Expression of active yeast pyruvate decarboxylase in *Escherichia coli*

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We have shown by appropriate modification of the translational signals and using the strong T7 RNA polymerase promoter $\phi 10$, that a cloned *Saccharomyces cerevisiae* pyruvate decarboxylase gene (*pdcl*) can be expressed in *Escherichia coli*. This protein, which migrated as a single band on SDS-polyacrylamide gels, was found to have a subunit molecular mass of approximately 62 kDa, similar to that of the enzyme produced by yeast. Polyclonal antibodies raised against purified yeast pyruvate decarboxylase recognized this bacterially produced protein. We found that this recombinant enzyme is active, indicating that the homotetramer encoded by the *pdcl* gene is functional.

Introduction

Pyruvate decarboxylase (PDC; EC 4.1.1.1) is a key enzyme in alcoholic fermentation, cleaving pyruvate to acetaldehyde and CO₂. Both thiamine diphosphate (ThDP) and Mg²⁺ are essential cofactors and the enzyme is only active as a tetramer. In *Saccharomyces cerevisiae*, the tetramer may be composed of identical or nearly identical subunits (Gounaris *et al.*, 1975; Zehender & Ullrich, 1985; Kuo *et al.*, 1986) but there is controversy as to their origin. The smaller β chain was originally suggested to be the result of proteolytic cleavage of the α chain. However, Zehender & Ullrich (1985) proposed that there were sufficient differences in the amino acid composition of the two chains to preclude this possibility. Kuo and co-workers (1986) isolated both α_4 and $\alpha_2\beta_2$ enzyme forms from brewing yeast, each of which is active.

One way to confirm that the homomeric form of PDC is active is to express the enzyme from a cloned gene in a heterologous host. Moreover, if successful, the same system could then be used to study structure–function relationships of this protein. However, we found that the natural yeast *pdcl* gene is not expressed well in *Escherichia coli*, presumably because it lacks suitable transcriptional and translational signals. Here we show that appropriate modification of those signals will give

high levels of expression in *E. coli*, and that the enzyme is active in this host.

Methods

Materials. Restriction enzymes and ligases were purchased from New England Biolabs or Toyobo and used according to the manufacturers' instructions. Nitrocellulose membranes (Hybond C-Extra), the Enhanced Chemiluminescence (ECL) Kit, and radionucleotides were purchased from Amersham, and commercial yeast PDC (P-5651) from Sigma. Oligonucleotides were synthesized on a Milligen 7500 DNA synthesizer, and were used without further purification.

Strains and plasmids. The T7 RNA polymerase/promoter expression system, consisting of plasmids pGP1-2 (which contains the phage T7 gene 1 for RNA polymerase under the control of λP_L and *cI857*) and pT7-7 (which contains the T7 ϕ promoter and the strong ribosome binding site from the T7 gene 10 protein inserted upstream of a multiple cloning site), was kindly supplied by S. Tabor (Harvard Medical School, USA) (Tabor & Richardson, 1985). pGP1-2 was supplied already transformed into *E. coli* K38 (HfrC1). *E. coli* DH5 α [F[–] ϕ 80d *lacZ*AM15 *endA1* *recA1* *hsdR17* (*r_K*, *m_K*) *supE44* *thi-1* λ^- *gyrA96* Δ (*lacZYA-argF*) U169] was used for transformation and plasmid preparation. YEp13-PDC1 (Schmitt *et al.*, 1983) was kindly supplied by S. Hohmann (Institut für Mikrobiologie, Technische Hochschule, Darmstadt, FRG). All strains were grown on Luria broth plates with appropriate antibiotics. Ampicillin and kanamycin were used at 75 μ g ml^{–1}. Cell growth was at 37 °C except for *E. coli* containing pGP1-2 which was grown at 30 °C.

Plasmid construction. Standard methods were employed for plasmid and M13 bacteriophage preparation, restriction enzyme digestion, ligation and transformation (Maniatis *et al.*, 1982). Plasmid constructions (Fig. 1) were transformed into *E. coli* DH5 α .

Protein expression in *E. coli*. The following protocol was modified from that described by Tabor & Richardson (1985). For expression of

Abbreviations: ECL, enhanced chemiluminescence; PDC, pyruvate decarboxylase; TBS, Tris-buffered saline; ThDP, thiamine diphosphate.

Fig. 1. Construction of plasmids pT7pdc1-A (a), pT7pdc1-B1 (b) and the nucleotide sequence of the 5' region of the *pdc1* gene in pT7pdc1-B1 (c). The ribosome-binding site (RBS) and the synthetic oligonucleotide sequence are underlined. MC site, multiple cloning site.

PDC in *E. coli*, derivatives of the plasmid pT7-7 were transformed into *E. coli* K38 containing pGP1-2. These cells were grown in T7 enrichment broth (2%, w/v, bacto-tryptone, 1%, w/v, yeast extract, 0.5% NaCl, 0.2% glycerol, 50 mM-potassium phosphate, pH 7.2) under ampicillin and kanamycin selection. Cells were grown at 30 °C to an OD₅₉₀ of 1.5, then heated to 42 °C for 30 min to induce the synthesis of T7 RNA polymerase. The temperature was then lowered to 30 °C and the cells incubated for a further 4 h to allow transcription from the T7 promoter in pT7-7 constructs and the consequent synthesis of pyruvate decarboxylase. The cells were collected by centrifugation at 4500 *g* for 10 min at 4 °C and then lysed in buffer containing 30 mM-K₂HPO₄, 2 mM-MgCl₂, 0.1% Nonidet P-40, 10 mM-2-mercaptoethanol, 0.1 mM-ThDP, 0.15 mg lysozyme ml⁻¹ and 2 µg DNAase I ml⁻¹. Lysates were centrifuged to remove cell debris, and then assayed for PDC activity or denatured at 95 °C for 3 min in cracking buffer (60 mM-Tris/HCl, pH 6.8, 1%, v/v, 2-mercaptoethanol, 10%, v/v, glycerol, 0.01% bromophenol blue). Proteins were separated by 12.5% (w/v) SDS-PAGE. Each lane contained 50 µg protein as determined using a Bicinchoninic Acid Protein Assay Kit (Sigma). Bands were visualized with Coomassie brilliant blue R250.

DNA sequencing. The sequence of the modified PDC gene, after insertion of the synthetic oligonucleotides, was confirmed by cloning appropriate restriction fragments into M13mp18/mp19 and sequencing using a T7 DNA Polymerase Sequencing Kit (Pharmacia-LKB).

Production of anti-yeast PDC antibodies. Antibodies against PDC were raised by injecting rabbits with 500 µg of a commercial preparation (Sigma) in complete Freund's adjuvant, followed by further injections of 250 µg enzyme in incomplete adjuvant every 4–6 weeks until reasonable titres were obtained. Serum was used without further purification for Western blot analyses.

Western blot analysis. Whole-cell extracts were separated by SDS-PAGE as above, then electroblotted onto nitrocellulose membranes using the carbonate buffer system described by Dunn (1986). Any

remaining protein binding sites on the membranes were blocked with Tris-buffered saline-(TBS) Tween buffer (150 mM-NaCl, 10 mM-Tris/HCl, 0.1% Tween 20, pH 7.4) containing 0.25% gelatin. After three 5 min washes in TBS-Tween, the filters were incubated for 2 h in rabbit anti-yeast PDC antiserum diluted 1:1000 in TBS-Tween containing 0.25% gelatin. The membranes were washed three times in TBS-Tween followed by a 1 h incubation in horseradish-peroxidase-linked goat anti-rabbit antibody diluted 1:1000 in TBS-Tween containing 5% (w/v) skimmed milk powder. After several brief washes in TBS-Tween, the filters were incubated in ECL chemiluminescent detection reagent for 1 min, drained, covered in plasticwrap, and exposed to Fuji RX100 film for 2 min at room temperature.

Determination of enzyme activity. PDC activity was determined in the soluble fraction of the *E. coli* lysates using 5 mM-pyruvate, 5 mM-MgCl₂, 0.1 mM-ThDP, 10 units alcohol dehydrogenase ml⁻¹, 0.15 mM-NADH, in 50 mM-MES buffer (pH 6.5) by measuring the conversion of NADH to NAD⁺ at 340 nm. One unit of activity is defined as the amount of activity required for the conversion of 1 µmol of NADH to NAD⁺ per min. This assay system does not distinguish PDC from lactate dehydrogenase. The observed activity was, therefore, corrected for controls performed in an identical manner but omitting alcohol dehydrogenase.

Results and Discussion

Construction of a yeast PDC expression system

The yeast *pdcl* gene was initially cloned into the multiple cloning site vector pUC18 as a 4.2 kb *Hind*III fragment from YEpl3-PDC1 which, although under *lac* promoter control, resulted in low levels of recombinant protein

Fig. 2. Expression of the cloned yeast *pdcl* gene in *E. coli*. Crude extracts from uninduced (30 °C) (–) and induced (42 °C) (+) cells containing the plasmids pT7*pdcl*-B1, pT7*pdcl*-A or the control pT7-7 were displayed on SDS-polyacrylamide gels and stained with Coomassie blue as described in Methods. The arrowhead shows the position of PDC. Partially purified yeast PDC was purchased from Sigma and included as a standard. Only the relevant portion of the gel (corresponding to a molecular mass range of approximately 30–100 kDa) is shown.

Fig. 3. Western blot analysis of yeast PDC expressed in *E. coli*. Lane designations are as for Fig. 2. The upper band of the doublet in the yeast PDC standard track appears to be due to a contaminant in the commercial preparation.

expression. The same fragment was then cloned into the plasmid pT7-7 (Tabor & Richardson, 1985) which placed it under the control of the strong T7 ϕ 10 promoter (pT7pdc1-A, Fig. 1), but again protein expression was poor. This low level of expression was thought to be the result of the distance of the *pdc1* initiation codon from the T7 promoter and the absence of an adjacent ribosome-binding sequence, since 1.2 kb of non-coding sequence separated the gene from the promoter in this construction. We then sought to improve expression by constructing a plasmid in which the upstream non-coding sequence was removed, and the *pdc1* gene was placed at the ϕ 10 translational start, within 9 bases of the ϕ 10 ribosome-binding sequence. The ϕ 10 initiation codon is contained within an *Nde*I site (CA/TATG), allowing coding regions with an *Nde*I site to be positioned in frame at the start codon. As the ATG of the *pdc1* gene was not contained within an *Nde*I site, an adapter was designed to create this restriction site and reform the 5' end of the gene:

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5' TATGCTGAAATTACTTTGGGTAAATATTTGTT 3'
3' ACAGACTTTAATGAAACCCATTTATAACAAGC5'

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This adapter had single stranded overhangs to facilitate cloning into *Nde*I and *Bst*BI sites. The oligonucleotides were annealed, ligated, then digested with *Bst*BI to yield double stranded dimers of 66 bp with *Bst*BI ends and an internal *Nde*I site. This process was monitored by (γ - 32 P)ATP end-labelling of the oligonucleotides.

*Bst*BI digestion of YEp13-PDC1 removed 1.2 kb of non-coding region upstream of the gene as well as the first 32 nucleotides from the 5'-end of the *pdc1* coding region. The 66-mer adapter was ligated to the 8.4 kb

*Bst*BI YEp13-PDC1 fragment and recircularized to form YEp8.4 (Fig. 1b). After *Nde*I digestion, the fragment from this plasmid was inserted into the *Nde*I site of pT7-7 to form pT7pdc1-B2 and the 3.2 kb of non-coding region 3' to the gene was removed by *Hind*III digestion [pT7pdc1-B1, Fig. 1(b)].

Restoration of the 5'-end of the *pdc1* gene and its positioning at the ϕ 10 translational start site was confirmed by DNA sequencing of the *Xba*I/*Hind*III fragment from pT7pdc1-B1 (Fig. 1c). The ribosome binding site and the *Nde*I restriction site of the plasmid are indicated.

Expression of PDC proteins in *E. coli*

Both pT7pdc1-A and pT7pdc1-B1 introduced into *E. coli* cells directed the synthesis of protein when cultured under inducing conditions. Analysis of whole-cell lysates from uninduced and induced cultures by Coomassie-blue-stained SDS-PAGE gels (Fig. 2) demonstrated the production by *E. coli* (pT7pdc1-B1) of significant amounts of a band which comigrated with purified yeast PDC, with an estimated subunit molecular mass of 62 kDa. A comparison of soluble and insoluble fractions showed that more than half of the PDC protein was in an insoluble form.

The identity of the recombinant protein was confirmed by comparison with the native yeast PDC using Western blot analysis. Fig. 3 clearly shows that the cloned gene product is recognized by antibody raised against commercial yeast PDC.

Enzyme activity in the soluble fraction was determined under uninduced and induced conditions as

Table 1. PDC activity in soluble fraction of *E. coli* lysates from cells transformed with various constructs

Construct	Activity [units (g cells) ⁻¹]	
	Uninduced	Induced
pT7-7	15	5
pT7 <i>pdcl</i> -A	7	87
pT7 <i>pdcl</i> -B1	45	249

described in Methods, and the results are shown in Table 1. These results suggest that the positioning of the *pdcl* gene directly downstream of the highly efficient ribosome-binding site enhanced its expression as suggested by Rosenberg *et al.* (1987) with regard to the efficiency of translation from genes expressed in this system. It is of interest that the pT7*pdcl*-B1 construct showed some PDC activity (18%) even under non-inducing conditions. Presumably the λ cI857 repressor has not blocked the P_L promoter tightly enough to totally inhibit synthesis of the T7 RNA polymerase, resulting in a background level of PDC expression in *E. coli* cells containing this construction.

The *pdcl* gene product, at the level of expression achieved here, did not appear to be detrimental to the *E. coli* host. A comparison was also made of the growth rate of *E. coli* containing either the control vector or the PDC construct under inducing conditions, in LB with and without 2% (w/v) glucose. Similar growth rates were achieved under all conditions.

Under anaerobic conditions the main competitors for pyruvate are the native *E. coli* enzymes pyruvate formate lyase and lactate dehydrogenase. The K_m for pyruvate for these two enzymes is 2.05 mM (Knappe *et al.*, 1974) and 4.4 mM (Tarmy & Kaplan, 1968), respectively. This is higher than the K_m for pyruvate of yeast (1.1–1.3 mM, Boiteux & Hess, 1970) or *Zymomonas mobilis* (0.9–1.0 mM, Thomas *et al.*, 1990) PDC. The higher affinity of PDC for its substrate explains the results of Brau & Sahm (1986) who found a reduction in the formation of lactate, formate and acetate with a corresponding increase in ethanol production in *E. coli* expressing the *Z. mobilis pdc* gene. A similar effect on the metabolism and by-product formation in *E. coli* may be occurring on expression of the yeast *pdcl* gene.

In this paper we have described an efficient expression system for the production of yeast PDC in *E. coli*. The results show that, whatever the origin and role of the different PDC subunits in yeast, the *pdcl* gene alone is

capable of yielding an active enzyme. We plan to investigate further the domain involved in the binding of the co-factor ThDP using site-directed mutagenesis to change specific nucleotides within a structural motif common to many ThDP-dependent enzymes (Hawkins *et al.*, 1989). The T7 expression system described herein will enable the rapid analysis of such alterations and, hence, definition of specific amino acids essential for catalysis, eventually leading to a more complete description of structure–function relationships in this protein.

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