Construction and expression of an ethanol production operon in Gram-positive bacteria

Lee A. Talarico, Malgorzata A. Gil, Lorraine P. Yomano, Lonnie O. Ingram and Julie A. Maupin-Furlow

Correspondence

Julie A. Maupin-Furlow
jmaupin@ufl.edu

Department of Microbiology and Cell Science, University of Florida, Gainesville, FL 32611-0700, USA

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Pyruvate decarboxylase (PDC), an enzyme central to homoethanol fermentation, catalyses the non-oxidative decarboxylation of pyruvate to acetaldehyde with release of carbon dioxide. PDC enzymes from diverse organisms have different kinetic properties, thermal stability and codon usage that are likely to offer unique advantages for the development of desirable Gram-positive biocatalysts for use in the ethanol industry. To examine this further, *pdc* genes from bacteria to yeast were expressed in the Gram-positive host *Bacillus megaterium*. The PDC activity and protein levels were determined for each strain. In addition, the levels of *pdc*-specific mRNA transcripts and stability of recombinant proteins were assessed. From this analysis, the *pdc* gene of Gram-positive *Sarcina ventriculi* was found to be the most advantageous for engineering high-level synthesis of PDC in a Gram-positive host. This gene was thus selected for transcriptional coupling to the alcohol dehydrogenase gene (*adh*) of *Geobacillus stearothermophilus*. The resulting Gram-positive ethanol production operon was expressed at high levels in *B. megaterium*. Extracts from this recombinant were shown to catalyse the production of ethanol from pyruvate.

INTRODUCTION

Pyruvate decarboxylase (PDC, EC 4.1.1.1) is central to homoethanol fermentation and catalyses the non-oxidative decarboxylation of pyruvate to acetaldehyde with release of carbon dioxide. Acetaldehyde generated from this reaction is reduced to ethanol by alcohol dehydrogenase (ADH, EC 1.1.1.1). These two enzymes (PDC and ADH) are sufficient to convert intracellular pools of pyruvate and NADH to ethanol. Currently, the portable production of ethanol (PET) operon used to engineer this pathway in Gram-negative bacteria consists of the *pdc* and *adh* genes from the Gram-negative *Zymomonas mobilis* (*Zm*) (Ingram *et al.*, 1987, 1998; Ohta *et al.*, 1991a, b). While this strategy has been highly successful in engineering mesophilic Gram-negative bacteria (Ingram *et al.*, 1998, 1999), advanced biocatalysts that withstand low pH, high temperature, high salt, high sugar, high ethanol and various other harsh conditions offer an opportunity to improve the commercial competitiveness of ethanol production (Ingram *et al.*, 1999; Dien *et al.*, 2003). Many of these qualities can be found in Gram-positive bacteria (Gold *et al.*, 1992). Unfortunately, modifying Gram-positive bacteria for ethanol production has had limited success. Ethanol production appears to be limited by the relatively low levels of activities obtained when the *Z. mobilis* *adh* and *pdc* genes are engineered into

lactic acid bacteria and *Bacillus* species (Barbosa & Ingram, 1994; Gold *et al.*, 1996; Hillman *et al.*, 1996; Nichols *et al.*, 2003).

Engineering a Gram-positive host for robust ethanol production has been limited, in part, by the availability of a suitable pool of *pdc* genes. Although *pdc* genes are widespread in plants, yeast and fungi, they are absent in animals and rare in bacteria (König, 1998). The *Z. mobilis* *pdc* gene has been the workhorse for Gram-negative biocatalysts. Recently, however, the gene sequences encoding functional PDCs from three additional bacteria have become available, including that of the Gram-positive *Sarcina ventriculi* (*Sv*), and Gram-negative *Acetobacter pasteurianus* and *Zymobacter palmae* (Talarico *et al.*, 2001; Raj *et al.*, 2001, 2002). Expression of these genes in recombinant *Escherichia coli* results in different levels of PDC protein; in particular, the levels of *S. ventriculi* PDC are reduced in comparison to the other PDCs (Talarico *et al.*, 2001; Raj *et al.*, 2001, 2002). This deficiency in *Sv*PDC protein is remedied by the inclusion of accessory tRNA genes (Talarico *et al.*, 2001; Raj *et al.*, 2002). Thus, the source of a *pdc* gene influences the levels of protein that are obtained in recombinant *E. coli*, which suggests that codon usage needs to be considered when engineering high-level PDC synthesis in Gram-positive hosts.

In this study, we have compared the expression of four different *pdc* genes in a recombinant Gram-positive

Abbreviations: ADH, alcohol dehydrogenase; PDC, pyruvate decarboxylase; PET, production of ethanol.

bacterium, *Bacillus megaterium*. Expression was highest for the *pdg* from *S. ventriculi*. By coupling the gene to *Geobacillus stearothermophilus adh* within a single operon, high levels of both enzymes were produced in active forms. Extracts from these cells converted pyruvate to ethanol, demonstrating the engineering of a functional pathway in a Gram-positive host.

METHODS

Materials. Biochemicals were purchased from Sigma. Other organic and inorganic analytical-grade chemicals were from Fisher Scientific. Restriction enzymes were from New England Biolabs. Oligonucleotides were from Qiagen Operon and Integrated DNA Technologies. *Bacillus megaterium* Protein Expression System was from MoBiTec. RNase-free water and solutions were from Ambion.

Bacterial strains and media. Strains and plasmids used in this study are listed in Table 1. *E. coli* DH5 α was used for routine recombinant DNA experiments. *B. megaterium* WH320 and derivatives were used for analysis of recombinant PDC protein and *pdg*-specific mRNA. Strains were grown in Luria-Bertani (LB) medium, unless otherwise indicated. Medium was supplemented with 2% (w/v) glucose and antibiotics (100 mg ampicillin l⁻¹, 30 mg kanamycin l⁻¹ or 15 mg tetracycline l⁻¹) as needed. All strains were grown at 37 °C and 200 r.p.m. Isolated colonies of *B. megaterium* were grown overnight in liquid medium and used as a 1.0% (v/v) inoculum into fresh medium, unless otherwise indicated.

Protoplast formation and transformation of *B. megaterium*. *B. megaterium* WH320 was grown to exponential phase to an OD₆₀₀ of 0.6 (1 cm path length), and protoplasts were generated according to the method of Puyet *et al.* (1987), with the following modifications. Cells were treated with lysozyme (10 µg ml⁻¹) for 20 min. Protoplasts were stored at -70 °C and transformed according to MoBiTec.

DNA isolation and cloning. Plasmid DNA was isolated and purified from *E. coli* using the QIAprep Spin Miniprep Kit (Qiagen). DNA was eluted from 0.8% (w/v) SeaKem GTG agarose (Cambrex Corp.) gels using the QIAquick gel elution kit (Qiagen). Similar strategies were used to generate the *B. megaterium* expression plasmids pJAM420, pJAM430, pJAM432 and pJAM435 (Fig. 1; Table 1). As an example, plasmid pJAM420 was constructed as follows. A *Bsp*HI-*Xho*I DNA fragment with the complete *pdg* gene of *S. ventriculi* strain Goodsir (ATCC 55887) was generated by PCR amplification from plasmid pJAM410 and cloned into the *Nco*I and *Xho*I sites of plasmid pET21d (Talarico *et al.*, 2001). The 1.9 kb *Xba*I-*Bsp*EI DNA fragment of the resulting pJAM419 was ligated into the *Spe*I and *Xma*I sites of plasmid pWH1520. This resulted in a pWH1520-based expression plasmid (pJAM420) that carried the *pdg* gene, along with the Shine-Dalgarno site and T7 transcriptional terminator of the original pET21d vector. The *pdg* gene was positioned to interrupt the *B. megaterium xylA* gene of plasmid pWH1520 and to generate a stop codon within *xylA* (*xylA'*). The Shine-Dalgarno site, originally from pET21d and upstream of the inserted *pdg*, was positioned directly downstream of the *xylA'* stop codon. This allowed for translational coupling of *xylA'* and *pdg*, in which the ribosomes would terminate at the stop codon of *xylA'* and reinitiate at the start codon of *pdg*. Sources of the *pdg* genes of plasmids pJAM430, pJAM432 and pJAM435 originated from *A. pasteurianus* strain NCIB 8619 (ATCC 12874) (Raj *et al.*, 2001), *Z. mobilis* strain CP4 (Conway *et al.*, 1987) and *PDC1* of *Saccharomyces cerevisiae* (Wei *et al.*, 2002) (Table 1). The fidelity of all cloned PCR products was confirmed by DNA sequencing by the Sanger dideoxy method

(Sanger *et al.*, 1977) using a LICOR sequencer (DNA Sequencing Facility, Department of Microbiology and Cell Science, University of Florida).

A Gram-positive PET operon was constructed as follows. The *Hind*III-*Mfe*I fragment of pLO11742 containing the *adh* gene from *G. stearothermophilus* (*Gsadh*) strain XL-65-6 was blunt-end ligated into the *Blp*I site of pJAM420 using Vent DNA Polymerase (New England Biolabs) (Table 1). This resulted in plasmid pJAM423, which was designed to couple the translation of *Svpdc* to *Gsadh*, with the *xylA'* promoter upstream of *Svpdc* and the T7 terminator following *Gsadh*.

Recombinant protein synthesis, protein electrophoresis and enzyme assays. PDC and ADH proteins were synthesized in recombinant *B. megaterium* using the expression plasmids described above. Cells were grown to an OD₆₀₀ of 0.3 (exponential phase), and transcription was induced from the *xylA'* promoter by addition of 0.5% (w/v) xylose for 3 h. Cells were harvested by centrifugation (5000 g, 10 min, 4 °C) and stored at -80 °C. For PDC activity assays, cell pellets (0.5 g) were thawed in 6 vols (wet w/v) of 50 mM sodium phosphate buffer, pH 6.5, containing 1 mM MgSO₄ and 1 mM thiamine pyrophosphate (TPP) (Buffer A). Cells were passed through a French pressure cell at 20 000 p.s.i. (138 MPa). For ethanol production and ADH activity assays, cells (from 100 ml culture) were resuspended in 3 ml Buffer A and lysed by sonication. Debris was removed by centrifugation (16 000 g, 20 min, 4 °C).

PDC activity was assayed by monitoring the pyruvic acid-dependent oxidation of NADH with ADH as a coupling enzyme at pH 6.5, as previously described (Conway *et al.*, 1987). Cell lysate (10 µl) was added to a final volume of 1 ml containing 0.15 mM NADH, 0.1 mM TPP, 50 mM pyruvate and 10 U ADH in 50 mM K-MES buffer, pH 6.5, with 5 mM MgCl₂. Control reactions without added ADH were performed to correct for NADH-oxidizing enzymes, such as lactate dehydrogenase, in cell lysate. ADH was assayed by monitoring the ethanol-dependent reduction of NAD⁺, as described elsewhere (Dekker, 1977). Cell lysate (10–30 µl) was added to a final volume of 1 ml containing 333 mM ethanol and 8.3 mM NAD⁺ in 50 mM sodium phosphate buffer, pH 6.5. NADH oxidation/NAD⁺ reduction was monitored in a 1 cm path length cuvette at 340 nm over a 5 min period using a SmartSpec 300 spectrophotometer (Bio-Rad). One unit of PDC/ADH activity is defined as the generation of 1 µmol NAD⁺/NADH per min under the conditions specified. Protein concentration was determined using Bio-Rad Protein assay dye with BSA as standard.

Ethanol production was monitored by gas chromatography as previously described (Beall *et al.*, 1991). Cell lysate (1.25 mg protein) was added to a final volume of 1 ml containing 40 mM NADH and carbon substrate in Buffer A and incubated in screw cap tubes (37 °C, 15 h). Carbon substrates included 150 mM pyruvate, 2% (w/v) xylose and 2% (w/v) glucose. The concentration of ethanol in aliquots of the reactions was determined by comparison to ethanol standards (0.1–10 g l⁻¹) with 1% (v/v) 1-propanol as the internal standard. Formaldehyde was included at 0.05% (v/v) to minimize bacterial growth in the standards.

Protein molecular masses were analysed by reducing and denaturing SDS-PAGE using 12% polyacrylamide gels that were stained by heating in the presence of Coomassie blue R-250 (Wong, 2000). Molecular mass standards were phosphorylase *b* (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa).

RNA isolation and quantification. *B. megaterium* strains expressing the various *pdg* genes were grown in triplicate to an OD₆₀₀ of 0.3, and recombinant transcription was induced for 15 min as described above. Total RNA was isolated using the RNeasy miniprep kit. Samples were treated with lysozyme and On-column DNase, as

Table 1. Strains, plasmids and primers

Strain, plasmid or PCR primer	Phenotype, genotype or primer sequence	Source
Strains		
<i>E. coli</i> DH5 α	F ⁻ <i>recA1 endA1 hsdR17</i> (r _k ⁻ m _k ⁺) <i>supE44 thi-1 gyrA relA1</i>	Gibco BRL
<i>B. megaterium</i> WH320	DSM319 <i>lac</i> ⁻ <i>xyl</i> ⁺	MoBiTec
Plasmids		
pET21d	Ap ^r ; <i>E. coli</i> expression vector	Novagen
pET24b	Km ^r ; <i>E. coli</i> expression vector	Novagen
pLOI276	Ap ^r ; complete <i>pdg</i> gene of <i>Z. mobilis</i> CP4 in plasmid pUC18	Conway <i>et al.</i> (1987)
pLOI1742	Ap ^r ; complete <i>adh</i> gene of <i>G. stearothermophilus</i> XL-65-6 in plasmid pUC18	L. P. Yomano and L. O. Ingram, unpublished results
pScPDC1	Ap ^r ; complete PDC1 gene of <i>Sac. cerevisiae</i> in pET22b	Wei <i>et al.</i> (2002)
pWH1520	Ap ^r Tc ^r ; <i>E. coli</i> – <i>B. megaterium</i> shuttle vector; designed for xylose-inducible expression of cloned genes in <i>B. megaterium</i>	Rygas & Hillen (1991)
pJAM304	Ap ^r ; pLITMUS28 derivative carries complete <i>pdg</i> gene of <i>A. pasteurianus</i> NCIB 8618 (ATCC 12874)	Raj <i>et al.</i> (2001)
pJAM419	Ap ^r ; pET21d derivative carries complete <i>pdg</i> gene of <i>S. ventriculi</i> Goodsir (ATCC 55887)	Talarico <i>et al.</i> (2001)
pJAM420	Ap ^r Tc ^r ; 1.9 kb <i>BspEI</i> – <i>XbaI</i> fragment of pJAM419 ligated with the <i>SpeI</i> – <i>XmaI</i> fragment of pWH1520; used for synthesis of SvPDC in <i>B. megaterium</i>	This study
pJAM423	Ap ^r Tc ^r ; 1.8 kb <i>HindIII</i> – <i>MfeI</i> fragment of pLOI1742 blunt-end ligated into the <i>BlnI</i> site of pJAM420; carries Gram-positive ethanol production operon; used for xylose-inducible synthesis of SvPDC and GsADH in <i>B. megaterium</i>	This study
pJAM429	Km ^r ; 1.9 kb fragment generated by PCR amplification using pJAM304 as template, For 5'-GGCC ATATGTATCTTGCAGA -ACG-3', Rev 5'-ATTAT CTCGAGTCAGGCCAGAGTGG -3' (<i>NdeI</i> and <i>XhoI</i> sites in bold); <i>NdeI</i> – <i>XhoI</i> fragment of PCR product ligated into <i>NdeI</i> and <i>XhoI</i> sites of pET24b; carries complete <i>Appdc</i> gene	This study
pJAM430	Ap ^r Tc ^r ; 1.9 kb <i>BspEI</i> – <i>XbaI</i> fragment of pJAM429 ligated into <i>SpeI</i> and <i>XmaI</i> sites of pWH1520; used for synthesis of ApPDC in <i>B. megaterium</i>	This study
pJAM431	Ap ^r ; 1.7 kb fragment generated by PCR amplification using pLOI276 as template, For oligo 5'-GGC CTCATGAGT TATACT-GTCGG-3', Rev oligo 5'-GATTT CTCGAGCTAGAGGAGC -TTG-3' (<i>BspHI</i> and <i>XhoI</i> sites in bold); <i>BspHI</i> – <i>XhoI</i> fragment of PCR product ligated into <i>NcoI</i> and <i>XhoI</i> sites of pET21d; carries complete <i>Zmpdc</i> gene	This study
pJAM432	Ap ^r Tc ^r ; 2.1 kb <i>XbaI</i> – <i>NgoMIV</i> fragment of pJAM431 ligated into <i>SpeI</i> and <i>XmaI</i> sites of pWH1520; used for synthesis of <i>ZmPDC</i> in <i>B. megaterium</i>	This study
pJAM435	Ap ^r Tc ^r ; 1.9 kb <i>BspEI</i> – <i>XbaI</i> fragment of pScPDC1 ligated into <i>SpeI</i> and <i>XmaI</i> sites of pWH1520; used for synthesis of ScPDC1 in <i>B. megaterium</i>	This study
RT primers*		
<i>Svpdc</i> For	5'-AATCGAAATGAAACCGCTAA-3'	This study
<i>Svpdc</i> Rev	5'-TGAGCTTGCAACCATTTCTTTTA-3'	This study
<i>Appdc</i> For	5'-CGCGCCCAACAGCAATGATCA-3'	This study
<i>Appdc</i> Rev	5'-GGGCGGAGTGAGCGTCGGTAAT-3'	This study
<i>Zmpdc</i> For	5'-TGGCGAACTGGCAGAAGCTATCA-3'	This study
<i>Zmpdc</i> Rev	5'-CGCGCTTACCCCATTTGACCA-3'	This study
<i>ScPDC1</i> For	5'-CACGGTCCAAAGGCTCAATACAA-3'	This study
<i>ScPDC1</i> Rev	5'-CCGGTGGTAGCGACTCTGTGG-3'	This study

*Abbreviations: RT, reverse transcriptase; For, forward primer; Rev, reverse primer; Sv, *S. ventriculi*; Ap, *A. pasteurianus*; Zm, *Z. mobilis*; Sc, *Sac. cerevisiae*.

Fig. 1. Strategy used to construct plasmids for expression of *S. ventriculi pdc* in recombinant *B. megaterium*. A similar approach was used to generate plasmids for expression of *Z. mobilis*, *A. pasteurianus*, and *Sac. cerevisiae pdc* genes in *B. megaterium*. Abbreviations: Ap^r, ampicillin resistance; Tc^r, tetracycline resistance; pT7 and T7T, T7 polymerase promoter and terminator, respectively; lacI, gene encoding lactose operon repressor; lacO, lactose operon operator; f1ori, filamentous bacteriophage f1 origin of replication; pBR ori, *E. coli* plasmid pBR322 origin of replication; pBC16 ori, *Bacillus cereus* plasmid pBC16 origin of replication; P_{xyl}, xylA promoter; xylR, gene encoding xylose repressor.

recommended by the supplier (Qiagen). Removal of DNA was confirmed by PCR using Jumpstart *Taq* Readymix in the absence of reverse transcriptase (Sigma). Quality and quantity of RNA were assessed by 0.8% (w/v) agarose gel electrophoresis and absorbance at 260 nm, respectively.

Total RNA (100 pg) served as template with the primers listed in Table 1 for quantitative real-time reverse transcriptase PCR (RT-PCR). Transcripts synthesized *in vitro* were used to generate standard curves of absolute copy number for each RT-PCR experiment. *In vitro* transcripts were generated from the *E. coli* *pd*c-expression vectors (pJAM419, pJAM429, pJAM431 and pScPDC1) with the MAXIscript T7 *in vitro* transcription kit (Ambion). Nuc-Away spin columns (Ambion) were used to remove unincorporated nucleotides. RT-PCR was performed using the QuantiTect SYBR Green 1-step RT-PCR kit (Qiagen) with an Icyler thermal cycler (Bio-Rad). Data with PCR efficiencies of 90–100% were analysed using the Icyler software version 3.0.6070 (Bio-Rad) and Microsoft Excel.

Pulse-chase. Recombinant *B. megaterium* strains expressing *Svpdc* and *Zmpdc* genes were grown in minimal medium (10 g sucrose, 2.5 g K_2HPO_4 , 2.5 g KH_2PO_4 , 1.0 g $(NH_4)_2HPO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 10 mg $FeSO_4 \cdot 7H_2O$, 7 mg $MnSO_4 \cdot H_2O$ in 985 ml deionized H_2O at pH 7.0) supplemented with tetracycline (MM Tet) using a 1% (v/v) inoculum. Cells were grown to an OD_{600} of 0.3 and recombinant gene transcription was induced for 15 min with 0.5% xylose. Cells were harvested by centrifugation (5000 g, 10 min, 25 °C) and resuspended in 2 ml MM Tet supplemented with 0.5% xylose and 50 $\mu Ci\ ml^{-1}$ ($1.85\ MBq\ ml^{-1}$) L-[^{35}S]methionine (DuPont-NEN). Cells were incubated for 15 min (37 °C, 200 r.p.m.) and harvested as above. Cell pellets were resuspended in MM Tet supplemented with 0.5% xylose and 5 mM L-methionine, with 15 mg chloramphenicol l^{-1} , and incubated (37 °C, 200 r.p.m.). Aliquots (0.5 ml) were withdrawn after 5, 10, 15, 30, 60, 90, 120, 150 and 180 min incubation and immediately added to 50 μl stop solution (75 mM NaCl, 25 mM EDTA, 20 mM Tris, pH 7.5, and 1 mg chloramphenicol ml^{-1}). Cells were incubated on ice (5 min), harvested at 16000 g (10 min, 25 °C) and stored at –80 °C. Cell pellets were subjected to three cycles of freeze–thaw (–80 °C and 0 °C) to weaken the cell membrane. Pellets were resuspended in lysis solution (75 mM NaCl, 25 mM EDTA, 20 mM Tris, pH 7.5, and 0.2 mg lysozyme ml^{-1}) and incubated (25 °C, 15 min). Samples (0.02 OD_{600} units per lane) were boiled (20 min) in SDS-PAGE loading dye (Bio-Rad) and separated by SDS-PAGE. Gels were dried and exposed to X-ray film. A VersaDoc model 1000 with Quantity One Software (Bio-Rad) was used for densitometric readings.

RESULTS

Construction of Gram-positive PDC expression plasmids

Our previous work suggested that codon usage influences the levels of PDC obtained in recombinant *E. coli* (Talarico *et al.*, 2001). To determine if this was the factor responsible for limiting PDC expression in Gram-positive bacteria, four PDC genes with different G+C content and codon usage were chosen for expression analysis. These included the *S. ventriculi* *pd*c gene (*Svpdc*), which is poorly expressed in *E. coli* and is the only known PDC from a Gram-positive bacterium. In addition, the *Sac. cerevisiae* PDC1 (*ScPDC1*) was analysed based on its close relationship to *Svpdc* (Talarico *et al.*, 2001; Raj *et al.*, 2001) and its use in

Fig. 2. PDC proteins synthesized in recombinant *B. megaterium*. After 3 h induction with 0.5% xylose, cell lysate (6 μg) was separated by reducing SDS-PAGE and stained with Coomassie blue R-250. Lane 1, molecular mass standards (5 μg). Lanes 2–6, cell lysate of *B. megaterium* WH320 transformed with: lane 2, plasmid pWH1520 (vector alone); lane 3, pJAM420 (carries *Svpdc*); lane 4, pJAM430 (carries *Appdc*); lane 5, pJAM432 (carries *Zmpdc*); lane 6, pJAM435 (carries *ScPDC1*). *SvpDC* (lane 3), *ZmpPDC* (lane 5) and *ScPDC1* (lane 6) proteins are indicated by arrowheads.

corn-to-ethanol production (Dien *et al.*, 2002). The *A. pasteurianus* *pd*c (*Appdc*) (Raj *et al.*, 2001) and *Z. mobilis* *pd*c (*Zmpdc*) were also included (Hoppner & Doelle, 1983; Bräü & Sahm, 1986; Bringer-Meyer *et al.*, 1986; Conway *et al.*, 1987; Neale *et al.*, 1987). The latter two genes are from Gram-negative bacteria and have high levels of expression and activity in Gram-negative hosts (Raj *et al.*, 2001, 2002).

To construct the expression plasmids, the *pd*c genes were initially cloned into pET vectors (Fig. 1, Table 1). DNA fragments containing each *pd*c gene and the Shine–Dalgarno and T7 terminator from the pET vector were cloned into the *B. megaterium* expression plasmid pWH1520. This generated a truncation of the *xylA* gene (*xylA'*), which encodes xylose isomerase and allowed for induction of *pd*c expression by xylose in *B. megaterium*.

Expression of PDC in recombinant *B. megaterium*

After induction of *pd*c expression in recombinant *B. megaterium*, the levels of PDC protein were estimated by Coomassie blue R-250-stained SDS-PAGE gels (Fig. 2). High levels of *SvpDC* were detected, and were estimated to account for 5% of protein in clarified cell lysate. In contrast, only low levels of the *ZmpPDC*, *ApPDC* and *ScPDC1* proteins were detected. To determine if the PDC proteins were

Table 2. PDC activity of *B. megaterium* strains transformed with *pdc* expression plasmids

Expression plasmid	Recombinant protein*	Specific activity [U (mg protein) ⁻¹] [†]	SD	Purified specific activity [U (mg protein) ⁻¹]
pWH1520	None	0.29	0.05	NA
pJAM420	<i>SvPDC</i>	5.29	0.23	65‡–103§
pJAM430	<i>ApPDC</i>	0.14	0.06	120 –134.2¶
pJAM432	<i>ZmPDC</i>	1.11	0.03	51.9#
pJAM435	<i>ScPDC1</i>	0.53	0.04	92**

*Abbreviations: *Sv*, *S. ventriculi*; *Ap*, *A. pasteurianus*; *Zm*, *Z. mobilis*; *Sc*, *Sac. cerevisiae*.

[†]PDC specific activity, determined for cell lysate using the ADH coupled assay.

References: ‡Talarico *et al.* (2001); §Lowe & Zeikus (1992); ||Neale *et al.* (1987); ¶Hoppner & Doelle (1983); #Ullrich *et al.* (1966); **Raj *et al.* (2002); NA, not applicable.

produced in an active form, the cell lysate of the recombinant *B. megaterium* strains was assayed for PDC activity (Table 2). Of the strains examined, those cells expressing *Svpdc* had the highest activity with 5.29 U (mg protein)⁻¹, while those encoding the *Zmpdc* and *ScPDC1* genes had five- to tenfold lower activity. No PDC activity was detected for the strain which encoded the *Appdc* gene. Thus, most if not all of the *SvPDC* in the cell lysate of recombinant *B. megaterium* was active, based on the specific activity of purified *SvPDC*, which is estimated to be 65–103 U (mg protein)⁻¹ (Lowe & Zeikus, 1992; Talarico *et al.*, 2001). The results demonstrate that *SvPDC* is not only produced in very high quantity, but is produced in an active form within the *B. megaterium* host cell.

It was previously reported that production of *SvPDC* in recombinant *E. coli* was limited to specific activities in cell lysate of 0.16 U (mg protein)⁻¹, even when using a BL21-CodonPlus-RIL strain augmented with accessory tRNAs for AUA and AGA codons (Talarico *et al.*, 2001). No tRNA augmentation was necessary for *SvPDC* production in recombinant *B. megaterium*, and yet there was a 33-fold increase in the specific activity in cell lysate compared to recombinant *E. coli*. This indicates that *B. megaterium* is a better host for the production of the *SvPDC* while it is suboptimal for the production of the Gram-negative PDCs *ZmPDC* and *ApPDC*, which are expressed more efficiently in *E. coli*. Previous studies have determined the specific activity of *ZmPDC* to be 6.2–8 U (mg protein)⁻¹ in cell lysate (Neale *et al.*, 1987; Raj *et al.*, 2002) when produced in recombinant *E. coli*, in contrast to the 1.1 U (mg protein)⁻¹ in *B. megaterium* for this study.

Analysis of PDC transcript levels

The factors responsible for low-level production of PDC protein in recombinant Gram-positive bacteria are unknown (Gold *et al.*, 1992, 1996; Barbosa *et al.*, 1994; Nichols *et al.*, 2003). In order to determine if transcript levels were limiting the production of PDC in Gram-positive hosts, we analysed the transcript levels specific for the various *pdc* genes when expressed in recombinant *B.*

megaterium. Total RNA was isolated and quantitative reverse transcriptase PCR was performed to determine if transcript levels correlated with PDC production. The transcript levels were similar for all four *pdc* genes, ranging from 12 to 24 % of total RNA, with the transcript for *Zmpdc* the lowest (12.2 ± 1.2 %) and *ScPDC1* the highest (24.5 ± 1.7 %). The levels of *Appdc*- and *Svpdc*-specific transcript were intermediate at 14.6 ± 2.1 and 21.6 ± 9.5 % of total RNA, respectively.

In contrast to *SvPDC* protein and its activity levels, which were at least fivefold higher than those of the other PDC proteins, there was not an abundance of *Svpdc* transcript compared to the other *pdc* transcripts. Thus, the *pdc*-specific mRNA levels did not correlate with the levels of PDC protein in the recombinant *B. megaterium* strains. These results indicate that the level of transcript is not the factor influencing protein levels of PDC in the cell. This is not unexpected, due to the use of the same inducible promoter, transcription terminator and vector for the construction of all four *pdc* gene expression plasmids. The reason the levels of *pdc*-specific transcript are so high for the four constructs may be due to the highly inducible *xylA* promoter used in this study. Alternatively, the method used to prepare total RNA may not completely release the rRNA from the thick cell wall of this Gram-positive bacterium.

PDC protein stability in recombinant *B. megaterium*

Gram-positive bacteria, particularly *Bacillus* species, are well known for an abundance of proteases (Wong, 1995). This is often a problem when producing heterologous proteins in these hosts (Wong *et al.*, 1994; Wong, 1995). To determine if protein degradation was responsible for limiting PDC production in *B. megaterium*, pulse-chase analysis was performed. The *SvPDC* and *ZmPDC* were chosen for analysis. After induction of *pdc* transcription (15 min), protein was labelled with L-[³⁵S]methionine (15 min) and chased with excess unlabelled L-methionine in the presence of the protein synthesis inhibitor chloramphenicol. This enabled the rate of protein degradation after induction of

Fig. 3. Induction of *S. ventriculi* PDC and *G. stearothermophilus* ADH in *B. megaterium*. Proteins were separated by reducing SDS-PAGE using 12% polyacrylamide gels and stained with Coomassie blue R-250. Lanes: 1, molecular mass standard (5 µg); 2, cell lysate (20 µg) of *B. megaterium* transformed with pWH1520 (vector alone) induced with xylose; 3, 4, cell lysate (20 µg) of *B. megaterium* WH320 transformed with pJAM423 (plasmid with Gram-positive PET operon) uninduced (lane 3) and xylose induced (lane 4).

pdg gene transcription to be monitored over a period of several hours. No significant degradation of either PDC protein was detected during the entire chase (data not shown). Thus, both of the recombinant PDCs examined (*SvPDC* and *ZmPDC*) were relatively stable, yet the amounts of the *SvPDC* protein and activity detected after 3 h induction were dramatically higher than those of the other PDCs (Fig. 1, Table 2). Protein degradation is, therefore, not a factor influencing the levels of active PDC protein in recombinant *B. megaterium*.

Construction of a portable ethanol production operon for Gram-positive bacteria

B. megaterium strain WH320 (the host used in this study) is capable of growth on xylose minimal medium. The strain also grows at temperatures up to 42 °C and at the relatively low pH of 5.0. Based on these characteristics, this strain appears to be a suitable candidate to perform preliminary tests on ethanol production with a portable pyruvate to ethanol (PET) operon, and may prove useful in large-scale ethanol production under acidic conditions. To construct a Gram-positive (G+) PET operon, the *adh* gene from *G. stearothermophilus* (*Gsadh*) was cloned behind the *Sypdc* gene in the *B. megaterium* pWH1520 expression vector. This vector was chosen based on successful use in the high-level synthesis of *SvPDC* (Fig. 2). The resulting plasmid pJAM423, which carried the G+ PET operon, was transformed into *B. megaterium*. After induction of the G+ PET operon with xylose, a considerable portion of the lysate of this strain was composed of the *SvPDC* and *GsADH* proteins (Fig. 3). Similar to *SvPDC*, the *GsADH* synthesized in this strain was active, with ADH activity levels of 1.8 U

(mg protein)⁻¹ in cell lysate. In contrast, no ADH activity was detected for the *B. megaterium* strain which carried the vector alone (pWH1520).

Based on these results, the *B. megaterium* strain which harboured the G+ PET operon (pJAM423) was further examined for its ability to produce ethanol during growth at low levels of xylose (0.5%). Approximately 20 mmol ethanol was produced per litre, 36% of the maximum theoretical yield.

The basis for such low yields was investigated by examining cell-free lysates of the *B. megaterium* strains with and without the G+ PET operon (i.e. pJAM423 versus pWH1520) in the presence of excess NADH. Although only trace levels of ethanol were observed with glucose and xylose, ethanol (72 mM) was produced from 150 mM pyruvate, a yield 11-fold that observed with the plasmid lacking ethanol genes (pWH1520).

These results demonstrate that the G+ PET operon of pJAM423 facilitates the production of high levels of *SvPDC* and *GsADH* in *B. megaterium* cells, and that these recombinant enzymes are fully functional in the conversion of pyruvate to ethanol. One explanation for the low levels of ethanol produced by whole cells of *B. megaterium* (pJAM423) is that the cytosolic pools of pyruvate may be limiting. Other metabolic pathways may compete for the available pyruvate and limit the ability of *SvPDC* to channel pyruvate ultimately to ethanol. This would be consistent with the differences in kinetic constants of *SvPDC* compared to other enzymes that utilize pyruvate. For example, *SvPDC* has a *K_M* for pyruvate 100-fold higher than those of the lactate dehydrogenases of *Bacillus* species (i.e. 5.7 mM versus 50 µM) (Jackson *et al.*, 1992; Raj *et al.*, 2002), which are likely to compete for pyruvate pools.

DISCUSSION

For the production of ethanol in Gram-positive bacteria to become a viable alternative for the production of fuels, it will be necessary to find a PDC that can be expressed at high enough levels to rapidly channel pyruvate to acetaldehyde. Until now, there has not been a PDC that has been expressed well in a recombinant Gram-positive bacterium (Gold *et al.*, 1992, 1996; Barbosa *et al.*, 1994; Nichols *et al.*, 2003).

In this study, *B. megaterium* expression vectors were designed in such a way as to transcribe all four *pdg* genes at similar rates by using the same *xylA* promoter, Shine-Dalgarno sequence and T7 terminator. Using this approach, the PDC protein of *S. ventriculi* was synthesized at high levels in the recombinant Gram-positive host. The *SvPDC* protein levels and activity were at least fivefold higher than those of the PDCs of *Z. mobilis*, *A. pasteurianus*, or *Sac. cerevisiae*. To assess the biological reason for these differences, quantitative reverse transcriptase PCR and pulse-chase experiments were performed. The recombinant Gram-positive host synthesized similar levels of *pdg*-specific

transcript, and PDC protein degradation was minimal. Thus, in the Gram-positive host examined in this study, protein synthesis limited the production of PDC proteins from yeast and Gram-negative bacterial genes.

It was previously demonstrated that the addition of accessory tRNAs is necessary for the enhancement of SvPDC protein levels in *E. coli* by tenfold (Talarico *et al.*, 2001). This is not the case when ApPDC and ZmPDC are expressed in *E. coli*. Both PDCs are produced at very high levels in this Gram-negative host without the addition of accessory tRNA. In *B. megaterium*, however, SvPDC is expressed at very high levels, while expression of ApPDC and ZmPDC is poor. The results of the expression of the PDC proteins in *E. coli* and *B. megaterium* indicate that codon usage of the *pdg* genes is one of the primary factors influencing synthesis of these proteins in Gram-positive hosts (Talarico *et al.*, 2001; Raj *et al.*, 2002). The contrasting codon usage of the *pdg* genes used in this study becomes evident when analysing the percentage G + C in the wobble position. *B. megaterium* has a wobble position percentage G + C of 30.8 %. The *Svpdc* gene has the lowest percentage G + C in the wobble position at 12.3 %, the *Appdc* gene has the highest at 74.2 %, and the *Zmpdc* and *ScPDC1* genes have similar percentages of 54.6 and 51.5 %, respectively. These values vary quite dramatically, and correspond with the general trend of efficiency of expression in *B. megaterium* demonstrated by this study. Other studies have shown that changing rare codons to codons optimal for the recombinant host can increase protein levels. For example, expression of *cyt2Aa1* of *Bacillus thuringiensis* in *Pichia pastoris* is improved (Gurkan & Ellar, 2003) and production of antigen 85A from *Mycobacterium tuberculosis* in *E. coli* is increased 54-fold (Lakey *et al.*, 2000).

Thus, future research is aimed at engineering Gram-positive hosts for ethanol production using the only known PDC that is expressed well in a Gram-positive host, SvPDC. Alternatively, a *pdg* gene with optimized codon usage could be synthesized for high-level production of alternative PDCs. SvPDC has qualities that make it unique among bacterial PDCs, including its substrate activation and elevated pH optimum (Lowe & Zeikus, 1992; Talarico *et al.*, 2001). However, in comparison to other PDCs, the K_M of SvPDC for pyruvate is over tenfold higher (Raj *et al.*, 2002).

The results of this study also demonstrate the generation of a Gram-positive operon that is functional in the synthesis of PDC and ADH proteins that actively convert pyruvate to ethanol. Our construction and expression of this G + PET operon using the SvPDC and *G. stearothermophilus* ADH have demonstrated that high-level synthesis of active PDC and ADH no longer limits ethanol production in Gram-positive biocatalysts. Optimization of the conversion of biomass to pyruvate and minimization of the pathways that compete for available pyruvate are now needed to ensure high yields of ethanol in recombinant Gram-positive bacteria.

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