Bioline). The *lct* promoter and SD sequence (Barstow et al. 1986) was amplified from *G. stearothermophilus* NCA1503 chromosomal DNA using the forward primer (PROM-F) 5′-CATCCGCTATATA TTAACGTGG-3′ and the reverse primer (PROM-R) 5′-ACT<u>GAGCTC</u>CCTCAATATAATGCGAACAC-3′ which incorporated a *Sac*I restriction site (underlined). The coding region (approx 1.5 kb) of the *pdc* gene from *Z. mobilis* was amplified from pLOI295 (Ingram and Conway 1988) by PCR using the forward primer (ZYM-F) 5′-TGAATA<u>GAGCTC</u>G TAAGCA**ATG**AGT-3′ which incorporates a *Sac*I site (underlined) and start codon (bold), and reverse primer (ZYM-R) 5′-ACTAGAGGAGCTTGTTAA CAGGC-3′. The *lct* promoter, SD, and *pdc* PCR product were purified and digested with *Sac*I. The *lct* promoter was then ligated to the *pdc* gene and the ligation products immediately amplified by PCR using PROM-F and either ZYM-R or PDC5-R primers. Products of the expected size were gel purified, phosphorylated using T4 polynucleotide kinase and ligated into the plasmid vector, which had been digested with *Pvu*II. The authenticity of all final constructs was confirmed by dye-terminator sequencing using Amplitaq DNA polymerase FS on an AB1373 automated sequencer (Applied Biosystems).

For electrocompetent *G. thermoglucosidasius* TN, cells were grown in TGP medium (17 g tryptone l^{-1}, 3 g soy peptone l^{-1}, 2.5 g K_2HPO_4 l^{-1}, 5 g NaCl l^{-1}, 4 g sodium pyruvate l^{-1}, 4 ml glycerol l^{-1} pH 7.3), washed and resuspended in TH buffer (272 mM trehalose, 8 mM HEPES, pH 7.5 with KOH) and electroporated in the same apparatus with a single 12 ms pulse at 25 µF, 1 kV and 400 Ω. Cells were immediately transferred to pre-warmed TGP and incubated at 50°C for 45 min, before plating on selective media. To protect against the known *Hae*III restriction activity, *G. thermoglucosidasius* LN was transformed with pBST22:ZYM produced in *E coli* HM2 (TOP10 pMETH), which expresses recombinant *Hae*III methylase (Green et al. 2002). This routinely gave a low transformation frequency of approximately 300 per µg DNA.

Quantitative assay for Pdc activity

Pdc activity was measured at 30°C by the spectrophotometric alcohol dehydrogenase-linked enzyme assay (Pohl et al. 1994). Results are the mean values of triplicate assays.

SDS-PAGE and western blotting

Protein samples were analyzed by SDS-PAGE as described by Laemmli (1970) using a discontinuous buffer system and a 12% (w/v) separating gel. Gels were either stained directly with Coomassie Blue, or electroblotted (Sartoblot II-S, Sartorius) onto pre-soaked (methanol for 10 s, then washed in water) PVDF membrane at 40 V for 1 h at 4°C. After incubation for 1 h with 10 g blocking reagent l^{-1}, the membrane was incubated for 1 h with 14 ml of anti-*Z. mobilis* Pdc goat IgG (a gift from Prof L Ingram, University of Florida, USA) diluted 1:20,000 in 5 g blocking solution l^{-1}. The membrane was then extensively washed (4 × 10 min) with 50 mM Tris, 150 mM NaCl pH 7.5 containing 1 g Tween l^{-1}, incubated for a further 1 h with a solution of anti-goat IgG conjugated with alkaline phosphatase (diluted 1:2,500 as above), washed as before and then visualised with the NBT/DCIP staining solution. All immunological reagents were from Roche Diagnostics.

Results

Cloning *pdc* in a shuttle vector

The *Z. mobilis pdc* gene was amplified by PCR from pLO1275 and cloned into the unique *Pvu*II site of the *Geobacillus*-*E. coli* shuttle vector pBST22, which expresses a thermostable neomycin phosphotransferase allowing selection with kanamycin at up to approx 68°C (Liao et al. 1986). To ensure expression under the required conditions a 300 bp region upstream of the *lct*A gene (note that both *lct* and *ldh* have been used for lactate dehydrogenase genes, with the latter now being more common) from *G. stearothermophilus* NCA1503 (Barstow et al. 1986), incorporating the Shine-Dalgarno sequence and presumably incorporating the *lct* promoter (a divergent open reading frame is present approx 300 bp upstream of *lct*A), was amplified and ligated upstream of the *pdc* gene. The final construct pBST22:ZYM was initially transformed into *E. coli* HM2.

Expression of Pdc in thermophiles

Together with the alcohol dehydrogenase from the pyruvate formate lyase pathway, expression of *pdc* in *G. thermoglucosidasius* TN should create an ethanol production route which would act as a drain for excess pyruvate production. Given that the conditions which lead to pyruvate excretion are also selective for lactate producing revertants, this would be difficult to demonstrate in steady state continuous cultures (confirmed experimentally). Therefore, two approaches were taken to demonstrate the presence of Pdc activity. Firstly, *G. thermoglucosidasius* TN pBST22:ZYM was grown anaerobically in chemostat culture ($D = 0.05\ h^{-1}$) under carbon-limited, steady state conditions which did not result in excess pyruvate accumulation at 52, 54, 56 or 58°C. The protein profiles of cell extracts from each of the cultures were examined on SDS-PAGE and subsequently probed by Western blotting using a polyclonal antibody against *Z. mobilis* Pdc (Fig. 1). This demonstrated that *G. thermoglucosidasius* TN pBST22:ZYM produced a cross-reactive polypeptide of the same size (60.8 kDa) as Pdc monomers at 52 and 54°C. However, at 56 and 58°C the polypeptide was less prevalent (note that despite differences in the loading of the negative control, the lanes for 56 and 58°C have higher loadings than those at 52 and 54°C). Pdc activity of 0.22 (±0.05) μmol min^{-1} mg^{-1} was detected, using the coupled assay, in samples taken from the 52°C culture but no activity was measurable with samples from higher temperatures.

The second test for Pdc expression was based on the argument that, if the accumulation of pyruvate results in selective conditions for lactate producing revertants, any metabolic activity which reduces pyruvate accumulation should delay the take-over by lactate producing cells. The experimental test consisted of establishing a carbon- (glucose-) limited continuous culture (at $D = 0.05\ h^{-1}$) under non-selective conditions and then switching the feed to a higher concentration of glucose (100 g l^{-1}). This imposes a gradual change from glucose limited to glucose excess conditions and the latter is known to result in take-over by lactate producing cells.

When this regime was imposed on *G. thermoglucosidasius* TN, extracellular pyruvate concentrations increased gradually up to a peak of 58 mM after 52 h, and then started to decline, concomitant with the onset and subsequent increase in lactate production (Fig. 2a).

With *G. thermoglucosidasius* TN pBST22:ZYM, while the cell densities were similar to those for

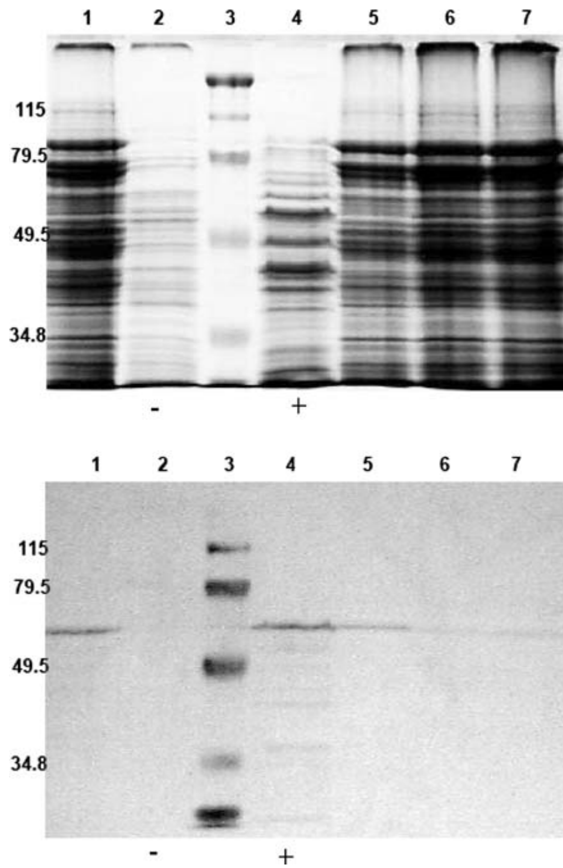

Fig. 1 (Top) SDS-PAGE and (bottom)Western blot using anti-*Zymomonas* PDC antibody of cell extracts from *G. thermoglucosidasius* TN pBST22:ZYM grown anaerobically in carbon limited chemostat culture at 52°C (lane 1), 54°C (lane 5), 56°C (lane 6) and 58°C (lane 7). Lane 3 contains molecular mass size markers while lane 2 contains extracts from *G. thermoglucosidasius* TN (negative control) and lane 4 contains extracts from *Z. mobilis* (positive control). Negative and positive controls are indicated below lanes 2 and 4 on both images

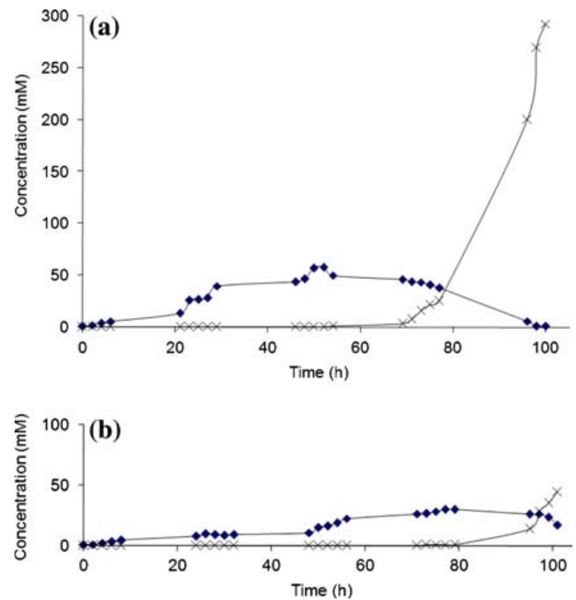

Fig. 2 Pyruvate (♦) and lactate (x) accumulation profiles during the selection of lactate producing revertants of (**a**) *G. thermoglucosidasius* TN and (**b**) *G. thermoglucosidasius* TN pBST22:ZYM in a gradient of increasing glucose concentration. Both strains were grown anaerobically in continuous culture ($D = 0.05\ h^{-1}$) at 52°C. At time zero the feed medium was changed from one containing 10 g glucose l^{-1}, on which steady state had been established, to an identical medium, but containing 100 g glucose l^{-1}. Similar profiles were obtained in duplicate runs of both **a** and **b**, although the actual onset of lactate appearance showed some variation

G. thermoglucosidasius TN, the extracellular pyruvate concentration was 16 mM after 52 h and continued to increase to 30 mM (Fig. 2b). Although ethanol concentrations were measured, these were not a good indicator of Pdc activity due to the interplay between Pdh and Pdc fluxes, as explained in the introduction, and were not significantly different from those of *G. thermoglucosidasius* TN. Lactate started to appear in this culture after 73 h, marginally before the peak in pyruvate concentrations (77 h). Thus the specific productivity of pyruvate was lower in the strain carrying the *pdc* construct, consistent with metabolism of pyruvate by an active Pdc. Interestingly a higher cell yield was also observed with *G. thermoglucosidasius* TN pBST22:ZYM (evident from the higher residual glucose levels, but similar cell densities). This is consistent with pyruvate excretion resulting in a redox imbalance in the cells, forcing more acetyl-CoA arising from the PFL pathway to be reduced to ethanol. Thus, the expected 1:1 ratio of acetate and ethanol, arising from PFL, shifts away from acetate production and, as the latter pathway involves ATP synthesis, lower ATP yields and, hence, cell yields result. As an example, at 52 h the concentrations of formate, acetate and ethanol in the *G. thermoglucosidasius* TN culture were 140, 47 and 234 mM, respectively, (from 83, 38 and 67 mM initially), demonstrating not only the shift from acetate production, but also the significantly increased flux to ethanol via Pdh under these conditions.

The effects described above were reproducible, although the actual onset of lactate appearance showed some variation. Given the difficulty in assessing a

quantitative contribution to Pdc, the lower pyruvate levels and later lactate appearance in *G. thermoglucosidasius* TN pBST22:ZYM cultures provides strong indirect evidence that Pdc is functional in vivo at 52°C.

Discussion

Pyruvate decarboxylase from *Z. mobilis* can clearly be expressed in *Geobacillus* spp. at the lower end of their growth temperature range. Despite the native (prefolded) enzyme being stable up to 60°C (Pohl et al. 1995) it is evident that the assembly of a functional Pdc in *Geobacillus* spp is more sensitive to temperature than the activity of a correctly assembled protein. A possible explanation for this discrepancy is that Pdc binding to one or both of its cofactors is a prerequisite to folding (Wittung-Stafshede 2002). Indeed, refolding studies with chemically denatured *Z. mobilis* Pdc clearly show that the presence of thiamine pyrophosphate (TPP) and Mg^{2+} is essential for monomers to refold correctly and that these subsequently form into active tetramers (Pohl et al. 1994). Although sufficient TPP and Mg^{2+} must be available in the cell to allow some correct folding of PDC at low temperatures, increasing temperature will progressively reduce the binding energy for formation of the initial complex of cofactor with unfolded protein. Above 54°C it is probable that all of the protein folds into an inactive structure and is subsequently degraded by endogenous proteases, which would explain the dramatic drop in antibody detectable Pdc monomer.

The thermostability of purified Pdc from *Z. mobilis* is also dependent on the presence of cofactors, although in this case the most important cofactor appears to be the TPP (Pohl et al. 1995). In the complete absence of TPP, there is a significant decline in activity at temperatures above 50°C, while in the complete absence of both cofactors activity starts to decline above 40°C (Pohl et al. 1995). Thus, even if the protein was capable of folding correctly at these temperatures, it is unlikely that the true in vivo thermostability would be as high as 60°C, and will clearly be highly dependent on intracellular free cofactor concentration.

The demonstration of Pdc activity in *Geobacillus* spp suggests that metabolic engineering to create a homo-ethanol pathway, similar to the work done with *E. coli* (Dien et al. 1998), is feasible but that improved Pdc assembly at high temperatures will be required to exploit the full temperature range of *Geobacillus* spp.

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