

Recombinant production of *Zymomonas mobilis* pyruvate decarboxylase in the haloarchaeon *Haloferax volcanii*

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Summary The unusual physiological properties of archaea (e.g., growth in extreme salt concentration, temperature and pH) make them ideal platforms for metabolic engineering. Towards the ultimate goal of modifying an archaeon to produce bioethanol or other useful products, the pyruvate decarboxylase gene of *Zymomonas mobilis* (*Zm pdc*) was expressed in *Haloferax volcanii*. This gene has been used successfully to channel pyruvate to ethanol in various Gram-negative bacteria, including *Escherichia coli*. Although the ionic strength of the *H. volcanii* cytosol differs over 15-fold from that of *E. coli*, gel filtration and circular dichroism revealed no difference in secondary structure between the ZmPDC protein isolated from either of these hosts. Like the *E. coli* purified enzyme, ZmPDC from *H. volcanii* catalyzed the nonoxidative decarboxylation of pyruvate. A decrease in the amount of soluble ZmPDC protein was detected as *H. volcanii* transitioned from log phase to late stationary phase that was inversely proportional to the amount of *pdc*-specific mRNA. Based on these results, proteins from non-halophilic organisms can be actively synthesized in haloarchaea; however, post-transcriptional mechanisms present in stationary phase appear to limit the amount of recombinant protein expressed.

Keywords: biotechnology, ethanol, halophile, metabolism, molecular biology, recombinant protein.

Introduction

Archaea encompass a diversity of organisms able to grow in a wide range of environments. Temperatures > 100 °C (Blochl et al. 1997, Kashefi and Lovley 2003), pH < 1 (Schleper et al. 1995, van de Vossenberg et al. 1998) and salt concentrations > 5 M (Martin et al. 2000) are just a few of the extremes in habitat where archaea are found. Although this diversity provides tremendous opportunities in metabolic engineering, it remains a relatively untapped source for such objectives (Schiraldi et al. 2003). Halophiles, hyperthermophiles, acidophiles and methanogens are ideal candidates for metabolic engineering because of their ability to survive in extreme environments and their unique metabolic pathways (Herbert 1992, Schiraldi et

al. 2003). Potential applications of archaea in biotechnology are far-reaching, ranging from bioremediation of toxic compounds to production of biopolymers with medicinal applications (Bertrand et al. 1990, Emerson et al. 1994, Hezayen et al. 2000, Margesin and Schinner 2001a, 2001b, Schiraldi et al. 2003). Archaea are presently employed in several industrial processes, including wastewater treatment, fermentation of food products and production of solar salt (Thongthai et al. 1992, Scherer et al. 2000, Javor 2002). As a group, however, the domain is still very much underrepresented in metabolic engineering. Current applications of archaea in biotechnology typically incorporate the properties of the native organism but do not use engineered strains for optimization of product formation.

Halophilic archaea are especially well suited for biotechnological applications because of established genetic exchange systems and the large amount of biomass attainable from these organisms (Blaseio and Pfeifer 1990, Holmes et al. 1994, Ng et al. 2000). Unique features of the haloarchaea also make them attractive candidates for industrial use, including cell lysis upon exposure to low salt concentrations, which reduces the cost of protein purification (Ventosa et al. 1998). In addition, haloarchaea are easily cultured with little risk of contamination due to the high salt concentrations in which they grow, resulting in easier maintenance of cultures (Margesin and Schinner 2001b).

This study was undertaken to produce a non-halophilic metabolic enzyme in a haloarchaeal host as part of an endeavor to better utilize this diverse group of organisms in biotechnology. Specifically, the pyruvate decarboxylase gene of *Zymomonas mobilis* was expressed in recombinant *Haloferax volcanii* with the long-term goal of engineering archaeal metabolism through recombinant technologies. Pyruvate decarboxylase (PDC) was employed based on its application in the industrial production of biofuels and (*R*)-phenylacetylcarbinol (Iding et al. 1998, MacDonald et al. 2001, Wyman 2001) and limited distribution in nature (Candy and Duggleby 1998, Raj et al. 2002). Currently, commercially viable, large-scale production of PDC-based products from lignocellulosic materials via simultaneous saccharification and fermentation (SSF) is

restricted (Ingram et al 1999). Engineering PDC production in robust microorganisms such as *H. volcanii*, which can use a variety of sugars (Rawal et al 1988) and withstand adverse environmental conditions (e.g., elevated temperature and high salt concentration), is expected to advance this area of biotechnology.

Materials and methods

Materials

Biochemicals were purchased from Sigma-Aldrich (St. Louis, MO). Other organic and inorganic analytical grade chemicals were from Fisher Scientific (Atlanta, GA). Restriction endonucleases and DNA-modifying enzymes were from New England BioLabs (Beverly, MA). SnakeSkin dialysis tubing was from Pierce (Rockford, IL). Hybond-P membranes used for immunoblot were from Amersham Pharmacia Biotech (Piscataway, NJ).

Strains and media

Bacterial and archaeal strains used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani medium (37 °C, 200 rpm) supplemented with 100 mg l⁻¹ ampicillin as needed. Unless otherwise indicated, *H. volcanii* strains were grown in ATCC 974 medium (42 °C, 200 rpm) supplemented with 0.1 mg l⁻¹ novobiocin as needed. For immunoblot and mRNA transcript analysis, cells were grown to log phase (optical density at 600 nm (OD₆₀₀) of ~0.2) and used as a 1% inoculum into fresh medium (10-ml cultures in 125-ml Erlenmeyer flasks). For analysis of ethanol and organic acid production, all media were supplemented with 1% (w/v) glu-

cose or glycerol. Cells were grown in 20-ml cultures in 250-ml screw cap Erlenmeyer flasks (37 °C, 200 rpm). *Haloferax volcanii* medium also included 250 g NaCl, 20 g MgSO₄, 2 g KCl, 0.2 g CaCl₂·2H₂O, 3 g trisodium citrate·2H₂O, 5 mg FeSO₄·7H₂O, 0.19 g MnSO₄·7H₂O and 10 g peptone (Fisher) per liter (Tawara and Kamo 1991) as well as 125 g NaCl, 50 g MgCl₂·6H₂O, 5 g K₂SO₄, 0.1 g CaCl₂·2H₂O, 1.0 g NH₄Cl, 0.2 g KH₂PO₄ and 0.2 g yeast extract per liter with 20 mM PIPES (piperazine-*N,N'*-bis(2-ethanesulfonic acid) and 20 mM MES (2-(*N*-morpholino)ethanesulfonic acid) buffers at pH 7.0 added separately (Oren and Gurevich 1994b). All media were adjusted to pH 6.9–7.0 with NaOH.

DNA purification and transformation

Plasmid DNA was isolated using a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). The DNA fragments were eluted from 0.8% (w/v) SeaKem GTG agarose (FMC Bioproducts, Rockland, ME) gels in 1× TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.5) using the QIAquick Gel Extraction Kit (Qiagen). *Haloferax volcanii* DS70 cells were transformed (Cline et al. 1989) with plasmid DNA isolated from *E. coli* GM2163.

Plasmids and cloning

Plasmids used in this study are summarized in Table 1. For expression of ZmPDC in *H. volcanii*, the Zm *pdC* gene was isolated from the pET21d-based plasmid pJAM431 by restriction enzyme digestion (*Xba*I and *Dra*III) and blunt-end ligated with the 9.9-kb *Bam*HI-to-*Kpn*I fragment of plasmid pBAP5010. The DNA fragment of pJAM431 used for ligation included not only the Zm *pdC* gene but also the Shine-Dalgarno sequence and T7 terminator of the original pET21d

Table 1. Plasmids and strains used in this study.

Plasmid or strain	Phenotype or genotype	Source
pET21d	Ap ^r ; expression vector	Novagen (Madison, WI)
pBAP5010	Ap ^r Nv ^r ; 11-kb shuttle expression vector derived from pBAP5009 by insertion of citrate synthase gene; includes P2 promoter of the rRNA operon of <i>H. cutirubrum</i>	Jolley et al. 1997
pLOI276	Ap ^r ; Zm <i>pdC</i> isolated from <i>Z. mobilis</i> genomic DNA and ligated into <i>Sma</i> I site of pUC18	Conway et al. 1987
pJAM431	Ap ^r ; fragment generated by PCR from pLOI276 cut with <i>Bsp</i> HI and <i>Xho</i> I and ligated into pET21d at the <i>Nco</i> I and <i>Xho</i> I sites	This study
pJAM202	Ap ^r Nv ^r ; 1152-bp <i>Xba</i> I-to- <i>Dra</i> III fragment of pJAM621 blunt-end ligated with a 9.9-kb <i>Bam</i> HI-to- <i>Kpn</i> I fragment of pBAP5010; <i>psmB-H6</i> oriented with rRNA P2; β-His expressed in <i>H. volcanii</i>	This study
pJAM206	Ap ^r Nv ^r ; 2155-bp <i>Xba</i> I-to- <i>Dra</i> III fragment of pJAM431 blunt-end ligated with a 9.9-kb <i>Bam</i> HI-to- <i>Kpn</i> I fragment of pBAP5010; Zm <i>pdC</i> oriented with rRNA P2; ZmPDC expressed in <i>H. volcanii</i>	This study
<i>E. coli</i> DH5α	F ⁻ <i>recA1 endA1 hsdR17</i> (r _k ⁻ m _k ⁺) <i>supE44 thi-1 gyrA relA1</i>	Life Technologies (Rockville, MD)
<i>E. coli</i> BL21(DE3)	F ⁻ <i>ompT [lon] hsd S_B</i> (r _B ⁻ m _B ⁻) (an <i>E. coli</i> B strain) with DE3, a λ prophage carrying the T7 RNA polymerase gene	Novagen
<i>E. coli</i> GM2163	F ⁻ <i>ara-14 leuB6 fhuA31 lacY1 tsx78 glnV44 galK2 galT22 mcrA dcm-6 his G4 rfbD1 rpsL 136 dam13::Tn9 xylA5 mtl-1 thi-1mcrB1hsdR2</i>	New England BioLabs (Beverly, MA)
<i>E. coli</i> KO11	<i>E. coli</i> B derivative; <i>pfl</i> ⁺ <i>pfl</i> ::(<i>pdC</i> ⁺ <i>adhB</i> ⁺ Cm ^r); selected for high Cm ^r ; hyperexpressive for <i>pdC</i> and <i>adh</i> ; <i>frd</i>	Ohta et al. 1991
<i>H. volcanii</i> DS70	Cured of pHV2	Wendoloski et al. 2001

expression vector. The start codon of the modified *pdc* gene was positioned 75 bp downstream of the *Halobacterium cutirubrum* rRNA P2 promoter. This resulted in the generation of shuttle expression plasmid pJAM206 for the synthesis of ZmPDC in *H. volcanii*.

Purification of ZmPDC

Protein purification steps were performed at room temperature unless otherwise indicated. Buffers were supplemented with 1 mM TPP, 1 mM MgCl₂ and 1 mM dTT. Centrifugations were at 16,000 g (20–30 min, 4 °C). Cells were harvested by centrifugation and stored at –70 °C. Cells were resuspended in 2.5 to 6 volumes (wet w/v) of 50 mM NaPO₄ buffer at pH 6.5 (Buffer A) and lysed by passage through a chilled French pressure cell at 20,000 psi followed by centrifugation. Dialysis was at 4 °C for 16 h (< 1 ml of sample against 3 l of buffer) followed by centrifugation. Samples were filtered (0.45-µm) before column chromatography.

(i) *Purification from recombinant E. coli (pJAM431)* Cells of *E. coli* BL21(DE3) were freshly transformed with pJAM431. Cells (1 l) were grown to log phase (OD₆₀₀ of 0.6 to 0.8), and *pdc* gene expression was induced with isopropyl-β-D-thiogalactopyranoside (1 mM) for 2 h. Cell lysate was subjected to thermal treatment (60 °C, 30 min). The sample was applied to a Q Sepharose Fast Flow 26/10 column (Pharmacia) equilibrated in Buffer A and eluted into Buffer A supplemented with 1 M NaCl. Fractions with PDC activity (1 M NaCl) were pooled and directly applied to a 5-ml Bio-scale hydroxyapatite type I column (Bio-Rad, Hercules, CA) equilibrated in Buffer A. ZmPDC was eluted with a linear gradient of 50 to 500 mM sodium phosphate buffer at pH 6.5 in 50 ml. Fractions with PDC activity (350–500 mM sodium phosphate) were pooled and applied to a Superdex 200 HR 10/30 column (Pharmacia) equilibrated in Buffer A. Column eluant with PDC activity was pooled, stored at 4 °C and used for biochemical and structural analyses.

(ii) *Purification from recombinant H. volcanii (pJAM206)* *Haloferax volcanii* (pJAM206) (10 l) was grown to log phase. Cell lysate was applied to a 5-ml Bio-scale hydroxyapatite type I column (Bio-Rad) equilibrated in Buffer A. ZmPDC was eluted with a linear gradient of 50 to 500 mM sodium phosphate at pH 6.5 in 85 ml. Hydroxyapatite fractions with PDC activity (250–450 mM sodium phosphate) were pooled and dialyzed against Buffer A supplemented with 1 M (NH₄)₂SO₄. Sample was applied to a 1 ml Phenyl-Sepharose FF (low sub) column (Pharmacia) and eluted with a linear gradient of 650 mM to 0 M (NH₄)₂SO₄ in 20 ml Buffer A. Fractions with PDC activity were concentrated with a Centricon 10 (Amicon, Billerica, MA). Sample (1 mg at 2.25 mg ml⁻¹) was applied to a Superdex 200 HR 10/30 column (Pharmacia) equilibrated in Buffer A. Fractions with PDC activity were applied to a 1-ml DEAE-cellulose column (Sigma) equilibrated in Buffer A. Column eluant with PDC activity was pooled, stored at 4 °C and used for biochemical and structural analyses.

Protein analysis and enzymatic assays

Protein concentrations were determined by the Coomassie

blue dye-binding assay with bovine serum albumin as the standard (Bio-Rad) (Bradford 1976). ZmPDC activity was monitored in a 1-ml coupled assay, which measured the pyruvate-dependent oxidation of NADH with yeast alcohol dehydrogenase (ADH) as described previously (Conway et al. 1987). One unit of activity (U) is defined as the amount of PDC that generates 1 µmol of acetaldehyde per min. Alcohol dehydrogenase activity was measured as the NAD-dependent oxidation of ethanol (0.05% (v/v)) as described previously (Neale et al. 1986). Yeast ADH was included as a positive control.

Molecular mass determinations

Subunit molecular masses were determined by SDS-PAGE using the standards phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa) (Bio-Rad). Native molecular masses were determined by Superdex 200 HR 10/30 (Pharmacia) equilibrated in 50 mM NaPO₄ buffer at pH 6.5 with 1 mM TPP, 1 mM MgCl₂ and 1 mM dTT. Molecular mass standards included: serum albumin (66 kDa), ADH (150 kDa), β-amylase (200 kDa) and apoferritin (443 kDa).

Circular dichroism

Circular dichroic spectra were obtained on a Jasco 500C spectropolarimeter (Easton, MD) at varying temperatures with a 1-mm path length, water-jacketed quartz cuvette. Instrument parameters were set to 1.0-nm bandwidth, 50-nm min⁻¹ scan rate and 0.2-nm resolution. Instrument calibration was performed with 0.06% (w/v) (1S)-(+)-10-camphorsulfonic acid and neodymium glass (Hennessey and Johnson 1982). Chicken egg white lysozyme at 7 µM concentration in 5 mM NaPO₄ buffer at pH 6.5 was used as a standard. The spectra of ZmPDC (100 to 160 nM) dialyzed into 5 mM NaPO₄ buffer at pH 6.5 were obtained from 250 to 195 nm. A mean of 10 accumulations was generated with the sample spectra taken for a minimum of three separate runs. Samples were allowed to equilibrate (5 min) at the set temperature before measurement. Noise reduction of spectra was performed with the Jasco J700 software package (Version 1.50.01). Secondary structure content of proteins was calculated from circular dichroism spectra as described previously (Chen et al. 1974, Deleage and Geourjon 1993, Sreerama and Woody 1993). α-Helical content was used for the comparison of the secondary structures, since current deconvolution methods are most reliable in the identification of α-helices compared with β-sheets and random coils (Wallace 2000).

Immunoanalysis

Chemiluminescent Western blots were performed with polyclonal goat anti-ZmPDC (1:1000) (Conway et al. 1987) and horseradish peroxidase-conjugated polyclonal anti-goat rabbit antibody (1:1000) (Southern Biotechnology Associates, Birmingham, AL) with ECL Plus according to the manufacturer (Amersham).

Quantitative RT-PCR analysis

Total RNA was purified from *H. volcanii* DS70 (pJAM206)

with the RNeasy MiniKit (Qiagen) protocol for the isolation of bacterial RNA with the following modifications. Cells were centrifuged (1 min at 18,000 g) and immediately resuspended in 100 μ l RNase-free water. An additional DNase treatment (Deoxyribonuclease I, amplification grade) was performed after RNA purification according to the supplier (Sigma). The total RNA concentration was determined by absorbance at 260 nm. Ribosomal RNA detected by ethidium bromide stain after gel electrophoresis served as the invariant control between total RNA samples prepared from log and stationary phase cells (OD_{600} of 1.1 and 3.0, respectively). One-step quantitative RT-PCR was performed with the one step QuantiTect SYBR Green RT-PCR Kit (Qiagen) and iCycler MyiQ Real-Time PCR Detection System (Bio-Rad). A dilution series of total RNA served as the template with the oligonucleotide primers 5'-TGGCGAACTGGCAGAAGCTATCA-3' and 5'-CGCGCTTACCCCATTTGACCA-3'. The reference standard was *Z. mobilis* *pdC*-specific mRNA generated by in vitro transcription with the *Hind*III DNA fragment of plasmid pJAM431 and the MAXIscripT In Vitro Transcription Kit (Ambion, Austin, TX). Reverse transcriptase-PCR included a reverse transcription step (50 °C, 30 min) and reverse transcriptase inactivation and DNA polymerase activation step (95 °C, 15 min). This was followed by 40 cycles of denaturation at 94 °C (15 s), annealing at 67 °C (30 s) and extension at 72 °C (30 s). A negative control incubated on ice (versus 50 °C) for the reverse transcriptase step was included to ensure that total RNA was not contaminated with genomic DNA. Data were analyzed with the MyiQ Single-Color Real-Time PCR Detection System software Version 1.0 (Bio-Rad). Reverse transcriptase-PCR was performed in triplicate with amplification efficiencies between 90 and 100%, and melt curve analysis was performed to verify uniformity of the product.

Analysis of ethanol and organic acid production

Ethanol production was monitored by gas chromatography as described previously (Beall et al. 1991). Ethanol standards ranging from 0.1 to 10 g l⁻¹, 0.05% (v/v) formaldehyde and 1% (v/v) isopropanol comprised the internal standard. Sterile medium supplemented with 8% (w/v) ethanol was included as a control to confirm that less than 10% of the ethanol volatilized during incubation at 37 °C for 5 days. Organic acids and glucose were measured as described previously (Causey et al. 2003) with a Hewlett-Packard HPLC (HP 1090 series II, Palo Alto, CA) equipped with a UV monitor (210 nm) and refractive index detector. Products were separated with a Bio-Rad HPX-87H column (10 μ l injection) with 4 mM H₂SO₄ as the mobile phase (0.4 ml min⁻¹, 45 °C).

Results and discussion

ZmPDC is active in high salt concentrations

Previous studies revealed that ZmPDC is relatively stable and active at high temperatures (50–60 °C) (Conway et al. 1987, Raj et al. 2002). Thus, the structural forces that stabilize ZmPDC at these high temperatures may also stabilize the enzyme at high salt concentrations (Keradjopoulos and Holldorf

1977). To investigate this, ZmPDC was purified from recombinant *E. coli* (ZmPDC_{Eco}) and assayed at increasing concentrations of NaCl and KCl (Figure 1). The enzyme retained nearly 50% activity when assayed in buffer supplemented with either 2 M NaCl or KCl. Furthermore, PDC retained 20% activity when assayed in the presence of 4 M salt. Pre-incubation of ZmPDC for 30 min in the salts prior to assay did not influence this relative activity. Based on these results, the ZmPDC is quite active at high salt concentrations and thus is an ideal candidate for channeling pyruvate to acetaldehyde in the high ionic strength cytosol of a haloarchaeon such as *H. volcanii*.

ZmPDC produced in recombinant *H. volcanii* is active

To engineer *H. volcanii* for ZmPDC production, the *pdC* gene was expressed from a plasmid (pJAM206) which included the ribosomal RNA P2 promoter from *Halobacterium cutirubrum* and T7 terminator from a pET plasmid to control transcription (Table 1). Immunoanalysis of log-phase *H. volcanii* (pJAM206) cells using anti-ZmPDC antibody revealed a cross-reacting protein of 60 kDa that was absent in the parent strain (data not shown). This 60-kDa protein corresponded in molecular mass to ZmPDC. A low, but detectable, level of PDC activity (0.12 U per mg protein) was present in *H. volcanii* (pJAM206) cell lysate but was not detected in the parent strain. These results indicated that the ZmPDC protein was produced and was active. The expression level was estimated to be 0.1% of soluble protein based on the specific activity of the cell lysate.

ZmPDC from *H. volcanii* is active and comparable in structure to that from *E. coli*

It has been shown previously that the ZmPDC purified from recombinant *E. coli* (ZmPDC_{Eco}) is comparable in biochemical properties to that of the native ZmPDC (Hoppner and Doelle 1983, Neale et al. 1987). Because of the ease of expres-

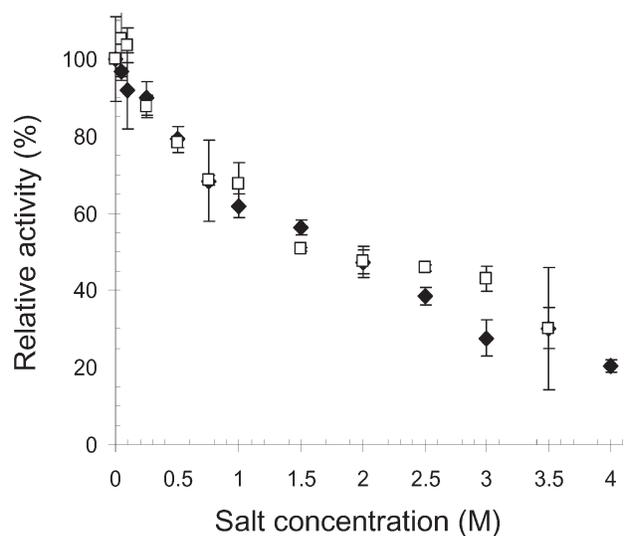


Figure 1. Effect of salt on the pyruvate decarboxylase (PDC) activity of *Z. mobilis* purified from recombinant *E. coli* (ZmPDC_{Eco}). Purified enzyme was incubated (15 min, 25 °C) and assayed in buffer supplemented with NaCl (◆) and KCl (□) at the concentrations indicated.

sion and purification from *E. coli*, the ZmPDC_{Eco} was used for comparison with ZmPDC purified from *H. volcanii* (ZmPDC_{Hvo}) (Figure 2). The ZmPDC_{Hvo} had 55 U mg⁻¹ of activity compared to 91 U mg⁻¹ of activity for the ZmPDC_{Eco} (Table 2). Both proteins purified as tetrameric complexes (240 kDa) based on gel filtration. Circular dichroism revealed no major differences in secondary structure between the ZmPDC purified from either organism (Figure 3). The circular dichroism spectra were identical with both exhibiting the characteristic minima at 208–209 nm and 222 nm and maximum at 195 nm consistent with a high degree of α -helical structure. The deconvolution methods utilized reflect this uniformity between the spectra, indicating little difference in the overall α -helical content. Additionally, the predicted α -helical content from the circular dichroism measurements (36–42.2%) correlates well with the published crystal structure estimate of 36.8% for ZmPDC_{Eco} (Dobritsch et al. 1998). Together these results reveal similar secondary structure between the ZmPDC_{Hvo} and ZmPDC_{Eco}.

The reason for the reduced (40%) activity of ZmPDC_{Hvo} compared with ZmPDC_{Eco} remains to be determined. The similarity of protein structure in the two preparations suggests that no perturbations to overall structure occurred when ZmPDC was expressed in *H. volcanii*. Instead, the 460-fold purification required for a homogeneous preparation of ZmPDC_{Hvo} compared with the 12.4-fold purification for ZmPDC_{Eco} (Table 2) may have contributed to a loss of the thiamine pyrophosphate (TPP) cofactor. Such a tetrameric form of ZmPDC, which retains its overall configuration but has lost cofactors and activity, has been reported (Candy et al. 1996). In fact, cofactor loss is a common problem when purifying TPP-dependent enzymes, which is why cofactors are typically included in all purification buffers and the pH is maintained below neutrality. Thus, although the ZmPDC purified from *H. volcanii* as a tetrameric complex, it does not preclude the possibility that a fraction of this preparation lacks the necessary cofactors.

Amount of soluble ZmPDC varies with the growth phase of *H. volcanii* (pJAM206)

To better understand and enhance expression of ZmPDC, the amounts of this foreign protein and its mRNA were monitored throughout growth of *H. volcanii* (pJAM206) on rich medium. The ZmPDC protein was detected in log phase and in the transition to the stationary phase but not in the stationary phase cells (Figure 4). Although transcription from rRNA promoters often declines in stationary phase (Aviv et al. 1996, Rosado and Gage 2003), the percentage of Zm *pdc*-specific mRNA to total RNA was actually threefold higher at this phase of growth ($1.38 \pm 0.19\%$) than at log phase ($0.42 \pm 0.05\%$). These results reveal a growth-phase-dependent decrease in the amount of ZmPDC protein that was inversely proportional to Zm *pdc*-specific mRNA. Thus, the reduced amount of ZmPDC protein in the stationary phase was not due to a lack of mRNA transcript. Instead, factors beyond transcription were responsible.

A variety of post-transcriptional factors may be responsible for the low amounts of ZmPDC protein in *H. volcanii*, includ-

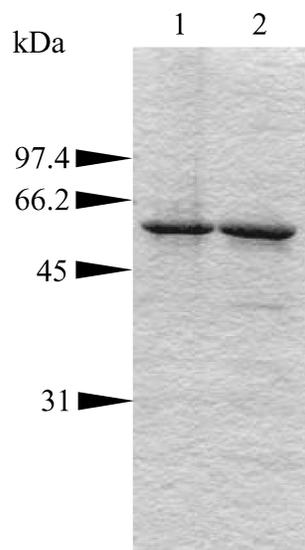


Figure 2. Comparison of *Z. mobilis* pyruvate decarboxylase purified from *H. volcanii* (pJAM206) (ZmPDC_{Hvo}; Lane 1) with that from *E. coli* (pJAM431) (ZmPDC_{Eco}; Lane 2). Protein (1 μ g) was separated by 12% reducing SDS-PAGE and stained with Coomassie blue.

ing proteolysis, inclusion body formation and reduced translation of the *pdc*-specific transcript. Our previous work indicates that differences in codon usage between *pdc* genes and the host organism can severely impair overall recombinant protein production (Talarico et al. 2001). Examination of the differences between the Zm *pdc* gene and *H. volcanii* genome suggest this may contribute to the low quantity of ZmPDC in this recombinant organism. The Zm *pdc* gene makes increased use of the codons: GCU, AAU, CAU, CCU, UAU and GUU compared with the *H. volcanii* genome. It is also possible that proteasome-mediated degradation is responsible for the low quantities of ZmPDC in stationary phase *H. volcanii*. Recent work indicates that the PanB and $\alpha 2$ proteins of this proteolytic system increase several-fold during the transition to stationary phase (Reuter et al. 2004).

Table 2. Purification of *Z. mobilis* pyruvate decarboxylase from recombinant *H. volcanii* and *E. coli* strains.

Purification step	Specific activity (U mg ⁻¹)	Fold purification	Yield (%)
<i>H. volcanii</i> (pJAM206)			
Lysate	0.12	1	100
Hydroxyapatite	0.56	4.5	15
Phenyl-sepharose	5.38	45	6
Superdex 200	29	240	5
DEAE-cellulose	55	460	2
<i>E. coli</i> (pJAM431)			
Lysate	7.33	1	100
Heat treatment	20.53	2.8	89
Q-sepharose	56.27	7.7	50
Hydroxyapatite	52.44	7.2	23
Superdex 200	91	12.4	8

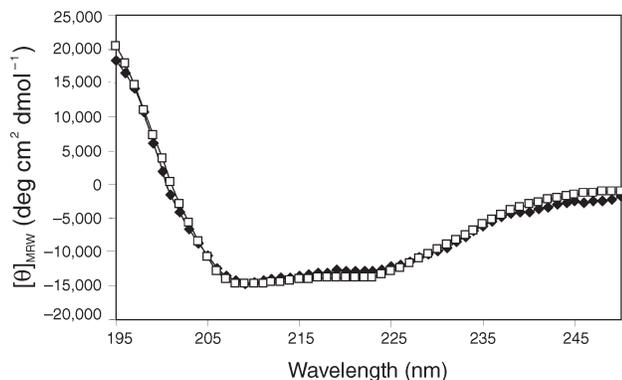


Figure 3. Circular dichroism (mean residue molar ellipticity, $[\theta]_{MRW}$) of *Z. mobilis* pyruvate decarboxylase purified from *H. volcanii* (pJAM206) (ZmPDC_{Hvo}) and from *E. coli* (pJAM431) (ZmPDC_{Eco}). Far-UV circular dichroism spectra of ZmPDC_{Eco} (◆) and ZmPDC_{Hvo} (□) at 100–160 nM in 5 mM sodium phosphate buffer at pH 6.5. The spectra shown are the mean of 10 accumulations with a minimum of three separate runs.

End-product formation by recombinant *H. volcanii* (pJAM206)

Pyruvate serves as a central intermediate in the metabolism of biomass and can be converted to a variety of end products, including ethanol. Pyruvate decarboxylase is central in channeling pyruvate to acetaldehyde, which is reduced to ethanol by ADH. Species of haloarchaea, including *H. volcanii*, are able to generate a pyruvate pool via the metabolism of glycerol and sugars (Tomlinson and Koch 1974, Rawal et al. 1988, Oren and Gurevich 1994a and 1994b). Breakdown of glucose follows a modified Entner-Doudoroff pathway in which glucose is first oxidized to gluconate. The phosphorylation step acts on 2-keto-3-deoxygluconate to form 2-keto-3-deoxy-6-phosphogluconate (KDPG), which is cleaved into glyceraldehyde-3-phosphate and pyruvate.

Oxidoreductases that catalyze the reduction of acetaldehyde to ethanol are widespread in nature (Jez et al. 1997) and have been identified in thermophilic and hyperthermophilic archaea (Radianingtyas and Wright 2003). Although ADH activity has not been reported in the haloarchaea, the partial genome sequence of *H. volcanii* reveals putative Zn-dependent ADHs

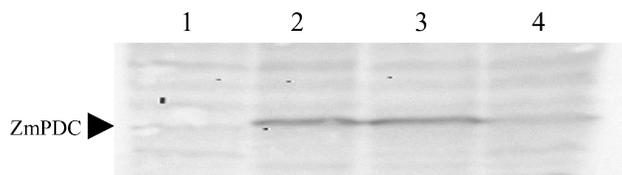


Figure 4. The *Z. mobilis* pyruvate decarboxylase (ZmPDC) protein in *H. volcanii* (pJAM206). The ZmPDC protein was detected in cell lysate (65 µg) by immunoblot. *Haloferax volcanii* DS70 grown to log phase was included as a control (Lane 1). *Haloferax volcanii* (pJAM206) was grown to log phase (Lane 2), the transition to stationary phase (Lane 3) and stationary phase (Lane 4) (OD_{600} of 0.848, 2.06 and 3.27, respectively).

Table 3. Ethanol production in recombinant *E. coli* and *H. volcanii* strains. Abbreviation: UD = undetectable ($< 0.1 \text{ g l}^{-1}$).

Strain	Ethanol (g l^{-1})
<i>E. coli</i> KO11	3.1
<i>E. coli</i> BL21(DE3) (pJAM431) without IPTG	0.34
<i>H. volcanii</i> DS70	UD
<i>H. volcanii</i> DS70 (pJAM206)	UD

(e.g., GenBank Accession no. T44975). Thus, it was possible that the recombinant *H. volcanii* strain expressing PDC would generate ethanol. Ethanol, however, was not detected in the spent medium of either parent or recombinant *H. volcanii* strains (Table 3). In contrast, ethanologenic strains of *E. coli* produced 0.34–3.1 g ethanol l^{-1} (Table 3). Further analyses of the *H. volcanii* strains revealed acetate and lactate were the major organic acids produced with no detectable amounts of ADH activity in cell lysates (data not shown). Thus, ethanol production may be limited not only by the amount of PDC but also by ADH in *H. volcanii* (pJAM206).

Conclusions

This study demonstrates that non-halophilic proteins can be expressed and purified from recombinant haloarchaea in a form that is active and has similar structure to that purified from non-halophilic sources. The synthesis and purification of an active non-halophilic bacterial protein from *H. volcanii* demonstrates the potential capabilities of genetic engineering archaea as well as halophiles, thus expanding the possibility for production of novel metabolites in these microorganisms. Future endeavors focused on the metabolic engineering of archaea will allow for a greater diversity of platform organisms to produce novel metabolites and improve production efficiencies of current industrial processes.

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