EXPRESSION OF PYRUVATE DECARBOXYLASE IN A GRAM POSITIVE HOST: *Sarcina ventriculi* PYRUVATE DECARBOXYLASE VERSUS OTHER KNOWN PYRUVATE DECARBOXYLASES

By

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This dissertation is dedicated to my mother, Sandra Lee, without whom none of this would be possible. I would also like to dedicate it to my husband, Timothy Blalock, for his love and encouragement during the course of this work

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TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVATIONS

ADH	alcohol dehydrogenase
Ap	*Acetobacter pasteurianus*
bp	base pairs
DNA	deoxyribonucleic acid
K_m	Michaelis Constant for enzyme activity
MES	2-[N-morpholino]ethanesulfonic acid
NADH	nicotinamide adenine dinucleotide
ORF	open reading frame
PAC	(*R*)-phenylacetylcarbinol (*R*-1-hydroxy-1-phenylpropane-2-one)
PDC	pyruvate decarboxylase
PET	portable ethanol operon
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
Sc	*Saccharomyces cerevisiae*
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Sv	*Sarcina ventriculi*
TPP	thiamine pyrophosphate
U	Unit of enzyme activity defined as the amount of enzyme that generates 1 μmol of product (acetaldehyde) per minute

V_{max}	maximal rate of enzyme activity
Zm	*Zymomonas mobilis*
Zp	*Zymobacter palmae*

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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The technology currently exists for bacteria to produce ethanol from inexpensive plant biomass. To enhance the commercial competitiveness of biocatalysts for the large-scale production of ethanol, a new host organism will need to be developed that can withstand many factors including low pH, high temperature, high ethanol concentrations, and various other harsh environmental conditions. Gram-positive bacteria naturally possess many of these qualities and would be ideal candidates for ethanol production; however, the use of the *pdc* and *adh* genes from the Gram-negative bacterium, *Zymomonas mobilis,* has met with only limited success. In order for this approach to be successful, a gene for pyruvate decarboxylase that is readily expressed in a Gram-positive host needs to be identified.

The *Sarcina ventriculi pdc* gene (*Svpdc)* is the first to be cloned and characterized from a Gram-positive bacterium. Comparative amino acid sequence analysis confirmed

that *Sv*PDC is quite distant from *Z. mobilis* PDC (*Zm*PDC) and plant PDC enzymes. Elucidation of the sequence of the *Svpdc* sequence also led to the identification of a new subfamily of PDCs.

The *Svpdc* gene was expressed at low levels in recombinant *E. coli* due to differences in the codon usage in the hosts and the *Sarcina ventriculi pdc*. Expression was improved by the addition of supplemental tRNA genes and facilitated the purification and biochemical characterization of the recombinant *Sv*PDC enzyme. This dramatic difference in codon usage suggested that the *Svpdc* gene was an ideal candidate for engineering high-level PDC production in low G+C Gram-positive bacteria. To confirm this, expression of *pdc* genes from distantly related organisms (i.e. *Z. mobilis, Acetobacter pasteurianus,* and *Saccharomyces cerevisiae*) were compared to that of the *Svpdc* in recombinant *Bacillus megaterium*. *Sv*PDC protein and activity levels were several-fold higher in recombinant *B. megaterium* compared to the other PDCs examined. Transcript levels using quantitative reverse transcriptase polymerase chain reaction and protein stability using pulse-chase indicated that *Sv*PDC was expressed at higher levels than other PDCs tested due to its optimal codon usage. This is the first PDC expressed at high levels in Gram-positive hosts.

CHAPTER 1
LITERATURE REVIEW

Industrial Importance of Pyruvate Decarboxylase

Pyruvate Decarboxylase Catalyzes the Production of Bioethanol

In 2001 the United States produced 1.77 billion gallons of fuel ethanol of which 90% was produced by fermentation of corn by yeast (1). The demand for fuel ethanol is expected to more than double in the next few years because it will replace the fuel oxygenate methyl tertiary butyl ether (MTBE), a known carcinogen which has been linked to ground water contamination and has proven difficult to remove from the environment (2). Fuel ethanol production in 2001 consumed over 5% of the corn crop, and it has been estimated that fuel ethanol production will reach more than 4 billion gallons per year by 2006 (1, 2). The use of corn as a feedstock for the production of ethanol has led to several problems. Because corn is also used for food, this feedstock has a higher price than alternative feedstocks that are considered waste products from various processes (3). The use of corn also leads to controversy over sacrificing a food product for fuel production (3). However, production of ethanol from non-food sources (bioethanol) can provide a useful alternative to the current method of disposing of lignocellulosic wastes such as rice straw and wood wastes that were historically burned but now must be disposed of in a more environmentally friendly and much more costly manner (3). Utilizing these waste products for bioethanol production not only provides

an inexpensive feedstock but benefits the environment by disposing of this material in an environmentally friendly manner and producing a clean burning fuel source (3).

Organisms traditionally used for ethanol fermentation do not have the ability to metabolize pentoses. Considerable research has been performed to identify naturally occurring organisms that can ferment pentoses (4). Because yeast have been the traditional organisms used for ethanol production and they produce high ethanol yields, research focused on identifying yeast that could metabolize pentose sugars (5). Several yeast strains have been identified that are capable of utilizing xylose for ethanol production: *Pachysolen tannophilus* (6-9), *Pichia stipitis* (10-14), and *Candida shehatae* (10, 11, 15). Unfortunately, these yeast produce only low levels of ethanol from xylose and exhibit a multitude of problems including low ethanol tolerance, utilization of the ethanol produced, inability to utilize and metabolize arabinose, and production of xylitol (6-15). Attempts have also been made to isolate yeast that ferment arabinose, but the yeast which have been isolated produce very low levels of ethanol (4.1 g/liter) (16).

Attempts to improve yeast strains for ethanol production have also been pursued by engineering recombinant strains. *S. cerevisiae* has been the primary focus of this research because the corn ethanol industry is already familiar with this organism, it produces high levels of ethanol, and it has been shown to be resistant to high levels of ethanol (5). Attempts to engineer a xylose utilization pathway from bacteria into *S. cerevisiae* has been unsuccessful (17-21). A more promising strategy has been to engineer the xylose-utilization genes from other yeast into *S. cerevisiae* (22, 23). One of the most successful recombinant strains of *S. cerevisiae* to utilize xylose has been a strain engineered with a plasmid that contained three xylose-metabolizing genes: a xylose

reductase gene and xylitol dehydrogenase gene from *Pichia stipitis*, and a xylulokinase gene from *S. cerevisiae* (23). This strain produced ethanol at 22 g/liter which was a vast improvement over strains expressing bacterial xylose utilization genes (23). While recombinant yeast have been engineered to utilize xylose, there have been no successful attempts to engineer arabinose utilization into these organisms.

Many bacteria naturally possess the ability to ferment hexose and pentose sugars, but produce a variety of fermentation endproducts (4). In bacteria, pentose and hexose sugars are metabolized to pyruvate. Ingram et al. (24) have demonstrated that funneling this pyruvate to ethanol is possible by the use of the pyruvate decarboxylase and alcohol dehydrogenase II from *Z. mobilis*. The low K_m of the *Z. mobilis* PDC (0.4 mM pyruvate) competes favorably with the other enzymes for pyruvate in the cell and causes large amounts of acetaldehyde to be made, which is then converted to ethanol by alcohol dehydrogenase (24). A portable ethanol production operon (PET) was generated that contained the *Z. mobilis* PDC transcriptionally coupled to the *Z. mobilis* alcohol dehydrogenase II (24). The PET operon was used to successfully engineer enteric bacteria for ethanol production including *Klebsiella oxytoca* (25), *Erwinia chrysanthemi* (26), *Enterobacter cloacae* (27), and many strains of *E. coli* (28, 29). The PET operon has also been successfully used to engineer a wide range of other organisms including tobacco (30, 31) and cyanobacteria (32). Attempts have also been made to engineer Gram-positive bacteria to produce ethanol using the PET operon, but this strategy has been unsuccessful due to poor expression of the *Z. mobilis* genes (33-35). The recent cloning and characterization of a PDC from the Gram-positive bacterium *Sarcina ventriculi* and its subsequent high level expression in the Gram-positive bacterium,

Bacillus megaterium, will enable the development of new Gram-positive biocatalysts for the production of ethanol (this study).

Pyruvate Decarboxylase Catalyzes the Production of PAC

In 1921, while examining the biotransformation of benzaldehyde to benzyl alcohol by fermenting Brewer's yeast (*Saccharomyces uvarum*), Neuberg and Hirsh discovered that after 3-5 days no sugar or benzaldehyde remained. Furthermore, the amount of benzyl alcohol produced was not proportional to the amount of substrate consumed (36). They later determined that the byproduct of this reaction was (*R*)-phenylacetylcarbinol (PAC) and named the enzyme that catalyzed its synthesis "carboligase" (37, 38).

The process of PAC formation by Brewer's yeast was patented in 1932, making it one of the first chiral intermediates to be produced on an industrial scale by biotransformation (39, 40). PAC is the first chiral intermediate in the production of L-ephedrine and pseudoephedrine, which are the major ingredients in several commonly used decongestants and antiasthmatics as well as having a possible use in control of obesity (41, 42).

Several studies have confirmed that the enzyme catalyzing the production of PAC is pyruvate decarboxylase (EC 4.1.1.1) (43-46). Pyruvate decarboxylase (PDC) catalyzes two different reactions: non-oxidative decarboxylation of α-ketoacids to the corresponding aldehyde (47-50) and the "carboligase" side reaction forming the hydroxyketones (51, 52). In the acycloin-type condensation reaction, an active aldehyde in the active site is condensed with a second aldehyde as a cosubstrate (40). The

cosubstrate is acetaldehyde *in vivo*, but can be another aldehyde when supplied externally (40). In the production of PAC, benzaldehyde is the cosubstrate fed to yeast cells (40).

Production of PAC by Yeast

The industrial production of PAC has historically utilized yeast cells, primarily *Saccharomyces* sp. and *Candida* sp. Many efforts to improve yeast PAC production have focused on increasing yields of PAC through alteration of the fermentation conditions and medium (53-55).

When *S. carlsbergensis* is grown on sucrose, acetaldehyde, and benzaldehyde the highest initial rate of biotransformation and the highest production of PAC were detected in the cells with the lowest PDC activity. This led to the suggestion that production of PAC is limited only by the intracellular pools of pyruvate and that biotransformation of PAC ceases due to low levels of pyruvate before benzaldehyde mediated inactivation of PDC occurs. Addition of pyruvate did not increase the rate of PDC synthesis but did increase the overall production of PAC (55).

The current industrial process for the production of PAC uses a two-stage fed-batch process. In the first stage, the yeast are grown under partial fermentative conditions to induce the production of PDC and allow intracellular accumulation of pyruvate. In the second stage, the biotransformation takes place with feeding of noninhibiting levels of benzaldehyde. Using this strategy a PAC accumulation level of 22 g/L has been reached (56).

This strategy, however, is hindered by side-reactions within the cells as well as the sensitivity of the cells to benzaldehyde and the fermentative products (57). Besides the PAC production, yeast cells also typically reduce up to 16% to 50% of the

benzaldehyde to benzyl alcohol (36-38). The production of benzyl alcohol is primarily due to the action of alcohol dehydrogenases and other oxidoreductases in the cell (58-60). Other byproducts are also produced including acetoin, benzoic acid, benzoin, butan-2,3-dione (diketone), *trans*-cinnamaldehyde, 2-hydroxypropiophenone, and 1-phenyl-propan-2,3-dione (acetylbenzoyl) (61, 62). In addition to the formation of these side-products, PAC is also enzymatically reduced to (1*R*, 2*S*)-1-phenyl-1,2-propane-diol (54).

At benzaldehyde concentrations above 16 mM the viability of the yeast cells is diminished, and PAC production is completely inhibited above 20 mM (63). If the level of benzaldehyde drops below 4mM, benzyl alcohol becomes the primary product (63). Comparison of intracellular and extracellular benzaldehyde levels shows that the membrane maintains a permeability barrier (9.4 mM), which results in lower levels of benzaldehyde in the cell and may protect intracellular proteins. At concentrations above 9.4 mM benzaldehyde, the barrier appears to falter and intracellular enzymes are inactivated (59). The yeast PDC, however, is resistant to denaturation by benzaldehyde at levels up to 66 mM benzaldehyde and is also fairly resistant to final PAC concentration (59). Thus it was concluded that the modification of cell permeability by benzaldehyde decreases PAC production by causing release of the cofactors necessary for the carboligation reaction (i.e. Mg^{2+} and TPP) and not by inactivation of PDC (59).

Because of these limitations, it would be beneficial to genetically engineer an organism for PAC production that is more resistant to benzaldehyde and does not catalyze multiple side reactions. Alternatively, a cell free system may be a viable alternative to the use of whole cells for PAC production.

Production of PAC in a Cell Free System

Utilization of isolated PDC for the biotransformation of pyruvate to PAC has only recently been pursued as an alternative to the use of whole cells. A distinct advantage to using a cell free system as opposed to cells as a source of PDC is that the oxidoreductases responsible for the conversion of benzaldehyde to benzyl alcohol as well as the cytotoxicity of benzaldehyde can be avoided (58, 59, 63, 64).

The first attempt to use partially purified PDC for the conversion of pyruvate to PAC compared the efficiencies of PDCs from *Z. mobilis* and *S. carlsbergensis* (65). This study proved that both PDCs can be used for production of PAC, however the *Z. mobilis* PDC has a much lower affinity for benzaldehyde (65).

In another study, a high concentration of benzaldehyde was used with partially purified PDC from *Candida utilis* (66). At a benzaldehyde levels of 200 mM, a PAC level of 190mM (28.6 g/L) was obtained which was considerably higher than previously reported values. Shin and Rogers (67) later determined that the factor limiting conversion of pyruvate and benzaldehyde to PAC was the inactivation of PDC by benzaldehyde. This inactivation was determined to be first order with respect to benzaldehyde and exhibited a square root dependency on time.

Stability of the PDC used for the production of PAC is an important factor in the success of the biotransformation. Previous studies have shown that *S. cerevisiae* PDC exhibits a high carboligase activity, but shows only low stability when isolated (65). The PDC from *Z. mobilis* has been shown to have low carboligase activity with respect to the yeast enzyme but high stability (65, 68). It was determined that mutating residues within the *Z. mobilis* PDC enhanced its carboligase activity (68-70). The Pohl lab (71, 72) used

the *Z. mobilis* PDC mutants to produce PAC in an enzyme-membrane reactor. This continuous reaction system utilized acetaldehyde and benzaldehyde in an equimolar ratio. At a substrate concentration of 50 mM of both aldehydes, a PAC volume production of 81 g $L^{-1}d^{-1}$ was obtained with higher yields possible by use of a series of membrane reactors.

Use of cell free systems for the production of PAC is relatively new, having only started in 1988 (65), as opposed to the biotransformation using whole cells which began in 1932 (40). At the moment, the most promising PDCs for production of PAC are variants of *Z. mobilis* PDC that enable benzaldehyde to access the active site (68-70). In cell free systems, the primary factor limiting production of PAC is the availability of PDC enzymes that can withstand the reaction conditions, mainly inactivation by aldehydes. Until recently, *Z. mobilis* PDC was the only known PDC from bacteria. This enzyme has been shown to be more stable when compared to the yeast PDCs and alteration of as little as one amino acid enhanced carboligase activity (70). Recently characterized PDCs from bacteria are likely to have beneficial qualities for the production of PAC.

Distribution of Pyruvate Decarboxylase

PDC has been identified in a wide variety of plants and fungi, but is rare in bacteria. The following section identifies the organisms known to encode PDC and describes the known function of the enzyme in that organism.

PDC in Fungi and Yeast

Several fungal PDCs have been identified. These PDCs from filamentous fungi appear to be active when the organism experiences anoxic conditions (73-75). It is

through PDC that the cell has the ability to regenerate NAD^+ through the production of acetaldehyde that is then converted to ethanol by alcohol dehydrogenase.

In *Neurospora crassa* PDC forms large cytoplasmic filaments that can measure 8-10 nm in length (73). The appearance of these filaments in the cell has been shown to correspond to increased levels of *pdc m*RNA and increased PDC activity levels within the cell (73). Disassembly of the filaments enables recovery of active PDC indicating that the filaments are an active storage form of the enzyme (73). This PDC is particularly interesting in that the amino acid sequence is more closely related to bacterial PDCs than to yeast PDCs while the kinetics are more similar to other fungal PDCs (73).

A gene encoding a putative *pdc* was isolated from a genomic DNA library of *Aspergillus parasiticus* (74). The *A. parasiticus* PDC deduced amino acid sequence was shown to have 37% similarity to the PDC1 from *Saccharomyces cerevisiae*, which was the highest to any PDC and showed that it is quite different from previously characterized PDCs (74). The organisms *A. parasiticus, Aspergillus niger,* and *Aspergillus nidulans* were tested for the production of ethanol in shake flask cultures. Ethanol was detected indicating a response to anoxic conditions even though they are obligate aerobes (74). Although this showed that *A. nidulans* produced ethanol under anoxic conditions (74), the reasearchers did not test for PDC activity in cell lysate. Lockington et al. (75) showed that mycelia subjected to anoxic stress had elevated levels of PDC activity. The gene for PDC was isolated and sequenced from *A. nidulans* (75) and the deduced amino acid sequence from this gene was shown to have highest similarity (37%) to the *A. parasiticus* PDC (75). This study showed that production of PDC in the cell is regulated at the level

of *m*RNA and that production of PDC is therefore the major determinant of ethanol production under anoxic conditions in *A. nidulans* (75).

Several PDCs from yeast have been identified and two are among the best studied of all PDCs (76). In yeast, PDC serves the same purpose as in most organisms, which is to replenish NAD^+ supplies under anaerobic conditions. In most yeast, fermentation and respiration both contribute to glucose catabolism under aerobic conditions. In *Saccharomyces cerevisiae* respiratory and fermentative pathways are mutually exclusive and the pyruvate produced during glycolysis is funneled by PDC almost entirely to acetaldehyde and then to ethanol by ADH (77). The majority of yeast, however, rely on respiration under aerobic conditions to regenerate NAD^+ (77).

Saccharomyces uvarum PDC has been extensively studied over the past two decades due to its various uses in industry, including use in breweries. Wild-type *S. uvarum* PDC exists in a mixture of isoforms consisting of an α_4 homotetramer composed of one type of subunit with a molecular weight of 59 kDa (78, 79)and an $\alpha_2\beta_2$ tetramer with two types of subunits with different molecular weights (β subunit is 61 kDa) (80). These subunits also differ in amino acid composition and sequence (81, 82). A high performance liquid chromatography separation procedure was used to obtain a single isoform (α_4) in a catalytically active state for crystallization (83). The first crystal structure of a PDC was obtained using crystallized form of this α_4 PDC (84). Deletion mutants of the gene coding for theβ-subunit have been used to produce the α_4 PDC protein for study (85). It was found that the α_4 enzyme is considerably less stable in aqueous solution than $\alpha_2\beta_2$ wild-type PDC having a rate of inactivation which is 5 times higher than the wild-type enzyme; however the kinetic features of the two isoforms are

the same (85). Some controversy currently exists over the substrate activation of α_4 PDC. A significant body of work led to the conclusion that the Cys221 residue is required for substrate activation of *S. uvarum* α_4 PDC by binding pyruvate leading to a conformational change in the enzyme (86-90). However, a crystal structure of *S. uvarum* PDC in the presence of the activator pyruvamide shows that this pyruvate analog does not interact with the Cys221 residue (91). Kinetic evidence in this study also suggests that Cys221 is not responsible for substrate activation (91). Further aspects of *S. uvarum* PDC activation will be discussed later in this chapter.

Saccharomyces cerevisiae has been extensively studied due to its various uses in industry, including industrial ethanol production (2). Nucleotide sequences of six PDC genes have been determined (92-98). Three of these genes have been identified as structural genes: *PDC1* (92, 99-101), *PDC5* (94, 102), and *PDC6* (95, 96). Wild-type *S. cerevisiae* PDC protein is composed of 85% from *PDC1* translation while 15% is from *PDC5* translation (102). If one of these two genes is deleted, translation of the other increases to compensate (102). A crystal structure of *S. cerevisiae* PDC1 in the inactive state was determined and was essentially the same as the *S. uvarum* PDC structure (103). For this reason, the *S. cerevisiae* PDC has been a central focus for understanding PDC structure-function because, unlike *S. uvarum* PDC, the nucleotide sequence has been determined (84, 91). The various site-directed mutagenesis studies performed on the *S. cerevisiae* PDC will be discussed later in this chapter.

A gene for PDC from *Kluveromyces lactis* was cloned, and it was determined that it was induced by glucose at a transcriptional level (104). The PDC protein encoded by this gene was purified and characterized, and it was determined that it was similar to *S.*

cerevisiae PDC with a few distinct differences (105). There is a very low binding affinity for pyruvate at the regulatory site (K_a = 207.00 mM); however, it is compensated by the fast isomerization (k_{iso} = 3.03) and low K_m value for pyruvate of 0.24 mM which is approximately 2-fold lower than that for *S. cerevisiae* PDC (K_m of 0.47 mM for pyruvate) (105).

While the PDC from *S. cerevisiae* has been studied extensively, the majority of other known yeast PDCs are not well characterized. PDC has been characterized from *Hanseniaspora uvarum* (106), *Zygosaccharomyces bisporus* (107), and genes for PDC have been sequenced from *Klyveromyces marxianus* (108) and *Pichia stipitis* (109).

PDC in Bacteria

Although study of PDC has been ongoing for many years, the main focus has been primarily on PDC from yeast. The discovery that ethanol formation in *Zymomonas mobilis* was catalyzed by PDC (110) and the later characterization of the protein (111-113) and gene (114-116) identified bacterial PDCs as a distinct group with unique properties that made them attractive for further research. The identification, cloning, and characterization of bacterial PDCs have been aggressively pursued in recent years and our knowledge of this previously unidentified group of PDCs is quickly expanding.

The PDC from *Z. mobilis* was the first bacterial PDC to be identified (110), characterized (111-113), and cloned (114-116) and has since become one of the most intensively studied PDC proteins. *Z. mobilis* PDC was the first PDC discovered that was not substrate activated (111). This enzyme has the highest specific activity of all PDCs (180 units per mg protein) and an extremely low K_m of 0.4 mM pyruvate (112). PDC from *Z. mobilis* is also the most stable PDC in the purified form of those tested (117).

This protein is readily expressed at high levels in *E. coli* (113, 114). A high resolution crystal structure of *Z. mobilis* PDC was obtained, and it was shown that the tight packing of the subunits in the dimers of the tetramer prevents large conformational changes and locks the enzyme in an active state (117). This crystal structure also showed how a previously characterized mutant, Trp392Ala, improved synthesis of PAC by *Z. mobilis* PDC (70) by relieving the steric hindrance caused by bulky amino acid side chains in the active site cavity (117). Extensive site-directed mutagenesis studies have been performed on *Z. mobilis* PDC (70, 110, 118-126). These studies will be discussed later in this chapter. The *Z. mobilis* PDC enzyme has been successfully used to engineer a wide variety of organisms for ethanol production (4, 30-32, 34, 127-129) and has also been modified for the efficient production of PAC in recombinant hosts (68, 70-72, 123).

Acetobacter pasteurianus utilizes PDC in a unique way (130). While all other known PDC proteins function only in anaerobic fermentation to ethanol, the *A. pasteurianus* PDC actually functions only in oxidative metabolism (130). In *A. pasteurianus*, this enzyme functions to cleave the central metabolite pyruvate into acetaldehyde and CO_2, after which the acetaldehyde is oxidized to the final product, acetic acid (130). Upon comparison of the deduced amino acid sequence, it was shown that the *A. pasteurianus* PDC is most closely related to the *Z. mobilis* PDC (130).

The most recently discovered bacterial PDC is from *Zymobacter palmae* (131). The *Z. palmae* PDC protein composed approximately 1/3 of the soluble protein when produced in recombinant *E. coli* (131). It was hypothesized that the high level of PDC protein produced is due to similar codon usage of this *pdc* gene and the *E. coli* genome (131). The K_m for pyruvate (0.24 mM) of the *Z. palmae* PDC is the lowest of all bacterial

PDCs and is equivalent to the lowest K_m for pyruvate reported for all PDCs (0.24 mM pyruvate for the PDC from *K. lactis*) (105, 131). This enzyme also has the highest V_{max} (130 units per mg protein) of recombinant bacterial PDC proteins purified using similar conditions (131). The high level of *Z. palmae* PDC produced in recombinant *E. coli* combined with the biochemical characteristics of this enzyme make it an exciting enzyme for the development of new biocatalysts for fuel ethanol production (131).

In 1992, Lowe and Zeikus (132) purified a PDC from *Sarcina ventriculi*. This was only the second PDC from bacteria to be characterized and unlike *Z. mobilis* PDC it was substrate activated (132). The gene for this PDC was cloned and expressed recombinantly in *E. coli* (133). Production of this protein in recombinant *E. coli* was low, probably due to large differences in codon usage, therefore augmentation with accessory *t*RNAs was necessary (133). The deduced amino acid sequence of *S. ventriculi* PDC differs from the *Z. mobilis* PDC and the *Sv*PDC appears to have diverged from a common ancestor that included most fungal PDCs and bacterial indole-3-pyruvate decarboxylases (133). The purified enzyme is biphasic with a K_m of 2.8 mM and 10 mM for pyruvate for the high and low affinity sites, respectively (133). Expression of *S. ventriculi* PDC is higher in *Bacillus megaterium* when compared to *S. cerevisiae* PDC1, *Z. mobilis* PDC, and *Acetobacter pasteurianus* PDC, indicating that it will be a useful tool in the engineering of Gram-positive bacteria for ethanol production (this study).

PDC in Plants

In plants, PDC serves to convert pyruvate to acetaldehyde. The acetaldehyde is then converted to ethanol by alcohol dehydrogenase. In this manner these two enzymes catalyze a pathway in which NAD^+ is regenerated under anaerobic conditions such as

during seed germination and in plant roots when submerged (134). Despite the large number of PDCs from plants, relatively few have been characterized in detail.

In 1976, Wignarajah and Greenway tested for the effect of anaerobiosis on the roots of *Zea mays* (135). In this study, they determined that flushing nitrogen gas through solutions for a period of 4 to 15 hrs increased activity levels of both alcohol dehydrogenase and PDC in the *Z. mays* roots. The PDC from *Z. mays* was later purified and characterized (136, 137). It had a K_m of 0.5 mM for pyruvate and a V_{max} of 96 units per mg protein. *Z. mays* PDC was shown to be substrate activated, and cooperative binding of pyruvate decreased as the pH decreased leading to the enzyme being less dependant on pyruvate for activation (136).

The PDC from *Pisum sativum* is one of the most thoroughly characterized plant PDCs (76, 138-143). Based on Southern hybridization experiment, *P. sativum* has three genes for putative-PDCs, of which only one has been sequenced (143). The purified enzyme is composed of two different subunits (65 kDa and 68 kDa), but it is still unknown whether the two subunits are transcriptional products of the same or different genes (142). The *P. sativum* PDC is activated by its substrate (140) and is ten times more stable than the PDC from the yeast, *S. carlsbergensis* (142). The active enzyme is a mixture of tetramers, octomers, and higher oligomers (139, 142).

Acetaldehyde is a predominant aldehyde in orange juice (144) and significantly influences flavor (145). PDC is the key enzyme for the formation of acetaldehyde in oranges (146). The PDC purified from orange fruit is mechanistically similar to yeast PDC, except that it has only one active site (147).

Ipomoea batatas (sweet potato) produces PDC in its roots (148-150). This PDC is substrate activated, has a K_m of 0.6 mM, and is inhibited by phosphate (149). Pyruvate decarboxylation is the rate-limiting step in alcoholic fermentations in sweet potato roots based on the finding that PDC activity is 21- to 28-fold less than ADH activity under aerobic conditions, but 6- to 8-fold less than ADH under anaerobic conditions (150).

PDC has also been characterized from *Triticum aestivum* (wheat) (81, 82, 151-154), *Oryza sativa* (rice) (155-160), and *Vicia faba* (fava bean) (161). PDC has been shown to be produced in but not characterized from *Capsicum annuum* (bell pepper) fruit (162), *Echinochloa crus-galli* (barnyard grass) (163), *Nicotiana tobacum* (tobacco) (164), *Vitis vinifera* (grape) (165), *Lycopersicon esculentum* (tomato) (166), *Lepidium latifolium* (167), *Populus deltoides* (Eastern cottonwood) (168), *Glycine max* (soybean) (168), and *Arabidopsis thaliana* (169, 170)

Structure of Pyruvate Decarboxylase

The crystal structures of *Z. mobilis* (117), *S. uvarum* (84, 91) and *S. cerevisiae* (103) PDCs have been invaluable when studying PDC proteins for use and engineering for industrial application. By comparison of deduced amino acid sequences and biochemical characteristics it has been shown that the *A. pasteurianus* and *Z. palmae* PDCs are more closely related to *Z. mobilis* PDC (130, 131); whereas, the *S. ventriculi* PDC is more closely related to *S. cerevisiae* PDC1 (133). Because the majority of the bacterial PDC proteins were only recently discovered (130, 131, 133) there has not been sufficient time for detailed structural analysis of these enzymes. However, the crystal structure and mutagenesis analysis of the well characterized *Z. mobilis, S. uvarum* and *S.*

cerevisiae PDC proteins can give important and useful information about the structure of the newly identified bacterial PDC proteins.

Subunits of PDC

The quaternary structure of most PDCs is a tetramer with an apparent molecular weight of 240 kDa (79, 105, 111, 130-133, 148, 151, 155, 171), with the exception of PDCs forming larger complexes: *A. pateurianus* (130), *Z. mays* (135), *P. sativum* (139, 142), *T. aestivum* (81), and *N. crassa* (73). The association of the subunits has been determined to be pH-dependant with optimal pH for catalytic activity and subunit association of between pH 5.0 and pH 6.7 (108, 113, 131, 132, 147, 155). Until recently it was believed that the tetramer was the only active conformation (172), but a recent study showed that both dimers and tetramers of *Sc*PDC1 had comparable specific activity (173). This study, however, determined a difference in the dissociation constant for the regulatory substrate by one order of magnitude among the two forms indicating that binding of the substrate to the regulatory site is influenced by oligomerization (173). In contrast, the subunit interactions of the *Z. mobilis* PDC are different than those of *S. cerevisiae* PDC1 (117). Unlike the *S. cerevisiae* PDC1, *Z. mobilis* PDC is not controlled by allosteric regulation. The reason for this difference is elucidated in the crystal structures (117). *Z. mobilis* PDC dimers are packed tightly together and lock the enzyme in an activated form so that large conformational changes are not possible or necessary for enzyme activity as they are in *S. cerevisiae* PDC1 (91, 117). This tight packing of the dimers also explains the extreme stability of the *Z. mobilis* PDC in comparison to the *S. cerevisiae* PDC1 (174). This data is also in agreement with the differences in the thermostabilities of the bacterial PDCs. The *Z. mobilis*, *A. pasteurianus*, and *Z. palmae*

PDCs have temperature optima of 60°C, while *S. ventriculi* PDC has a temperature optimum of 32°C and is completely inactive at 60°C (131). Structural differences in the subunit interactions may be responsible for the instability of *S. ventriculi* PDC at high temperatures. Analysis of *S. ventriculi* PDC thermostability throughout a range of pH shows that the enzyme is more stable between pH 5.0 and pH 5.5 indicating that protonation of an amino acid side chain may stabilize the subunit interactions at high temperatures (this study).

Cofactors of PDC

Both Mg^{2+} and thiamin diphosphate (TPP) are required cofactors for the action of PDC (175, 176). It has been demonstrated that TPP dissociates from PDC under alkaline conditions, but it is difficult if not impossible to remove Mg^{2+} completely from the enzyme (137, 177-179). Mg^{2+} can be replaced by other divalent cations, such as Mn^{2+}, Ni^{2+}, Co^{2+}, and Ca^{2+} (176). The substitution of Mg^{2+} with these other cations does not affect the V_{max} of the enzyme, but it does affect the stability of the reconstructed holoenzyme (180, 181). A TPP derivative retaining the N-1'-4'amino system functions properly with full binding capacity therefore proving that this is the functional group necessary for activity of the PDC (182, 183). The *Z. mobilis* PDC retains its tetrameric state even after the TPP and Mg^{2+} cofactors are removed (178). This is also true of *S. ventriculi* PDC, *Z. palmae* PDC, and *S. cerevisiae* PDC1, but not of *A. pasteurianus* PDC (131). *A. pasteurianus* PDC forms both tetramers and octomers of similar specific activity and dissociates into dimers after cofactor extraction (131). Tetrameric configuration and activity are restored upon addition of the cofactors (131). The dissociation of the subunits is consistent with the behavior of other PDCs upon cofactor

removal (76). Residues responsible for binding the cofactors, as determined through X-ray crystallography studies, are conserved throughout yeast and bacterial PDC proteins (91, 103, 131).

Kinetics of PDC

There are currently two distinct groups of PDC proteins based on kinetics. All known PDC proteins, except those from Gram-negative bacteria, are allosterically regulated (76). The substrate activation behavior of *S. cerevisiae* PDC has been studied in detail through site-directed mutagenesis and crystal structure analysis (47, 86-91, 103, 184-191). Initial studies of the *S. uvarum* PDC determined that a cysteine residue was most likely responsible for the substrate activation behavior. In these studies, irreversible activation of the enzyme, exhibited by disappearance of the lag phase in product formation, was achieved by utilization of thiol specific reagents (80, 192-194). Use of a PDC1-PDC6 fusion protein that contained Cys221 as its only cysteine residue suggested that the Cys221 residue was responsible for the substrate activation behavior of *S. cerevisiae* PDC (86). Site-directed mutagenesis of the Cys221 and/or Cys222 to serine showed that the enzyme could no longer be activated by the substrate (87). Steady state kinetics studies were also used to bolster the argument for Cys221 as the site of substrate activation (88, 89). Although crystal structures of *S. uvarum* and *S. cerevisiae* PDC were determined in the presence and absence of effectors (84, 103, 187, 195), these crystal structures were not of high enough quality to determine where the activator molecules bound the enzyme . More recently, Lu et al. obtained a high resolution crystal structure of *S. uvarum* PDC in the presence of pyruvamide and determined that pyruvamide did not bind at or near Cys221 (91). This study also used kinetics to show that the Cys221Ser

was in fact still substrate activated (91); however, this data was later refuted by Wei et al. (90) who used solvent kinetic isotope effect to reaffirm that their original assertion that Cys221Ser does shift the enzyme into an active conformation. Lu et al. (91) determined the residues that bind pyruvamide in the regulatory site of the crystal structure of PDC1 as Tyr157 and Arg 224. Sergeinko et al. (191), however, argues that pyruvamide should not be considered to form an active conformation of the enzyme and may actually represent an inhibitory mode of binding. It is interesting to note that plant PDCs and the *S. ventriculi* PDC are substrate activated, yet the Cys221 equivalent is not conserved in these proteins while equivalent residues for Tyr157 and Arg224 are conserved (121, 131, 133).

The Gram-negative bacterial PDC proteins are the only known PDCs that exhibit Michaelis-Menten kinetics (111-113, 130, 131). These PDCs also have high affinity for the substrate pyruvate with a K_m of 0.24 mM pyruvate for *Z. palmae* PDC, 0.39 mM pyruvate for *A. pasteurianus* PDC, and 0.43 mM pyruvate for *Z. mobilis* PDC (131). The Gram-negative bacterial PDCs also have the highest V_{max} values of all PDCs (68, 131). The low K_m and high Vmax of *Z. mobilis* PDC have already been exploited successfully to engineer biocatalysts for fuel ethanol production (4).

Catalytic Residues of PDC

All crystal structures of TPP dependent enzymes have a glutamate residue close to the N-1' of TPP that promotes the ionization of the C-2 proton of TPP (121). Candy et al. demonstrated that substitution of Glu50 with either aspartate or glutamine yields an enzyme with 3.0% and 0.5% remaining catalytic activity of the wild-type enzyme, respectively (119). Each of these mutants also displays a decreased affinity for both

cofactors (119). The equivalent glutamate in yeast, Glu51, is also essential for catalytic activity (196). Only 0.04% catalytic activity of the wild-type enzyme remains upon substitution of Glu51 with glutamine and binding of TPP to the protein is slow (196). The *Z. mobilis* PDC crystal structure reveals that amino acid side chains Asp27, His113, His114, Thr388, and Glu473 are in the vicinity of the active site and are conserved among PDC proteins (117). This data corresponds well with the crystal structure data and site-directed mutagenesis studies of the *S. cerevisiae, Z. mobilis*, and *S. uvarum* PDCs (91, 120, 122, 195, 197).

Alternative Substrates of PDC

As discussed previously in this chapter, Neuberg and Hirsch (36, 38) first discovered that yeast could catalyze the formation of PAC when benzaldehyde was added to the medium. This reaction was later determined to be catalyzed by PDC (43-46). It has since been shown that the yeast PDCs are much more efficient at carboligase reactions than the PDC from *Z. mobilis* (65). The reason for this difference is believed to be the size of the active site cleft which is smaller in the *Z. mobilis* PDC than its yeast counterparts (117). Bruhn et al. found that the mutation of only one amino acid increased carboligase activity by *Z. mobilis* PDC by 4-fold when compared to wild-type (70). The crystal structure of *Z. mobilis* PDC showed other large side chains that were possible sites for mutagenesis to increase carboligase activity (117). Pohl et al. have since made these mutations and found a wide variety of carboligase activities catalyzed by these PDC variants, including one in which the stereochemistry has been changed to form (*S*)-phenylacetylcarbinol (123).

PDC from Brewer's yeast catalyzes the formation of acetoin through two separate mechanisms (198-200). Acetoin is produced by the aldol-type condensation reaction between two molecules of acetaldehyde or by the addition of acetaldehyde to an intermediate formed between pyruvate and thiamin pyrophosphate (198-200). Besides pyruvate, yeast PDCs have been shown to accept longer aliphatic α-keto acids like α -keto butanoic acid, α -keto pentanoic acid, branched aliphatic α -keto acids, as well as α -keto-phenylpropanoic acid (benzoylformate) and various phenyl-substituted derivatives of the latter (69, 201, 202). Only C4 and C5-keto acids have been shown to be substrates for PDC from *Z. mobilis* (65).

Study Rationale and Design

Engineering Gram-positive bacteria for ethanol production has been difficult due to the absence of suitably expressed *pdc* genes. A PDC was previously purified and characterized from the Gram-positive bacterium *S. ventriculi;* however the gene was not cloned (132). It was expected that *S. ventriculi* PDC will be expressed at high levels in Gram-positive hosts due to its origination from a Gram-positive bacterium. To test this possibility the *pdc* gene from *S. ventriculi* was cloned, sequenced, and characterized. *Sv*PDC was expressed in recombinant *E. coli* and the protein was biochemically characterized. *Sv*PDC was expressed in a Gram-positive host, *B. megaterium*. *Sv*PDC production in *B. megaterium* was analyzed and optimal conditions for *Sv*PDC activity in *B. megaterium* were determined. Expression analysis and optimization of a variety of PDCs (i.e. *Z. mobilis, A. pasteurianus, S. cerevisiae,* and *S. ventriculi*) in *B. megaterium* were performed to determine the optimal PDC for ethanol production in Gram-positive bacterial hosts.

CHAPTER 2
CLONING AND EXPRESSION OF *pdc*, AND CHARACTERIZATION OF PYRUVATE DECARBOXYLASE FROM *Sarcina ventriculi*

Introduction

PDC (EC 4.1.1.1) serves as the key enzyme in all homo-ethanol fermentations. This enzyme catalyzes the non-oxidative decarboxylation of pyruvate to acetaldehyde and carbon dioxide using Mg^{2+} and thiamine pyrophosphate (TPP) as cofactors. Acetaldehyde is subsequently reduced to ethanol by alcohol dehydrogenase (ADH, EC1.1.1.1) during the regeneration of NAD^+. PDC is widespread among plants, absent in animals, and rare in prokaryotes. Prior to this study, the only bacterial *pdc* gene described was from the Gram-negative α-proteobacterium *Zymomonas mobilis* (114-116, 203). *Z. mobilis* PDC was purified to homogeneity, crystallized, and extensively characterized (121). This enzyme has also been purified from an unusual Gram-positive organism, *Sarcina ventriculi* (132).

S. ventriculi is an obligate anaerobe that grows from pH 2 to pH 10, fermenting hexose and pentose sugars to produce acetate, ethanol, formate, CO_2 and H_2 (204, 205). In this organism, the relative production of ethanol and acetate vary with environmental pH. Under acidic conditions where acetic acid is toxic to cells, ethanol is the primary product (205). At neutral pH and above, a near equimolar mixture of ethanol and acetate are produced with low levels of formate (206). These changes in fermentation profiles

have been attributed to changes in the levels of two enzymes that metabolize pyruvate, PDC and pyruvate dehydrogenase (205, 206).

The properties of the *S. ventriculi* PDC are very different from those of the *Z. mobilis* enzyme. Unlike the Michaelis-Menten kinetics of *Z. mobilis* PDC (111, 116), the *S. ventriculi* enzyme is activated by pyruvate (132), similar to PDC enzymes from yeast and higher plants. *S. ventriculi* PDC was reported to have an unusually high K_m for pyruvate (13 mM) compared to K_m values of 0.3 mM to 4.4 mM for other PDC enzymes (111, 116, 207). The phenylalanine content of purified *S. ventriculi* PDC was reported to be 4-fold to 5-fold higher than that of other PDC enzymes suggesting significant differences in primary structure (132).

To further examine the unusual nature of the *S. ventriculi* PDC, this gene was cloned, sequenced, and expressed in recombinant *E. coli.* This approach provided the primary amino acid sequence and facilitated PDC purification for further kinetic and biophysical characterization.

Materials and Methods

Materials

Biochemicals were purchased from Sigma Chemical Co. (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, Mass.). Oligonucleotides were from Sigma-Genosys (The Woodlands, Tex.). Digoxigenin-11-dUTP (2'-deoxyuridine-5'-triphosphate coupled by an 11-atom spacer to digoxigenin), alkaline phosphatase conjugated antibody raised against digoxigenin, and nylon membranes for colony and plaque hybridizations were

from Roche Molecular Biochemicals (Indianapolis, Ind.). Positively charged membranes for Southern hybridization were from Ambion (Austin, Tex.).

Bacterial Strains and Media

Table 2-1 lists the *E. coli* strains used in this study including strains TB-1 and DH5α that were used for routine recombinant DNA experiments. *E. coli* strain SE2309 was used to create a genomic DNA library in plasmid pBR322. *E. coli* strains ER1647, LE392, and BM25.8 were used in conjunction with λBlueSTAR for a genomic DNA library. *E. coli* strains BL21(DE3), BL21-CodonPlus-RIL, and BL21-CodonPlus-RIL/pSJS1240 were used to examine the expression of the *S. ventriculi pdc* gene from plasmid pJAM419. *E. coli* strains were grown in Luria-Bertani (LB) medium and supplemented with antibiotics as appropriate (30 mg of chloramphenicol per liter, 100 mg of carbenicillin per liter, 100 mg of ampicillin per liter, and/or 50 mg of spectinomycin per liter). *S. ventriculi* strain Goodsir was cultivated as described previously (205).

DNA Isolation

Plasmid DNA was isolated and purified using a Quantum Prep Plasmid Miniprep Kit from BioRad (Hercules, Ca.). DNA fragments were eluted from 0.8% SeaKem GTG agarose (FMC Bioproducts, Rockland, Me.) using either Ultrafree-DA filters from Millipore (Bedford, Md.) or the QIAquick gel extraction kit from Qiagen (Valencia, Ca.). *S. ventriculi* genomic DNA was isolated and purified as described previously (208).

Cloning of the *S. ventriculi pdc* Gene

A degenerate oligonucleotide (5'-AARGARGTNAAYGTNGARCAYA-TGTTYGGNGT-3') was synthesized based on the N-terminal amino acid sequence of PDC purified from *S. ventriculi* (132)(where, R is A or G; N is A, C, G, or T; Y is C or T). This oligonucleotide was labeled at the 3'-end using terminal transferase with

digoxigenin-11-dUTP and dATP as recommended by the supplier (Roche Molecular Biochemicals) and was used to screen genomic DNA from *S. ventriculi*.

For Southern analysis, genomic DNA was digested with *Bgl*I, *Eco*RI, or *Hin*cII, separated by 0.8% agarose electrophoresis, and transferred to positively charged nylon membranes (209). Membranes were equilibrated at 58ºC for 2 h in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 1% blocking reagent (Roche Molecular Biochemicals), 0.1% *N*-lauroylsarcosine, and 0.02% SDS. After the probe (0.2 pmol per ml) and Poly(A) (0.01mg per ml) were added, membranes were incubated at 58ºC for 18.5 h. Membranes were washed twice with 2 X SSC containing 0.1% SDS (5 min per wash) at 25ºC and twice with 0.5 X SSC containing 0.1% SDS (15 min per wash) at 58ºC. Signals were visualized using colorimetric detection according to supplier (Roche Molecular Biochemicals).

For generation of a genomic library in plasmid pBR322, *S. ventriculi* chromosomal DNA was digested with *Hin*cII and fractionated by electrophoresis. The 2.5- to 3.5-kb *Hin*cII DNA fragments were ligated into the *Eco*RV site of pBR322 and transformed into *E. coli* SE2309. Colonies were screened with the degenerate oligonucleotide by colorimetric detection. By this method, plasmid pJAM400 that carries a *Hin*cII fragment containing 1,350 bp of the *pdc* gene was isolated.

The λBlueSTAR Vector System (Novagen) was used to create an additional genomic library to facilitate isolation of the full-length *pdc* gene from *S. ventriculi*. Genomic DNA was digested with *Bcl*I, separated by electrophoresis in 0.8% agarose, and the 6.5- to 8.5-kb fragments were ligated with the λBlueSTAR *Bam*HI arms. *In vitro* packaging and plating of phage was performed according to the supplier (Novagen). A

DNA probe was generated using an 800-bp *Eco*RI fragment of the *pdc* gene from pJAM400 that was labeled with digoxigenin-11-dUTP using the random primed method as recommended by the supplier (Roche Molecular Biochemicals). Plaques were screened using colorimetric detection. Cre-*loxP*-mediated subcloning was used to circularize the DNA of the positive plaques by plating λBlueSTAR phage with *E. coli* BM25.8 that expresses Cre recombinase (Novagen). The circularized plasmid pJAM410 was then purified and electroporated into *E. coli* DH5α.

For generation of a *pdc* expression vector, the promoterless *pdc* gene was subcloned into pET21d after amplification from pJAM413 (Table 2-1) by the polymerase chain reaction (PCR). Primers were designed for directional insertion using *Bsp*HI (oligo 1) and *Xho*I (oligo 2) restriction sites. The resulting fragment was ligated into compatible *Nco*I and *Xho*I sites of pET21d (Novagen) to produce pJAM419 (Figure 2-1). The fidelity of the *pdc* gene was confirmed by DNA sequencing.

Nucleotide and Protein Sequence Analyses

DNA fragments of plasmids pJAM400 and pJAM410 (Figure 2-1) were subcloned into plasmid vector pUC19 for determining the *pdc* sequence using the dideoxy termination method (210) and a LI-COR (Lincoln, Neb.) automated DNA sequencer (DNA Sequencing Facility, Department of Microbiology and Cell Science, University of Florida). The nucleotide sequence of the *S. ventriculi pdc* gene and surrounding DNA was deposited in the GenBank database (accession number AF354297).

Genepro 5.0 (Riverside Scientific, Seattle, WA), ClustalW version 1.81 (211), Treeview version 1.5 (212), and MultiAln (213) were used for DNA and/or protein

sequence alignments and comparisons. Deduced amino acid sequences were compared to protein sequences available in the GenBank, EMBL, and SwissProt databases at the National Center for Biotechnology Information (Bethesda, Md.) using the BLAST network server (214). The Dense Alignment Surface (DAS) method was used for the prediction of transmembrane α-helices (215).

Production of *S. ventriculi* PDC in Recombinant *E. coli*

Plasmid pJAM419 was transformed into *E. coli* BL21-CodonPlus-RIL containing plasmid pSJS1240 (Table 2-1). Expression of the *pdc* gene in this plasmid is regulated by the bacteriophage T7 RNA polymerase-promoter system (Novagen). Freshly transformed cells were inoculated into LB medium containing ampicillin, spectinomycin, and chloramphenicol and grown at 37ºC (200 rpm) until cells reached an $O.D._{600nm}$ of 0.6 to 0.8 (mid-log phase). Transcription/translation of *pdc* was initiated by the addition of 1 mM isopropyl-γ-D-thiogalactopyranoside (IPTG). Cells were harvested after 2-3 h by centrifugation at 5000 × *g* (10 min, 4ºC) and stored at –70ºC or in liquid nitrogen.

Purification of the *S. ventriculi* PDC Protein

All purification buffers contained 1 mM TPP and 1mM $MgSO_4$ unless indicated otherwise. Recombinant *E. coli* cells (14.8 g wet wt) were thawed in 6 volumes (wt/vol) of 50 mM Na-PO_4 buffer at pH 6.5 (Buffer A) and passed through a French pressure cell at 20,000 lb per in^2. Cell debris was removed by centrifugation at 16,000 × *g* (20 min, 4ºC). Supernatant was removed and filtered through a 45 μm filter membrane. Filtrate (692 mg protein) was applied to a Q Sepharose Fast Flow 26/10 column (Pharmacia) that was equilibrated with Buffer A containing 300 mM NaCl. The *Sv*PDC did not bind and eluted in the wash. The wash fractions containing PDC activity (326 mg protein) were

precipitated with 80% $(NH_4)_2SO_4$. Protein was dissolved in Buffer A, dialyzed against buffer A (4°C, 16 h), and filtered (.45 μm membrane). The filtrate (287 mg) was applied to a Q Sepharose column equilibrated with Buffer A and developed with a linear NaCl gradient (0 to 400 mM NaCl in 220 ml of Buffer A). PDC active fractions eluted at 230 to 300 mM NaCl and were pooled. The pooled sample (23 mg) was applied to a 5 ml Bio-scale hydroxyapatite type I column (BioRad) that was equilibrated with 5 mM Na-PO_4 buffer at pH 6.5 (Buffer B). The column was washed with 15 ml Buffer B and developed with a linear Na-PO_4 gradient (5 to 500 mM Na-PO_4 at pH 6.5 in 75 ml). Protein fractions (11.4 mg) with PDC activity eluted at 200 to 300 mM Na-PO_4 and were pooled. For further purification, portions of this material (0.25 to 0.5 mg protein per 0.25 to 0.5 ml) were applied to a Superdex 200 HR 10/30 column (Pharmacia) equilibrated in 50 mM Na-PO_4 at pH 6.5 with 150 mM NaCl and 10% glycerol in the presence or absence of 1 mM $MgSO_4$ and 1 mM TPP.

Activity Assays

PDC activity was assayed by monitoring the pyruvate-dependent reduction of NAD^+ with baker's yeast alcohol dehydrogenase (ADH) (Sigma) as a coupling enzyme at pH 6.5 as previously described (115), with the following modifications. Buffered enzyme (100 μl) was added to a final volume of 1 ml containing 0.15 mM NADH, 0.1 mM TPP, 0 to 25 mM pyruvate, and 10 U ADH in 50 mM potassium-MES (2-[N-morpholino]ethanesulfonic acid) buffer with 5 mM $MgCl_2$ at pH 6.5. Since this assay does not distinguish PDC from NADH oxidizing enzymes such as lactate dehydrogenase, activity of cell lysate was estimated by correcting for control reactions performed in the absence of added ADH. One unit of enzyme activity is defined as amount of enzyme that

oxidizes 1 μmol of NADH per min. Thermostability was determined by incubating purified PDC in 50 mM Na-PO_4 buffer at pH 6.5 with 1 mM TPP and 1 mM $MgCl_2$ for 90 min and then assaying for activity with 10 mM pyruvate under standard conditions. Protein concentration was determined using Bradford protein reagent with bovine serum albumin as the standard (BioRad).

Molecular Mass and Amino Acid Sequence Analyses

Subunit molecular mass was estimated by reducing and denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% polyacrylamide gels which were stained with Coomassie blue R-250. The molecular weight standards for SDS-PAGE were: phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). For determination of native molecular mass, samples were applied to a Superdex 200 HR 10/30 column equilibrated with 50 mM Na-PO_4 buffer at pH 6.5 with 150 mM NaCl, 10% glycerol, and no added cofactors. Molecular mass standards included: serum albumin (66-kDa), alcohol dehydrogenase (150 kDa), α-amylase (200 kDa), apoferritin (443 kDa), and thyroglobulin (669 kDa).

The N-terminal sequence was determined for PDC protein purified from recombinant *E. coli*. The protein was separated by SDS-PAGE and electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P). The sequence was determined by automated Edman degradation at the protein chemistry core facility of the University of Florida Interdisciplinary Center for Biotechnology Research.

Results and Discussion

PDC Operon in *S. ventriculi*

The N-terminal amino acid sequence of the PDC protein purified from *S. ventriculi* (132) was used to generate a degenerate oligonucleotide for hybridization to genomic DNA. This approach facilitated the isolation of a 7.0-kb *Bcl*I genomic DNA fragment from *S. ventriculi*. The fragment was further subcloned in order to sequence both strands of a 3,886 bp *Hin*cII-to-*Hin*cII region that hybridized to the oligonucleotide probe (Figure 2-1). Analysis of the DNA sequence revealed an open reading frame (ORF) of 1,656 bp encoding a protein with an N-terminus identical to that of the previously purified *S. ventriculi* PDC (Figure 2-2). The ORF is therefore designated *pdc*. A canonical Shine-Dalgrano sequence is present 7 bp upstream of the *pdc* translation start codon. In addition, a region 82 to 110 bp upstream of *pdc* has limited identity to the eubacterial –35 and –10 promoter consensus sequence. Downstream (43 bp) of the *pdc* translation stop codon is a region predicted to form a stem-loop structure followed by an AT-rich region, consistent with a ρ-independent transcription terminator. Thus, the *S. ventriculi pdc* appears to be transcribed as a monocistronic operon like the *Z. mobilis pdc* gene (115).

A partial ORF was identified 722 bp upstream of *pdc* which encodes a 177 amino acid protein fragment (ORF1*) (Figure 2-1). ORF1* has identity (28-29 %) to several hypothetical membrane proteins (GenBank accession numbers CAC11620, CAC24018, CAA22902) and is predicted to form several transmembrane spanning domains (data not shown).

PDC Protein Sequence in *S. ventriculi*

The *S. ventriculi pdc* gene apparently encodes a protein of 552 amino acids (including the N-terminal methionine) with a calculated pI of 5.16 and anhydrous molecular mass of 61,737 Da. Consistent with other *Z. mobilis* and fungal PDC proteins, the N-terminal extension of up to 47 amino acids that is common to plant PDC proteins is not conserved in the *S. ventriculi* PDC protein. Although the *pI* of the purified *S. ventriculi* PDC protein has not been experimentally determined, the calculated pI is consistent with the acidic pH optimum of 6.3 to 6.7 for stability and activity of this enzyme (132). The amino acid composition of the protein deduced from the *S. ventriculi pdc* gene is similar to that determined for the *S. cerevisiae PDC1* and *Z. mobilis pdc* genes (Table 2-2). A notable exception is the alanine composition of *Z. mobilis* PDC, which is 1.8- to 2.2-fold higher than the composition of *S. cerevisiae* PDC1 and *S. ventriculi* PDC. Although the phenylalanine composition of the deduced *S. ventriculi* PDC protein is consistent with the other PDC proteins, it is almost 3.6-fold less than the composition previously reported for the purified *S. ventriculi* enzyme (132). The reason for this discrepancy remains to be determined.

The amino acid sequence of *S. ventriculi* PDC was aligned with the sequences of the yeast (Sc) PDC1 and *Z. mobilis* (Zm) PDC proteins, both of which have been analyzed by X-ray crystallography (76, 84, 103, 117) (Figure 2-3). The conserved motif of TPP-dependent enzymes identified by Hawkins et al. (216) and known to be involved in Mg^{2+}-TPP cofactor binding is highly conserved in all three PDC proteins. Other amino acid residues located within a 0.4 nm distance of the Mg^{2+} and TPP binding site of the two crystallized PDC proteins are also conserved in the *S. ventriculi* PDC protein.

These include residues with similarity to the aspartate (SceD444, ZmoD440) and asparagine residues (SceN471, ZmoN467) that are involved in binding Mg^{2+}. The *S. ventriculi* PDC appears to be more similar to the yeast PDC than to that of *Z. mobilis* in binding the diphosphates of TPP where serine and threonine side chains (SceS446 and T390) as well as the main chain nitrogen of isoleucine (SceI476) are conserved. This contrasts with the *Z. mobilis* enzyme, which utilizes a main chain nitrogen of aspartate (ZmoD390) instead of the threonine hydroxyl group (SceT390) for binding the β-phosphate. *S. ventriculi* PDC residues are also similar to the aspartate, glutamate, threonine, and histidine residues (SceD28, E477, T388, H114 and H115; ZmoD27, E473, T388, H113, and H114) which may potentially interact with intermediates during the decarboxylation reaction mediated by *Z. mobilis* and yeast PDCs. Furthermore, the isoleucine (Sc and Zm I415) side chain which appears to stabilize the V conformation of TPP through Van der Waals interactions as well as the glutamate (SceE51, ZmoE50) which may donate a proton to the N1' atom of TPP are conserved in the *S. ventriculi* PDC protein sequence.

A notable exception in conservation is the yeast C221 residue (Figure 2-3), which is highly conserved among the majority of fungal PDC enzymes but is not conserved in either bacterial or plant PDC proteins. Based on site directed mutagenesis, chemical modification, and kinetic studies, this C221 residue has been proposed to be a primary binding site of the regulatory substrate molecule and the starting point of a signal transfer pathway to the active site TPP in the yeast enzyme (87, 89, 193). Consistent with these previous results the yeast C221 is positioned in a large cavity formed at the interface among all three PDC domains including the α or PYR (residues 1 to 189), β or R

(residues 190 to 356), and γ or PP (residues 357 to 563) domains (84). However, recent high-resolution structural analysis of the brewer's yeast PDC crystallized in the presence of pyruvamide, a pyruvate analogue, enabled localization of the activator binding site and revealed that cysteine does not play a direct role in this binding (91). Additionally, kinetic studies using stopped-flow techniques revealed that the C221A variant of yeast PDC was still substrate activated, and the lag phase of product formation did not disappear with progressive thiol oxidation (91). Instead, tyrosine (Y157) and arginine (R224) residues form hydrogen bonds with the amide group of pyruvamide. Both of these residues are conserved in the *S. ventriculi* PDC and not found in the *Z. mobilis* enzyme which displays Michaelis-Menten kinetics. These results suggest that residues of the *S. ventriculi* PDC protein may allosterically bind the substrate activator with a mechanism common to the majority of fungal PDC proteins. Interestingly, these two residues that bind pyruvamide in the yeast enzyme are not universally distributed among the substrate activated PDC enzymes, most notably the plant PDCs.

Phylogenetic analysis was performed to compare the PDC proteins and other TPP-dependent enzymes including indole-3-pyruvate decarboxylase (IPD), the E1 component of pyruvate dehydrogenase (E1), acetolactate synthase (ALS), and transketolase (TK) (Figure 2-4). The comparison reveals that all of these proteins are related in primary sequence and that there is a significant clustering of the sequences into families based on specific enzyme function. Of these, the *S. ventriculi* PDC appears most closely related to eubacterial IPD proteins as well as the majority of fungal PDC proteins. In contrast, the *Z. mobilis* PDC protein is most closely related to plant PDCs in addition to a couple of out-grouping fungal PDCs. Thus, it appears that the IPD protein family

has close evolutionary roots with the PDC family and specifically the *S. ventriculi* PDC protein. In addition, the distant relationship of the *S. ventriculi* and *Z. mobilis* PDC proteins is consistent with the biochemical differences between these two enzymes (111, 116, 132, 207). In contrast to *Z. mobilis* PDC which may have originated by the horizontal transfer of a plant *pdc* gene, *S. ventriculi* PDC appears to have diverged early during evolution and last shared a common ancestor with most eubacterial IPD and fungal PDC enzymes.

The unique nature of the *S. ventriculi* PDC enabled us to search for previously unknown PDC-like proteins (Figure 2-5). A new subfamily of hypothetical PDC proteins from Gram-positive bacteria has now been identified for further study due to their similarity to the newly identified *S. ventriculi* PDC. This subfamily includes PDC proteins from Gram-positive organisms including two bacilli, *Bacillus anthracis* and *Bacillus cereus*.

Production of *S. ventriculi* PDC Protein

Unlike *Z. mobilis pdc*, the codon usage of the *pdc* of *S. ventriculi* dramatically differs from that of *E. coli* (Table 2-3). In particular, the *pdc* gene of *S. ventriculi* requires elevated use of $tRNA_{AUA}$ and $tRNA_{AGA}$ both of which are relatively rare in *E. coli*. This is in contrast to the *Z. mobilis pdc* gene, which does not use the AUA codon and has only minimal use of the AGA codon. This suggests that production of the *S. ventriculi* PDC protein in recombinant *E. coli* may be limited by mRNA translation.

To further investigate this, the levels of the tRNA genes that are rare in *E. coli* were modified during *pdc* expression by including multiple copies of these genes on the

chromosome (*E. coli* strain BL21-CodonPlus-RIL) and/or on a complementary plasmid (pSJS1240) (Table 2-1). These modified *E. coli* strains were transformed with plasmid pJAM419 which carries the *S. ventriculi pdc* gene positioned 8 bp downstream of an optimized Shine-Dalgrano consensus sequence and controlled at the transcriptional-level by T7 RNA polymerase promoter and terminator sequences from plasmid vector pET21d. Detectable levels of PDC activity (0.16 U per mg protein at 5 mM pyruvate) were observed after induction of *pdc* transcription in *E. coli* host strains with additional chromosomal and/or plasmid copies of the *ileU/ileX*, *argU* and *leuW* genes encoding the rare $tRNA_{AUA}$, $tRNA_{AGG/AGA}$, and $tRNA_{CUA}$. A 5- to 10-fold increase in the level of a 58-kDa protein with a molecular mass comparable to the *S. ventriculi* PDC (Figure 2-6) was produced in these strains compared to a similar *E. coli* strain without added tRNA genes [BL21(DE3)] (data not shown). These results suggest that the *S. ventriculi* PDC protein is synthesized in recombinant *E. coli* and that the high percentage of AUA and AGA codons of the *pdc* gene probably limits translation.

Interestingly, additional proteins of 43 and 27 kDa were also observed when the PDC protein was synthesized in *E. coli* compared to control strains (Figure 2-6). The origin of these proteins remains to be determined. They may be fragments of PDC generated by proteolysis or truncated PDC produced from errors in translation/transcription. Alternatively, increased production of PDC may increase the levels of acetaldehyde, which can be toxic to the cell, and may subsequently induce the levels of proteins in response to this stress.

Properties of the *S. ventriculi* PDC Protein from Recombinant *E. coli*

The *S. ventriculi* PDC protein was purified over 136-fold from a recombinant *E. coli*. The N-terminal amino acid sequence of this protein (MKITIAEYLLXR, where X is

an unidentified amino acid) was identical to the sequence of PDC purified from *S. ventriculi* (132). Both PDC proteins have an N-terminal methionine residue, which suggests that this residue is not accessible for cleavage by either the *S. ventriculi* or *E. coli* aminopeptidases.

The thermostability of the purified *S. ventriculi* PDC was examined in the presence of 1 mM cofactors TPP and Mg^{2+} at pH 6.5. Enzyme activity was stable up to 42˚C but was abolished after incubation for 60 to 90 min at temperatures of 50˚C and above. This is consistent with the significant loss of PDC activity observed when a thermal treatment step (60º C for 30 min) was included in the purification (data not shown). In contrast, methods used to purify *Z. mobilis* and other PDC proteins (76) typically incorporate thermal treatment to remove unwanted proteins. These results suggest that the recombinant *S. ventriculi* PDC protein is not as thermostable as other PDC proteins including that of *Z. mobilis.*

PDC proteins have been shown to bind TPP and Mg^{2+} cofactors with high affinity at slightly acidic pH (76). Consistent with this, the recombinant *S. ventriculi* PDC retains full activity after incubation at 37˚C for 90 min in the presence of 25 mM EGTA or EDTA in pH 6.5 buffer without cofactors. This is similar to the PDC protein purified directly from *S. ventriculi* which is fully active after similar treatment with metal chelators.

The recombinant *S. ventriculi* enzyme displays sigmoidal kinetics (Figure 2-7) suggesting substrate activation similar to the fungal and plant PDC proteins (76). This contrasts with the *Z. mobilis* PDC which is the only PDC protein known to display Michaelis-Menten kinetics. The recombinant *S. ventriculi* PDC has a K_m of 2.8 mM for

pyruvate and V_{max} of 66 U per mg protein for acetaldehyde production (Figure 2-7). This K_m value is almost 5-fold less than the K_m (13 mM) observed for the PDC purified from *S. ventriculi* (132); however, it is within the range of K_m values determined for many of the fungal and plant PDCs including those purified from *S. cerevisiae* (1 to 3 mM)(184, 217), *Zygosaccharomyces bisporus* (1.73 mM) (107), orange (0.8 to 3.2 mM) (147), and wheat germ (3 mM) (81). The K_m value for the recombinant PDC protein is several-fold higher than those values reported for the PDCs of *Z. mobilis* (0.3 to 0.4 mM) (112, 113) and rice (0.25 mM)(155).

The reason for the apparent discrepancy in affinity for pyruvate between the PDC purified from *S. ventriculi* and that from recombinant *E. coli* may in part be due to the type of buffer used in the enzyme assay (sodium hydrogen maleate vs. potassium-MES buffer pH 6.5, respectively). The *Z. mobilis* PDC, which was reported to have a K_m of 4.4 mM for pyruvate was determined in Tris-maleate buffer at pH 6 (111) while the K_m values of 0.3 to 0.4 mM were from assays using potassium-MES buffer at pH 6 (112) and sodium citrate buffer at pH 6.5, respectively (113). It is also possible that the different methods used for purification of the *S. ventriculi* PDC protein may have influenced its affinity. In our study, the cofactors TPP and Mg^{2+} were included in the buffers for all purification steps. This differs from the initial steps used for purification of PDC from *S. ventriculi* (132) which may have resulted in partial loss of cofactors and decreased affinity of the enzyme for its substrate, pyruvate. If so, it is more likely TPP than Mg^{2+} since metal chelators do not influence the activity of either the PDC purified from recombinant *E. coli* (described above) or the enzyme purified from *S. ventriculi* at pH 6.5 (132). An additional possibility is that synthesis of PDC in *E. coli* may have modified the

affinity of the enzyme through misincorporation of amino acids due to a high percentage of rare codons in the gene.

At pH 6.5, the recombinant PDC forms a 235-kDa homotetramer consisting of a 58-kDa protein, as determined by Superdex 200 gel filtration chromatography and SDS-12% PAGE electrophoresis (see methods). Exclusion of the cofactors from the buffer during gel filtration chromatography at pH 6.5 did not alter the tetramer configuration or enzyme activity, suggesting that the cofactors are tightly bound. The configuration of the PDC complex is consistent with that purified from *S. ventriculi* as well as the majority of those isolated from fungi, plants, and *Z. mobilis*. There are however, plant PDCs which have been reported to form larger complexes including the PDC from *Neurospora crassa* which forms aggregated filaments of 8-10 nm (73) as well as the PDC from *Pisum sativum* which forms up to 960 kDa complexes (142).

Conclusion

Based on this study, the *S. ventriculi* PDC protein appears to share similar primary sequence structure to TPP-dependent enzymes and is highly related to the fungal PDC and eubacterial IPD enzymes. The close relationship of the *S. ventriculi* and fungal PDC structures is consistent with the similar biochemical properties of these enzymes. Both types of enzymes display substrate cooperativity with similar affinities for pyruvate. The structure and biochemistry of the *S. ventriculi* PDC, however, dramatically contrast with the only other bacterial PDC (*Z. mobilis*) that has been characterized. The *Z. mobilis* PDC is closely related to plants in primary structure; however, it is the only PDC enzyme known to display Michaelis-Menten kinetics.

This study also demonstrates the synthesis of active, soluble *S. ventriculi* PDC protein in recombinant *E. coli*. Only two other genes, the *Z. mobilis pdc* and *S. cerevisiae PDC1* genes, have been reported to synthesize PDC protein in recombinant bacteria (114, 115, 218). Of these, at least 50% of the *S. cerevisiae* PDC1 forms insoluble inclusions in *E. coli* and thus has not been useful in engineering bacteria for high-level ethanol production (218). Due to codon bias, accessory tRNA is essential for efficient production of *S. ventriculi* PDC in recombinant *E. coli*. However, the low G+C codon usage of the *S. ventriculi pdc* gene should broaden the spectrum of bacteria that can be engineered as hosts for high-level production of PDC protein and the engineering of homo-ethanol pathways (4). The *S. ventriculi* PDC is unique among previously characterized bacterial PDCs. This has enabled the identification of a new subfamily of PDC-like proteins from Gram-positive bacteria that will broaden the host range of future endeavors utilizing Gram-positive bacterial hosts.

Table 2-1. Strains and plasmids used for production of PDC from *S. ventriculi* in *E. coli*.

Strain or plasmid	Phenotype, genotype, description, PCR primers	Source
S. ventriculi Goodsir	American Type Culture Collection 55887	American Type Culture Collection (Manassas, Va.)
E. coli TB-1	F$^-$ *ara* Δ(*lac-proAB*) *rpsL* (Str^r) [ϕ80*lac* Δ(*lacZ)M15*] *thi hsdR*(r_k^- m_k^+)	New England BioLabs (Beverly, Mass.)
E. coli DH5α	F$^-$ *recA1 endA1 hsdR17*(r_k^- m_k^+) *supE44 thi-1 gyrA relA1*	Life Technologies (Rockville, Md.)
E. coli SE2309	F$^-$ e14$^-$(McrA$^-$) *endA1 supE44 thi-1 relA1? rfbD1? spoT1?* Δ(*mcrC-mrr*)*114::IS10 pcnB80 zad2084*::Tn*10*	provided by K. T. Shanmugam (Univ. of Fl.)
E. coli ER1647	F$^-$ *fhuA2*Δ(*lacZ*) *r1 supE44 trp31 mcrA1272::*Tn*10*(Tet^r) *his-1 rpsL104* (Str^r)*xyl-7 mtl-2 metB1* Δ(*mcrC-mrr*)*102::*Tn*10*(Tet^r) *recD1040*	Novagen (Madison, Wi.)
E. coli LE392	F$^-$ e14-(McrA$^-$) *hsdR514*(r_k^- m_k^+) *supE44 supF58 lacY1 or* Δ(*lacIZY*)*6 galK2 galT22 metB1 trpR55*	Novagen
BM25.8	*supE thiD*(*lac-proAB*) [F' *traD36 proA*$^+$*B*$^+$ *lacI*^{q}*ZΔM15*] λimm^{434}(kan^R) P1 (Cm^R) *hsdR* ($r_{K12}^-$$m_{K12}^+$)	Novagen
E. coli BL21-CodonPlus-RIL	F$^-$ *ompT hsdS*(r_B^- m_B^-) *dcm*$^+$ Tet^r *gal* λ (DE3) *endA* Hte [*argU ileY leuW* Cam^r] (an *E.coli* B strain)	Stratagene (La Jolla, Ca.)
E. coli BL21(DE3)	F$^-$ *ompT gal*[*dcm*] [*lon*] *hsdS*$_B$ ($r_B^-$$m_B^-$; an *E. coli* B strain) with λDE3, a λ prophage carrying the T7 RNA polymerase gene	Novagen
pBR322	Ap^r, Tc^r; cloning vector	New England Biolabs
pUC19	Ap^r; cloning vector	New England Biolabs
pBlueSTAR-1	Ap^r; plasmid derived from λBlueSTAR-1	Novagen
pET21d	Ap^r; expression vector	Novagen
pSJS1240	Sp^r; derivative of pACYC184 with *E. coli ileX* and *argU*	(219)
pJAM400	Ap^r; two 3-kb *Hin*cII fragments of *S. ventriculi* genomic DNA ligated into the *Eco*RV site of pBR322; carries only 1350 bp of *pdc*	This study
pJAM410	Ap^r; 7-kb fragment of *S. ventriculi* genomic DNA with the complete *pdc* gene in pBlueSTAR-1	This study

Table 2-1. Continued.

Strain or plasmid	Phenotype, genotype, description, PCR primers	Source
pJAM411	Ap^r; 6-kb *Swa*I fragment from pJAM410 ligated into the *Hin*cII site of pUC19; carries 2103 bp of pBlueSTAR-1 vector and 4 kb of *S. ventriculi* genomic DNA with the complete *pdc*	This study
pJAM413	Ap^r; 3-kb *Sac*II fragment of pJAM411 with the complete *pdc* gene in the *Hin*cII site of pUC19	This study
pJAM419	Ap^r; 1.7-kb *Bsp*HI-to-*Xho*I fragment generated by PCR amplification using pJAM410 as a template, oligo1, 5'-ggcc**tcatga**aaataacaattgcag-3', and oligo2, 5'-gcggg**ctcgag**attagtagttattttg-3'(*Bsp*HI and *Xho*I sites indicated in bold); ligated with *Nco*I-to-*Xho*I fragment of pET21d; carries complete *pdc* with its start codon positioned 8 bp downstream the Shine-Dalgrano sequence of pET21d	This study

Table 2-2. Amino acid composition of PDC proteins. Composition expressed as % residues per mol enzyme predicted from the gene sequence (g) or chemically determined from the purified enzyme (e). Abbreviations: Sv, *Sarcina ventriculi* PDC; Sc, *Saccharomyces cerevisiae* PDC1; Zm, *Zymomonas mobilis* PDC; ND, not determined. References: Sv(e) (132), Sv(g) (this study), Sc(g) (99), Zm (g) (113).

Amino	Composition (mol%)			
Acid	Sv(e)	Sv(g)	Sc(g)	Zm(g)
Asx	8.7	9.8	10.2	10.2
Glx	10.7	12.0	8.9	8.6
Ser	5.5	6.5	6.0	4.2
Gly	7.3	7.1	7.8	8.1
His	1.1	1.6	2.0	2.1
Arg	4.1	4.0	2.7	3.0
Thr	6.1	6.5	7.7	4.6
Ala	7.2	6.9	8.2	15.0
Pro	2.7	2.7	4.6	4.8
Tyr	3.2	4.0	3.1	3.9
Val	6.8	8.0	7.5	7.8
Met	2.1	2.7	2.4	1.9
Ile	6.1	7.1	6.6	4.9
Leu	7.8	8.2	9.7	8.8
Phe	16.2	4.7	4.2	3.2
Lys	4.4	6.9	6.2	6.3
Cys	ND	0.9	1.1	1.2
Trp	ND	0.5	1.3	1.2

Table 2-3. Codon usage of *S. ventriculi* (Sv) and *Z. mobilis* (Zm) *pdc* genes.

Amino Acid	Codon	Zm*	Sv	*E. coli*†
Ala	GCA	28.1	32.5	20.1
	GCC	35.1	0	25.5
	GCG	12.3	1.8	33.6
	GCU	73.8	34.4	15.3
Arg	**AGA**	**1.8**	**39.8**	**2.1**
	CGC	15.8	0	22.0
	CGG	1.8	0	5.4
	CGU	12.3	0	20.9
Asn	AAC	47.5	19.9	21.7
	AAU	10.5	27.1	17.7
Asp	GAC	28.1	3.6	19.1
	GAU	10.5	47.0	32.1
Cys	UGC	28.1	1.8	6.5
	UGU	10.5	7.2	5.2
Gln	CAA	0	28.9	15.3
	CAG	17.6	0	28.8
Glu	GAA	61.5	85.0	39.4
	GAG	3.5	5.4	17.8
Gly	GGA	1.8	50.6	8.0
	GGC	19.3	0	29.6
	GGG	0	0	11.1
	GGU	56.2	19.9	24.7
His	CAC	8.8	3.6	9.7
	CAU	14.1	12.7	12.9
Ile	**AUA**	**0**	**39.8**	**4.4**
	AUC	35.1	9.0	25.1
	AUU	14.1	21.7	30.3
Leu	CUA	0	7.2	3.9
	CUC	19.3	0	11.1
	CUG	33.4	0	52.6
	CUU	12.3	14.5	11.0
	UUA	1.8	59.7	13.9
	UUG	5.4	0	13.7
Lys	AAA	33.4	63.3	33.6
	AAG	31.6	5.4	10.3
Met	AUG	21.1	27.1	27.9
Phe	UUC	31.6	18.1	16.6
	UUU	0	28.9	22.3
Pro	CCA	3.5	18.1	8.4
	CCC	3.5	0	5.5
	CCG	29.9	1.8	23.2
	CCU	10.5	7.2	7.0

Table 2-3. Continued

Amino Acid	Codon	Zm*	Sv	*E. coli*†
Ser	AGC	14.1	12.7	16.1
	AGU	7.0	12.7	8.8
	UCA	1.8	32.5	7.2
	UCC	15.8	0	8.6
	UCU	5.3	7.2	8.5
Thr	ACA	0	34.4	7.1
	ACC	28.1	0	23.4
	ACG	10.5	0	14.4
	ACU	8.8	30.7	9.0
Trp	UGG	12.3	5.4	15.2
Tyr	UAC	12.3	7.2	12.2
	UAU	26.4	32.5	16.2
Val	GUA	0	36.2	10.9
	GUC	26.4	0	15.3
	GUG	7.0	0	26.4
	GUU	43.9	43.4	18.3

*Codon usage for amino acids represented as frequency per thousand bases. Stop codons are not indicated. CGA and AGG codons for Arg, UCG for Ser, and GGG for Gly were not used for either of the *pdc* genes. Abbreviations: Zm, *Zymomonas mobilis*; Sv, *Sarcina ventriculi*.

†Average usage in frequency per thousand bases for genes in *E. coli* K-12. Highlighted are codons for accessory tRNAs essential for high-level synthesis of *S. ventriculi* PDC in recombinant *E. coli*.

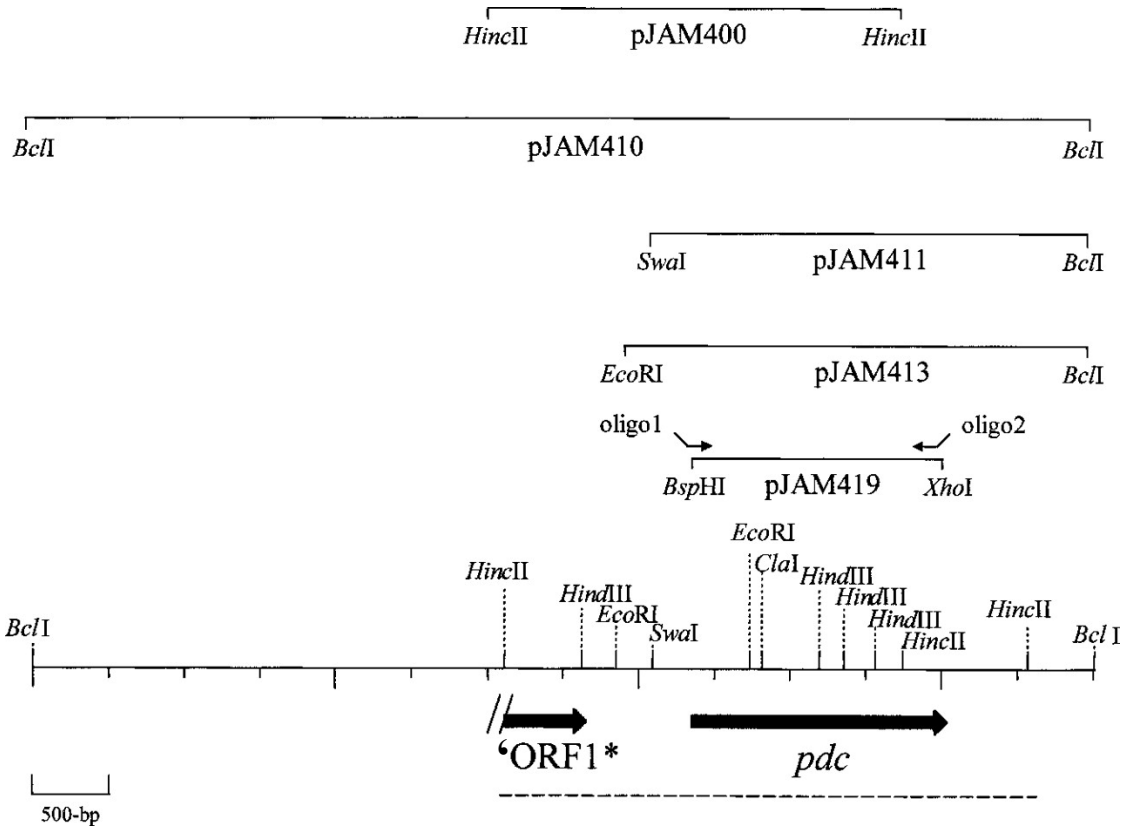

Figure 2-1. A partial map of restriction endonuclease sites for a 7-kb *Bcl*I genomic DNA fragment from *S. ventriculi*. Plasmids used in this study include pJAM410 which carries the complete 7-kb *Bcl*I fragment as well as pJAM400, pJAM411, and pJAM413 which were used for DNA sequence analysis. Plasmid pJAM419 was used for expression of the *S. ventriculi pdc* gene in recombinant *E. coli*. The location of the *pdc* gene and 'ORF1* are shown directly below the physical map with large arrows indicating the direction of transcription. The dashed line below the physical map indicates the 3,886 bp *Hin*cII-to-*Hin*cII region sequenced. Abbreviations: *pdc*, pyruvate decarboxylase gene; 'ORF1*, partial open reading frame of 177 amino acids with no apparent start codon.

```
            -35                    -10
AAATTTAAAAATAACATCAGATAAATCGTTTATATTAATTTTTACTAAAAGCTATTTAAA   60
          ttgaca-------N17-------tataat
                                                    SD
GGTGTATTATATATACATAGTTTATCTTATAAATAAAAAATGAATTGGAGGAAATACATA  120

ATGAAAATAACAATTGCAGAATACTTATTAAAAAGATTAAAAGAAGTAAATGTAGAGCAT  180
M  K  I  T  I  A  E  Y  L  L  K  R  L  K  E  V  N  V  E  H    20
M  K  I  I  I  A  E  Y  L  L  K  R  L  K  E  V  N  V  E  H
ATGTTTGGAGTTCCTGGAGATTATAACTTAGGATTTTTAGATTATGTTGAAGATTCTAAA  240
M  F  G  V  P  G  D  Y  N  L  G  F  L  D  Y  V  E  D  S  K    40
M  F  G  V  P  G  D  Y  N  L  G  F  L  D  Y  V
GATATTGAATGGGTTGGAAGCTGTAATGAACTTAATGCAGAATATGCAGCAGATGGATAT  300
D  I  E  W  V  G  S  C  N  E  L  N  A  E  Y  A  A  D  G  Y    60

GCAAGACTTAGAGGATTTGGTGTAATACTTACAACTTATGGAGTTGGTTCACTTAGTGCA  360
A  R  L  R  G  F  G  V  I  L  T  T  Y  G  V  G  S  L  S  A    80

ATAAATGCTACAACAGGTTCATTTGCAGAAAATGTTCCAGTATTACATATATCAGGTGTA  420
I  N  A  T  T  G  S  F  A  E  N  V  P  V  L  H  I  S  G  V   100

CCATCAGCTTTAGTTCAACAAAACAGAAAGCTAGTTCACCATTCAACTGCTAGAGGAGAA  480
P  S  A  L  V  Q  Q  N  R  K  L  V  H  H  S  T  A  R  G  E   120

TTCGACACTTTTGAAAGAATGTTTAGAGAAATAACAGAATTTCAATCAATCATAAGCGAA  540
F  D  T  F  E  R  M  F  R  E  I  T  E  F  Q  S  I  I  S  E   140

TATAATGCAGCTGAAGAAATCGATAGAGTTATAGAATCAATATATAAATATCAATTACCA  600
Y  N  A  A  E  E  I  D  R  V  I  E  S  I  Y  K  Y  Q  L  P   160

GGTTATATAGAATTACCAGTTGATATAGTTTCAAAAGAAATAGAAATCGACGAAATGAAA  660
G  Y  I  E  L  P  V  D  I  V  S  K  E  I  E  I  D  E  M  K   180

CCGCTAAACTTAACTATGAGAAGCAACGAGAAAACTTTAGAGAAATTCGTAAATGATGTA  720
P  L  N  L  T  M  R  S  N  E  K  T  L  E  K  F  V  N  D  V   200

AAAGAAATGGTTGCAAGCTCAAAAGGACAACATATTTTAGCTGATTATGAAGTATTAAGA  780
K  E  M  V  A  S  S  K  G  Q  H  I  L  A  D  Y  E  V  L  R   220

GCTAAAGCTGAAAAAGAATTAGAAGGATTTATAAATGAAGCAAAAATCCCAGTAAACACT  840
A  K  A  E  K  E  L  E  G  F  I  N  E  A  K  I  P  V  N  T   240
```

Figure 2-2. Nucleic acid and predicted amino acid sequence of the *S. ventriculi pdc* gene. DNA is shown in the 5'- to 3'-direction. Predicted amino acid sequences are shown in single-letter code directly below the first base of each codon. The N-terminal sequence previously determined for the purified PDC protein is shown directly below the sequence predicted for PDC. A putative promoter is double underlined with the –35 and –10 eubacterial promoter consensus sequence indicated in lower-case letters below the DNA sequence. A presumed ribosome-binding site is underlined. The translation stop codon is indicated by an asterisk. A stem-loop structure which may facilitate ρ-independent transcription termination is indicated by arrows below the DNA sequence.

Figure 2-2. Continued.

```
TTAAGTATAGGAAAGACAGCAGTATCAGAAAGCAATCCATACTTTGCTGGATTATTCTCA  900
L  S  I  G  K  T  A  V  S  E  S  N  P  Y  F  A  G  L  F  S    260

GGAGAAACTAGTTCAGATTTAGTTAAAGAACTTTGCAAAGCTTCTGATATAGTTTTACTA  960
G  E  T  S  S  D  L  V  K  E  L  C  K  A  S  D  I  V  L  L    280

TTTGGAGTTAAATTCATAGATACTACAACAGCTGGATTTAGATATATAAATAAAGATGTT 1020
F  G  V  K  F  I  D  T  T  T  A  G  F  R  Y  I  N  K  D  V    300

AAAATGATAGAAATTGGTTTAACTGATTGTAGAATTGGAGAAACTATTTATACTGGACTT 1080
K  M  I  E  I  G  L  T  D  C  R  I  G  E  T  I  Y  T  G  L    320

TACATTAAAGATGTTATAAAAGCTTTAACAGATGCTAAAATAAAATTCCATAACGATGTA 1140
Y  I  K  D  V  I  K  A  L  T  D  A  K  I  K  F  H  N  D  V    340

AAAGTAGAAAGAGAAGCAGTAGAAAAATTTGTTCCAACAGATGCTAAATTAACTCAAGAT 1200
K  V  E  R  E  A  V  E  K  F  V  P  T  D  A  K  L  T  Q  D    360

AGATATTTCAAACAAATGGAAGCGTTCTTAAAACCTAATGATGTATTAGTTGGTGAAACA 1260
R  Y  F  K  Q  M  E  A  F  L  K  P  N  D  V  L  V  G  E  T    380

GGAACATCATATAGTGGAGCATGTAATATGAGATTCCCAGAAGGATCAAGCTTTGTAGGT 1320
G  T  S  Y  S  G  A  C  N  M  R  F  P  E  G  S  S  F  V  G    400

CAAGGATCTTGGATGTCAATTGGATATGCTACTCCTGCAGTTTTAGGAACTCATTTAGCT 1380
Q  G  S  W  M  S  I  G  Y  A  T  P  A  V  L  G  T  H  L  A    420

GATAAGAGCAGAAGAAACATTCTTTTAAGTGGTGATGGTTCATTCCAATTAACAGTTCAA 1440
D  K  S  R  R  N  I  L  L  S  G  D  G  S  F  Q  L  T  V  Q    440

GAAGTTTCAACAATGATAAGACAAAAATTAAATACAGTATTATTTGTAGTTAACAATGAT 1500
E  V  S  T  M  I  R  Q  K  L  N  T  V  L  F  V  V  N  N  D    460

GGATATACAATTGAAAGATTAATCCACGGACCTGAAAGAGAATATAACCATATTCAAATG 1560
G  Y  T  I  E  R  L  I  H  G  P  E  R  E  Y  N  H  I  Q  M    480

TGGCAATATGCAGAACTTGTAAAAACATTAGCTACTGAAAGAGATATACAACCAACTTGT 1620
W  Q  Y  A  E  L  V  K  T  L  A  T  E  R  D  I  Q  P  T  C    500

TTCAAAGTTACAACTGAAAAAGAATTAGCAGCTGCAATGGAAGAAATAAACAAAGGAACA 1680
F  K  V  T  T  E  K  E  L  A  A  A  M  E  E  I  N  K  G  T    520

GAAGGTATTGCTTTTGTTGAAGTAGTAATGGATAAAATGGATGCTCCAAAATCATTAAGA 1740
E  G  I  A  F  V  E  V  V  M  D  K  M  D  A  P  K  S  L  R    540

CAAGAAGCAAGTCTATTTAGTTCTCAAAATAACTACTAATATATATTATATATAAATAAA 1800
Q  E  A  S  L  F  S  S  Q  N  N  Y  *                        552

AATTAAAAAAGATTGTAAATTAAATTTAAAGGTGACTTCTATTAATAGAGGTCATCTTTTT 1860
                          →  →  →  →  ←  ←  ←

ATGCTTATAAGTTTAATTTTATAAAATACAATTAGTAATTAAACACTTTATAAGAAAAAA 1920
←
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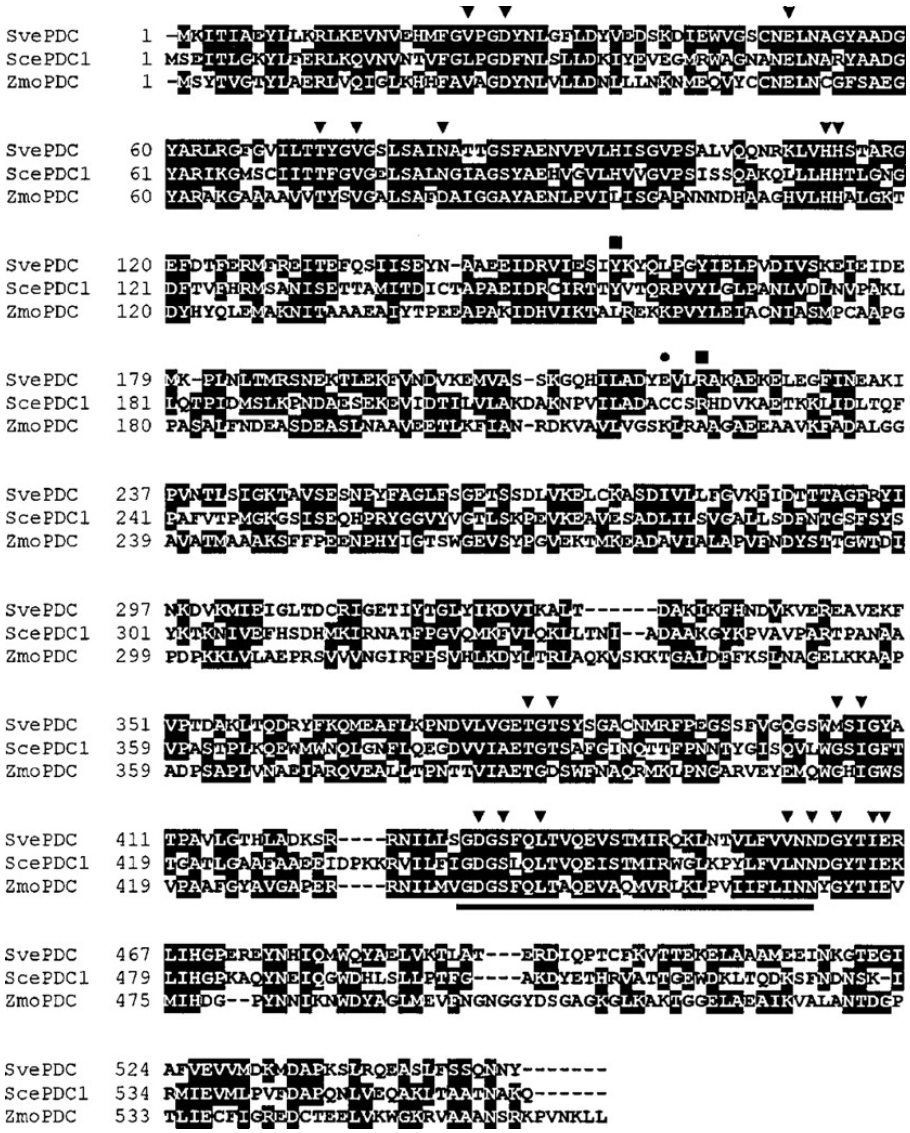

Figure 2-3. Multiple amino acid sequence alignment of *S. ventriculi* PDC with other PDC protein sequences. Abbreviations with GenBank or SwissProt accession numbers: Sce, S. cerevisiae P06169; Sve, S. ventriculi; Zmo, Z. mobilis P06672. Identical amino acid residues are shaded in inverse print. Functionally conserved and semi-conserved amino acid residues are shaded in gray. Dashes indicate gaps introduced in protein sequence alignment. Indicated above the sequences are amino acid residues within a 0.4 nm distance of the Mg2+ and TPP binding site of yeast PDC1 (84)(▼), the Cys221 residue originally postulated to be required for pyruvate activation of yeast PDC1 (●), and the Tyr157 and Arg224 residues which form hydrogen bonds with allosteric activators such as pyruvamide (■). The underlined sequence is a conserved motif identified in TPP-dependent enzymes (216).

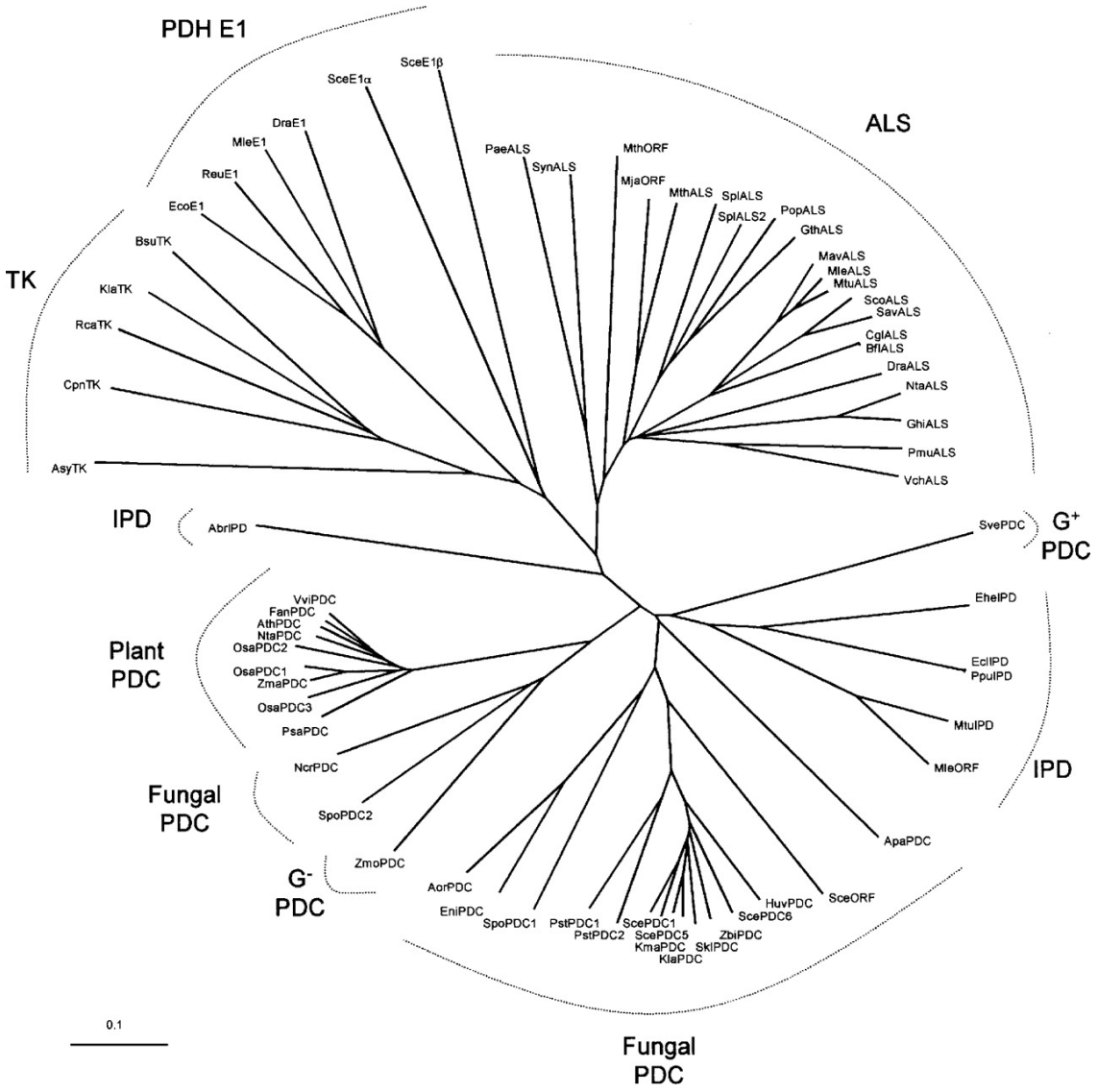

Figure 2-4. Relationships between selected PDCs. The dendrogram shown above summarizes the relationships between selected PDCs and other thiamine pyrophosphate-dependent enzymes. Deduced protein sequences were aligned using ClustalX. Amino acid extensions at the N- or C-terminus as well as apparent insertion sequences were removed. Remaining regions containing approximately 520 to 540 amino acids were compared. Treeview was used to display these results as an unrooted dendrogram. Protein abbreviations: PDC, pyruvate decarboxylase; IPD, indole-3-pyruvate decarboxylase; ORF, open reading frame; ALS, acetolactate synthase; PDH E1 or E1, the E1 component of pyruvate dehydrogenase; TK, transketolase. Organism abbreviations and GenBank or SwissProt accession numbers: Abr, *Azosprillum brasilense* P51852, Aor, *Aspergillus oryzae* AAD16178; Apa, *Aspergillus parasiticus* P51844; Asy, *Ascidia sydneiensis samea* BAA74730; Ath, *Arabidopsis thaliana* BAB08775; Bfl, *Brevibacterium flavum* A56684; Bsu, *Bacillus subtilis* P45694; Cgl, *Corynebacterium glutamicum* P42463;

Cpn, *Chlamydophila pneumoniae* H72020; Dra, *Deinococcus radiodurans* A75387 (ALS), A75541 (E1); Ecl, *Enterobacter cloacae* P23234; Eco, *E. coli* CAA24740; Ehe, *Erwinia herbicola* AAB06571; Eni, *Aspergillus* (*Emericella*) *nidulans* P87208; Fan, *Fragaria* x *ananassa* AAG13131; Ghi, *Gossypium hirsutum* S60056; Gth, *Guillardia theta* NP_050806; Huv, *Hanseniaspora uvarum* P34734; Kla, *Kluyveromyces lactis* Q12629 (PDC), Q12630 (TK); Kma, *Kluyveromyces marxianus* P33149; Mav, *Mycobacterium avium* Q59498; Mja, *Methanococcus jannaschii* Q57725; Mle, *Mycobacterium leprae* CAC31122 (ORF), 033112 (ALS), CAC30602 (E1); Mth, *Methanobacterium thermoautotrophicum* A69081 (ORF), C69059 (ALS); Mtu, *Mycobacterium tuberculosis* E70814 (IPD), 053250 (ALS); Ncr, *N. crassa* P33287; Nta, *Nicotiana tabacum* P51846 (PDC), P09342 (ALS); Osa, *Oryza sativa* P51847 (PDC1), P51848 (PDC2), P51849 (PDC3); Pae, *Pseudomonas aeruginosa* G83123; Pmu, *Pasteurella multocida* AAK03712; Pop, *Prophyra purpurea* NP_053940; Ppu, *Pseudomonas putida* AAG00523; Psa, *P. sativum* P51850; Pst, *Pichia stipitis* AAC03164 (PDC1), AAC03165 (PDC2); Rca, *Rhodobacter capsulatus* JC4637; Reu, *Ralstonia eutropha* Q59097; Sav, *Streptomyces avermitilis* AAA93098; Sce, *S. cerevisiae* P06169 (PDC1), P16467 (PDC5), P26263 (PDC6), Q07471 (ORF), NP_011105 (E1α), NP_009780 (E1β); Sco, *Streptomyces coelicolor* T35828; Skl, *Saccharomyces kluyveri* AAF78895; Spl, *Spirulina platensis* P27868; Spo, *Schizosaccharomyces pombe* Q09737 (PDC1), Q92345 (PDC2); Sve, *S. ventriculi* AF354297; Syn, *Synechocystis* sp. BAA17984; Vch, *Vibrio cholerae* A82375; Vvi, *Vitis vinifera* AAG22488; Zma, *Zea mays* P28516; Zbi, *Zygosaccharomyces bisporus* CAB65554; Zmo, *Z. mobilis* P06672. Scale bar represents 0.1 nucleotide substitutions per site.

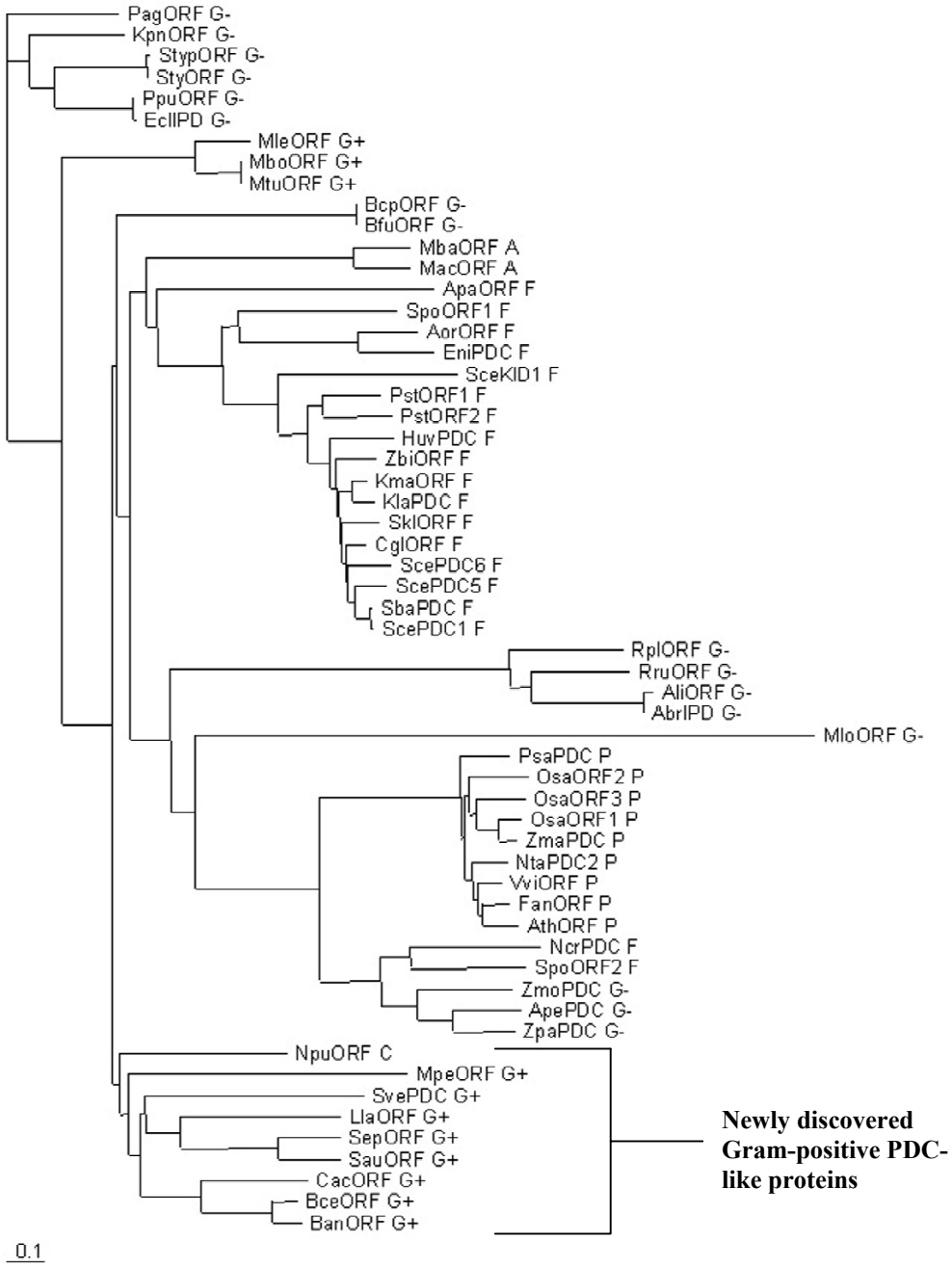

Figure 2-5. Relationships between pyruvate decarboxylase (PDC), indole pyruvate decarboxylase (IPD), α-ketoisocaproate decarboxylase (KID), and homologues (ORF). Abbreviations: Abr, *Azospirillum brasilense*; Ali, *Azospirillum lipoferum*; Aor, *Aspergillus oryzae*; Apa, *Aspergillus parasiticus*; Ape, *A. pasteurianus*; Ath, *Arabidopsis thaliana*; Ban, *Bacillus anthracis*; Bce, *Bacillus cereus*; Bcp, *Burkholderia cepacia*; Bfu, *Burkholderia fugorum*; Cac, *Clostridium acetobutylicum*; Cgl, *Candida glabrata*; Ecl, *Enterobacter cloacae*; Eni, *Emericella nidulans*; Fan, *Fragaria* x *ananassa*; Huv, *Hanseniaspora uvarum*; Kla, *Kluyveromyces*

lactis; Kma, *Kluyveromyces marxianus*; Kpn, *Klebsiella pneumoniae*; Lla, *Lactococcus lactis*; Mac, *Methanosarcina acetovorans*; Mba, *Methanosarcina barkeri*; Mbo, *Mycobacterium bovis*; Mle, *Mycobacterium leprae*; Mlo, *Mesorhizobium loti*; Mpe, *Mycoplasma penetrans*; Mtu, *Mycobacterium tuberculosis*; Ncr, *Neurospora crassa*; Npu, *Nostoc punctiforme*; Nta, *Nicotiana tabacum*; Osa, *Oryza sativa*; Pag, *Pantoea agglomerans*; Ppu, *Pseudomonas putida*; Psa, *Pisum sativum*; Pst, *Pichia stipitis*; Rpl, *Rhodopseudomonas palustris*; Rru, *Rhodospirillum rubrum*; Sau, *Staphylococcus aureus*; Sba, *Saccharomyces bayanus*; Sep, *Staphylococcus epidermidis*; Sty, *Salmonella typhimurium*; Styp, *Salmonella typhi*; Sce, *S. cerevisiae*; Skl, *Saccharomyces kluyveri*; Spo, *Schizosaccharomyces pombe*; Sve, *S. ventriculi*; Vvi, *Vitis vinifera*; Zma, *Zea mays*; Zbi, *Zygosaccharomyces bisporus*; Zmo, *Z. mobilis*; Zpa; *Z. palmae*; G+, Gram-positive; G-, Gram-negative; C, cyanobacteria; A, archaea; P, plants; F, fungi and yeast; Bar, 0.1 nucleotide substitutions per site; ORF, open reading frame with no enzyme information.

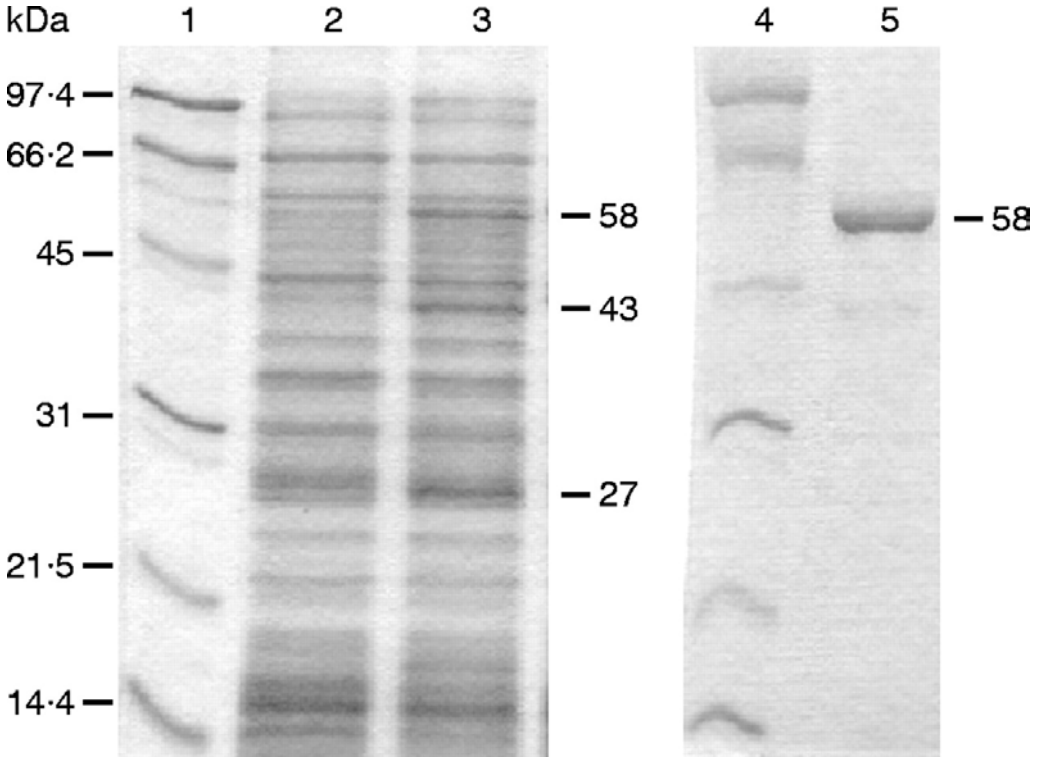

Figure 2-6. *S. ventriculi* PDC protein synthesized in recombinant *E. coli*. Proteins were analyzed by reducing SDS-PAGE using 12% polyacrylamide gels and stained with Coomassie blue R-250. Lanes 1 and 4, Molecular mass standards (5 μg). Lanes 2 and 3, Cell lysate (20 μg) of IPTG-induced *E. coli* BL21-CodonPlus-RIL/pSJS1240 transformed with pET21d or pJAM419, respectively. Lane 5. *S. ventriculi* PDC protein (2 μg) purified from recombinant *E. coli*.

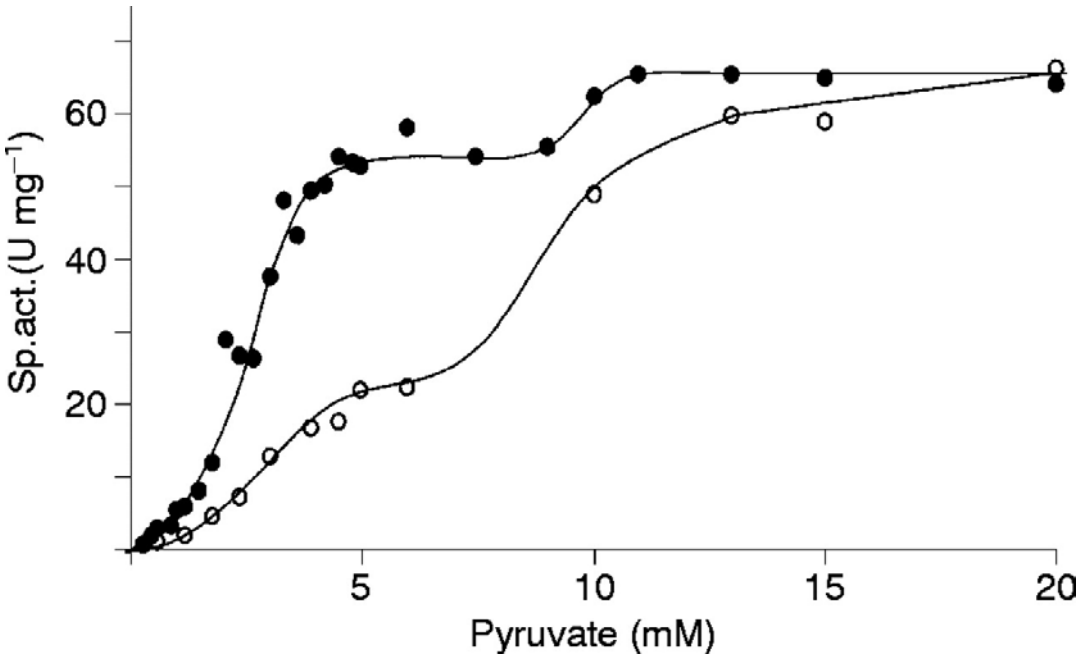

Figure 2-7. Pyruvate dependant activity of the *S. ventriculi* PDC purified from recombinant *E. coli*. The data represent mean results from triplicate determinations of PDC activity by the ADH coupled assay using 1 μg of purified enzyme in 1 ml final assay volume as described in methods section. *Sv*PDC assayed in K-MES (●) and Maleate buffer (O).

CHAPTER 3
OPTIMIZATION OF *Sarcina ventriculi* PDC EXPRESSION IN A GRAM POSITIVE HOST

Introduction

Our previous work has shown that the PDC from the Gram-positive bacterium *S. ventriculi* (*Sv*PDC) was poorly expressed in *E. coli* (133). Addition of accessory tRNAs was necessary for a ten-fold increase in protein production. The elevated levels of protein produced upon addition of accessory tRNAs facilitated the purification of the *Sv*PDC. While the protein produced and purified from *E. coli* enabled the initial characterization of *Sv*PDC, it was not the optimal host due to low levels of SvPDC produced.

Therefore, it was necessary to determine if there was a host that had similar codon usage to that of *Sv*PDC so that limited tRNAs would not hinder over expression of the protein. Because *S. ventriculi* is a low-G+C Gram-positive bacterium, it was reasoned that a low-G+C Gram-positive host would be most suitable for expression of this protein in large quantities.

The Gram-positive bacterium *Bacillus megaterium* WH320 was examined as a potential host for engineering high-level synthesis of PDC. *B. megaterium* has several advantages over other bacilli including the availability of a shuttle plasmid (pWH1520) for xylose inducible expression of foreign genes cloned downstream of the *xylA* promoter. Another advantage is that the alkaline proteases, often responsible for the degradation of foreign proteins in recombinant bacilli, are not produced in *B. megaterium*

(220, 221). In contrast to *E.* coli, the tRNAs for AUA and AGA are abundant in *B. megaterium* suggesting that factors limiting PDC production will be optimal in this host.

In this study, *Sv*PDC was over expressed in and purified from *B. megaterium*. The biochemical characteristics and optimum conditions for activity of the *Sv*PDC enzyme purified from *B. megaterium* were determined. Due to the expression of SvPDC in *B.* megaterium, a plasmid was also constructed containing a Gram-positive ethanol production operon in which production of *Sv*PDC and *Geobacillus stearothermophilus* alcohol dehydrogenase (ADH) (222) are transcriptionally coupled and expression of these proteins was demonstrated.

Materials and Methods

Materials

Biochemicals were purchased from Sigma Chemical Company (St. Louis, MO). Other organic and inorganic analytical-grade chemicals were purchased from Fisher Scientific (Marietta, GA). Restriction enzymes were from New England Biolabs (Beverly, MA). Oligonucleotides were obtained from QIAgen Operon (Valencia, CA). *Bacillus megaterium* Protein Expression System was purchased from MoBiTec (Marco Islands, FL). Rnase-free water and solutions were obtained from Ambion (Austin, TX).

Bacterial Strains and Media

Strains and plasmids used in this study are listed in Table 3-1. *E. coli* DH5α was used for routine recombinant DNA experiments. *B. megaterium* WH320 was used for protein production. Growth and transformation of *B. megaterium* were performed according to the manufacturer (MoBiTec). All strains were grown in Luria-Bertani (LB) medium supplemented with antibiotics as appropriate (ampicillin 100 mg per liter, or tetracycline 12.5 mg per liter) at 37°C and 200 rpm.

DNA Isolation

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 (BioWhittaker Molecular Applications) gels using the QIAquick gel elution kit (QIAgen).

Cloning of the *Sarcina ventriculi pdc* Gene Into Expression Vector pWH1520

Plasmid pJAM420 was constructed using the following methods. The *Bsp*EI-to-*Xba*I fragment of plasmid pJAM419 was ligated with 7.7-kb *Spe*I-to-*Xma*I fragment of

plasmid vector pWH1520. This resulted in generation of the *B. megaterium* expression plasmid pJAM420 that carried the *S. ventriculi pdc* gene, along with the Shine-Dalgrano site and T7 transcriptional terminator of the original pET21d vector. The *pdc* gene was positioned to interrupt the *B. megaterium xylA* gene (*xylA'*) of plasmid pWH1520 and to generate a stop codon within *xylA'*. The Shine-Dalgrano site of the inserted *pdc* gene was positioned directly downstream of the *xylA'* stop codon to allow for translational coupling in which the ribosomes would presumably terminate at the stop codon for *xylA'* and then reinitiate at the *pdc* start codon.

Gram-positive Ethanol Operon (PET).

To construct the Gram-positive PET operon, the *Hin*dIII-to-*Mfe*I fragment of pLOI1742 containing the *adh* gene from *G. stearothermophilus* (222) was blunt-end ligated into the *Blp*I site of pJAM420 using Vent Polymerase (New England Biolabs). This resulted in the generation of plasmid pJAM423 which was designed to facilitate the translational coupling of the *S. ventriculi pdc* gene with the *G. stearothermophilus adh* gene. The *xylA* promoter is upstream of the *Svpdc* and the terminator now follows the *adh* gene.

Protoplast Formation and Transformation of *B. megaterium*.

A 1.0% (v/v) inoculum of *B. megaterium* WH320 cells was grown in LB to an OD_{600nm} of 0.6 units (early-log phase). Protoplasts were formed according to Puyet et al. (223) with the following variations. Cells were treated with 10 μg per ml lysozyme for 20 min at 37°C. Protoplasts were stored at –70°C. Transformation of the protoplasts was performed according to the *B. megaterium* protein expression kit manual (MoBiTec).

Production of *Sv*PDC In Recombinant Hosts.

Production of *Sv*PDC in *E. coli* was performed as previously described (133). *Sv*PDC protein was synthesized in *B. megaterium* WH320 cells using pJAM420. A 1.0% (v/v) overnight inoculum of recombinant *B. megaterium* cells was grown in LB supplemented with tetracycline to an OD_{600nm} of about 0.3 units (early-log phase). Transcription from the *xylA'* promoter was induced with 0.5% (w/v) xylose for 3 h. Cells were harvested by centrifugation at 5000 × *g* (10 min, 4°C) and stored at -70°C.

Purification of the *S. ventriculi* PDC Protein.

All purification buffers contained 1 mM TPP and 1mM $MgSO_4$ unless indicated otherwise. Purification of *Sv*PDC from *E. coli* was performed as previously described (133). Recombinant *B. megaterium* cells (15 g wet wt) were thawed in 6 volumes (w/v) of 50 mM Na-PO_4 buffer at pH 6.5 (Buffer A) and passed through a French pressure cell at 20,000 lb per in^2. Cell debris was removed by centrifugation at 16,000 × *g* (20 min, 4ºC). Supernatant was filtered through a .45 μm membrane. Filtrate (372.3 mg protein) was applied to a Q Sepharose Fast Flow 26/10 column (Pharmacia) that was equilibrated with Buffer A. A linear gradient was applied from 0 mM to 400 mM NaCl. Fractions containing PDC activity eluted at 250 to 300 mM NaCl were pooled. Pooled fractions were applied to a 5 ml Bio-scale hydroxyapatite type I column (BioRad) that was equilibrated with 5 mM Na-PO_4 buffer at pH 6.5 (Buffer B). The column was washed with 15 ml Buffer B and developed with a linear Na-PO_4 gradient (5 to 500 mM Na-PO_4 at pH 6.5 in 75 ml). Protein fractions with PDC activity were eluted at 300 to 530 mM Na-PO_4 and were pooled (1mg protein per ml). For further purification, portions of this material (0.25 to 0.5 ml) were applied to a Superdex 200 HR 10/30 column (Pharmacia)

equilibrated in 50 mM Na-PO_4 at pH 6.5 with 150 mM NaCl and 10% glycerol in the presence or absence of 1 mM $MgSO_4$ and 1 mM TPP.

Activity Assays and Protein Electrophoresis Techniques.

PDC activity was assayed by monitoring the pyruvic acid-dependant reduction of NAD^+ with alcohol dehydrogenase (ADH) as a coupling enzyme at pH 6.5, as previously described (224). Sample was added to a final volume of 1 ml containing 0.15 mM NADH, 0.1 mM TPP, 50.0 mM pyruvate, and 10 U ADH in 50 mM K-MES buffer at pH 6.5 with 5 mM $MgCl_2$. The reduction of NAD^+ was monitored in a 1 cm path length cuvette at 340 nm over a 5 min period using a BioRad SmartSpec 300 (BioRad). Protein concentration was determined using BioRad Protein assay dye with bovine serum albumin as the standard according to supplier (BioRad).

The pH optimum of *Sv*PDC was assayed in buffers suitable to maintain the desired pH. The temperature optimum of *Sv*PDC was assayed using a Beckman DU640 (Beckman) spectrophotometer with a circulating water bath.

Thermostability of *Sv*PDC was assayed by incubating purified enzyme in lysis buffer at a concentration of 0.02 μg of protein per μl for 90 min. After incubation, samples were assayed at room temperature.

Molecular masses were estimated by reducing and denaturing SDS-PAGE using 12% polyacrylamide gels. Proteins were stained using the Rapid Fairbanks method (225). The 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).

Results

*Sv*PDC Expression Vector for *B. megaterium*.

Previous studies have shown that *Sv*PDC is poorly expressed in *E. coli* (133). Because *Sv*PDC is from a Gram-positive bacterium, we decided to use a Gram-positive bacterial expression system for high-level production of *Sv*PDC. A fragment of plasmid pJAM419, used previously for expression of *Sv*PDC in *E. coli* (133), was isolated that contained a Shine-Dalgrano sequence, the *S. ventriculi pdc* gene, and the T7 terminator. This fragment was cloned into pWH1520 in such a way that the xylose isomerase gene (*xylA*) of pWH1520 was truncated to form a stop codon after 30 codons. The Shine-Dalgrano sequence upstream of *Sv*PDC was positioned so that a xylose-inducible transcriptional coupling occurred between *xylA'* and *Svpdc.* The resulting plasmid, pJAM420, was used for expression of *Sv*PDC in *B. megaterium*.

Production and Purification of *Sv*PDC from *B. megaterium.*

Based on SDS-PAGE, expression of *Sv*PDC is notably higher when expressed in *B. megaterium* compared to *E. coli* (Figure 3-1). The high levels of *Sv*PDC protein produced in *B. megaterium* facilitated a 22-fold purification of the protein from this host, with 8.35 mg purified protein from 15 g of cells (wet wt.) (Table 3-2). This is in contrast to purification of *Sv*PDC from *E. coli*, which began with 14.8 g of cells and only yielded 0.2 mg purified protein (unpublished data).

Purified *Sv*PDC from *B. megaterium* was determined to be a 235 kDa homotetramer of 58 kDa subunits as determined by Superdex 200 gel filtration chromatography and SDS-12% PAGE electrophoresis. These results correlate with previous studies (132, 133).

Determination of Optimum Conditions for *Sv*PDC Activity.

It is important when assaying any enzyme to determine its optimum conditions. PDCs are routinely assayed at pH 6.5 (178, 218) and pH 6.0 (94, 112). While these pH values are acceptable for the *Z. mobilis* and *S. cerevisiae* PDC proteins, they may give misleading kinetic values for *Sv*PDC. Our research found that the recombinant *Sv*PDC from *B. megaterium* had a pH optimum in the range of pH 6.5 to pH 7.4 (Figure 3-2). This pH optimum is quite high when compared to the PDCs from *Z. mobilis* (pH 6.0), *S. cerevisiae* PDC1 (pH 5.4-5.8), *A. pasteurianus* (pH 5.0-5.5), and *Z. palmae* (pH 5.5-6.0) (113, 131, 226). The pH optimum of *Sv*PDC purified from recombinant *E. coli* has previously been shown to be between pH 6.3 to 6.7 (131), which is higher than the other bacterial PDCs and also different to the pH optimum determined for the *B. megaterium* purified protein.

The temperature optimum of the *Sv*PDC from *B. megaterium* was determined to be 32°C (Figure 3-3). This temperature differs greatly from the *Z. mobilis, Z. palmae,* and *A. pasteurianus* PDCs, which have temperature optima of 60°C (131). There is, however, an approximately 2.5-fold increase in activity of the *Sv*PDC at 32°C compared to room temperature. This increase in activity is comparable to that observed when the Gram-negative bacterial PDCs were assayed at their optimal temperatures (131).

Kinetics of *Sv*PDC Produced in *B. megaterium.*

The recombinant *Sv*PDC from *B. megaterium* displayed sigmoidal kinetics (Figure 3-4). The recombinant *Sv*PDC from *B. megaterium* had a K_m of 3.9 mM for pyruvate and a V_{max} of 98 U per mg of protein when assayed at pH 6.5 and room temperature. When assayed at optimal conditions, 32°C and pH 6.72, there was an

increase in both K_m (6.3 mM for pyruvate) and V_{max} (172 U per mg protein). These results suggest that a change in conformation mediated by an increase in pH and/or temperature reduces the affinity of the enzyme for pyruvate but increases the overall activity of the enzyme. Further study is necessary to determine the cause of this phenomenon.

Thermostability of *Sv*PDC Produced in *B. megaterium*.

Previous studies showed that *Sv*PDC is not as thermostable as the other bacterial PDC proteins (131, 133). In order to determine if production of the *Sv*PDC protein in a sub optimal host was responsible for this, thermostability of *Sv*PDC produced in *E. coli* was compared to that produced in *B. megaterium* (Figure 3-5). When assayed for thermostability, the *Sv*PDC produced in *B. megaterium* retained 30% activity after incubation at 50°C while the protein purified from *E. coli* only had 0.95% residual activity after incubation at 50°C. These results indicate that *Sv*PDC is more thermostable when produced in *B. megaterium* compared to *E. coli*. Misincorporation of amino acids due to use of rare codons and/or misfolding of the *Sv*PDC protein may have occurred when the enzyme was produced in *E. coli* and may account for this reduction in thermostability.

During the biochemical characterization of *Sv*PDC, we discovered that pH had a drastic effect on the thermostability of this enzyme (Figure 3-6). While the optimal pH for activity of the *Sv*PDC is pH 6.72, this is not optimal for its thermostability. At pH 6.5 the *Sv*PDC enzyme has only 3% of original activity remaining after incubation at 60°C while samples incubated at pH 5.0 to pH 5.5 have 94% to 97% activity remaining. It was

also determined that *Sv*PDC retained 100% activity when stored at pH 5.5 for two weeks compared to 62 % when stored at 4°C at pH 6.5 (data not shown).

Generation of a Gram-positive Ethanol Production Operon.

B. megaterium WH320 is capable of growth when tested in xylose minimal medium. The strain is also able to grow at temperatures up to 42°C and at a low pH of 5.0. This strain appears to be a suitable candidate to perform preliminary tests on ethanol production with a portable pyruvate to ethanol operon (PET) and may prove useful in large-scale ethanol production under acidic conditions. To construct a Gram-positive PET operon, the *adh* gene from *G. stearothermophilus* (222) was cloned behind the *S. ventriculi pdc* gene in the *B. megaterium* pWH1520 expression vector. This vector was chosen based on successful overproduction of *S. ventriculi* PDC (Figure 3-7). The resulting PET plasmid, pJAM423, was transformed into *B. megaterium.* After xylose induction, a considerable portion of the cell lysate of this strain was composed of the *S. ventriculi* PDC and *G. stearothermophilus* ADH proteins (Figure 3-7). The ethanol production of this construct was tested in the presence of 0.5% xylose. HPLC analysis showed that ethanol production was doubled from that of a strain with pWH1520 alone, but levels were still quite low (20mM)(data not shown). PDC has already been shown to be very active in cell lysate, but further analysis needs to be performed to determine if the ADH is active.

Discussion

The *Sv*PDC protein is poorly expressed in recombinant *E. coli* (133). Therefore, we reasoned that a host more similar to *S. ventriculi* might express this PDC at higher levels. *B. megaterium* was chosen as a host because it has several benefits over other

Gram-positive expression systems. These include a xylose inducible expression vector and absence of alkaline proteases that are often responsible for degradation of foreign proteins (220, 221). Augmentation of the host, *B. megaterium*, with accessory *t*RNAs was not necessary for high-level *Sv*PDC production. This high yield of *Sv*PDC protein facilitated the 22-fold purification. The *Sv*PDC protein was more active when produced in *B. megaterium* compared to *E. coli*. We believe that the difference in activity is primarily due to differences in the rate of misincorporation of amino acids based on codon usage.

The *Sv*PDC protein produced in *B. megaterium* has a higher V_{max} (98 U per mg protein) at RT than when produced by *E. coli* (66 U per mg protein). The *Sv*PDC produced in *B. megaterium* is also more thermostable than the *E. coli* produced protein. Choosing the correct host appears to have affected the quality of *Sv*PDC protein that was recovered. These results indicate that differences can occur in the biochemical properties of recombinant protein based on host.

In this study, we discovered that the pH of the incubation buffer has an effect on the thermostability of *Sv*PDC. Low pH stabilized *Sv*PDC at higher temperatures. These results suggest that residues of *Sv*PDC gain a charge between pH 5.0–5.5 that allows the tetramer conformation to remain stable at higher temperatures. This is an important discovery because it gives insight into residues that can be altered in future experiments in order to engineer *Sv*PDC to be more thermostable at cytosolic pH.

The current portable production of ethanol (PET) operon consists of the *pdc* and *adh* genes from *Zymomonas mobilis*, a Gram-negative organism (24, 25, 129, 227). Past research to engineer a Gram-positive host for ethanol production has focused on using

this PET operon, but these attempts have met with limited success (33-35, 228) primarily due to poor expression of the PDC. We have shown that *Sv*PDC is expressed at high levels in *B. megaterium*, a Gram-positive host. Our construction and expression of the Gram-positive ethanol production operon using the *Sv*PDC and *G. stearothermophilus* ADH has demonstrated that recombinant PDC and ADH production no longer limit ethanol production in Gram-positive biocatalysts.

Our research shows that selection of host for recombinant production of proteins can affect the quality and stability of the recombinant protein. We have also demonstrated that *Sv*PDC has qualities that make it unique among bacterial PDCs, including its substrate activation and elevated pH optimum. *Sv*PDC is the only bacterial PDC that is not thermostable, but our results indicate that alteration of charged residues may facilitate the engineering of thermostable *Sv*PDC variants. Lastly, we have created a Gram-positive ethanol production operon that will be useful in engineering future Gram-positive hosts for ethanol production.

Table 3-1. Strains, plasmids, and primers used in Chapter 3.

Strain or Plasmid	Phenotype or genotype, PCR primers	Source
E. coli DH5α	F- *recA1 endA1 hsdR17* (r_k^- m_k^+) *supE44 thi-1 gyrA relA1*	GibcoBRL (Gathers-burg, Md.)
E. coli BL21-CodonPlus-RIL	F^- *ompT hsdS*(r_B^- m_B^-) *dcm*$^+$ Tet^r *gal* λ (DE3) *endA* Hte [*argU ileY leuW* Cam^r] (an *E.coli* B strain)	Stratagene (La Jolla, CA.)
B. megaterium WH320	*lac*$^-$ *xyl*$^+$	MoBiTec
pSJS1240	Sp^r; derivative of pACYC184 with *E. coli ileX* and *argU*	(219)
pET21d	Ap^r; expression vector for replication in *E. coli*	Novagen
pWH1520	Ap^r Tc^r; shuttle expression vector for replication in *E. coli* and *B. megaterium*	(220)
pJAM419	Ap^r; pET21d derivative encoding *Sv*PDC	(133)
pJAM420	Ap^r Tc^r; 1.9-kb *Bsp*EI-to-*Xba*I fragment of pJAM419 ligated with the *Spe*I-to-*Xma*I fragment of pWH1520; used for synthesis of SvPDC in *B. megaterium*	This study
pLOI1742	Plasmid containing the *G. stearothermophilus adh* gene	L. Yomano
pJAM423	Ap^r Tc^r; 1.8-kb *Hin*dIII-to-*Mfe*I fragment of pLOI1742 blunt-end ligated into the *Blp*I site of pJAM420; xylose-inducible Gram-positive ethanol production operon	This study

Table 3-2. Purification of SvPDC from *B. megaterium*.

Step	Protein (mg)	Sp. Act. (U per mg protein)	Purification Fold	Percent Yield
Cell Lysate	372.3	3.85	1.00	100
Q-Sepharose	38.26	20.34	5.28	54
Hydroxyapatite	15.70	24.83	6.45	27
Superdex 200	8.35	84.18	21.87	49

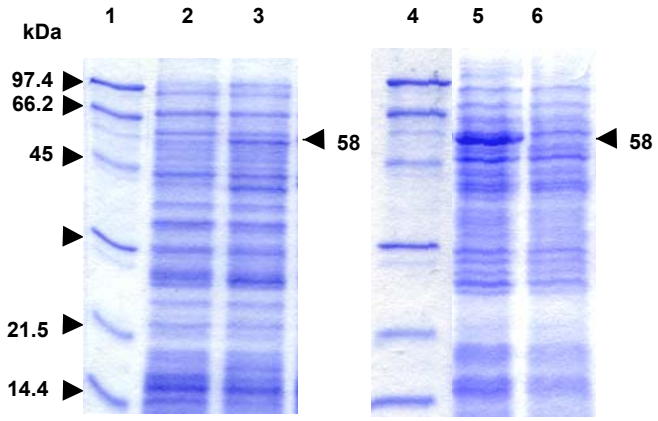

Figure 3-1. *S. ventriculi* PDC protein synthesized in recombinant *E. coli* and *B. megaterium*. Proteins were analyzed by reducing SDS-PAGE using 12% polyacrylamide gels and stained with Coomassie blue R-250. Lanes 1 and 4, Molecular mass standard (5 µg). Lanes 2 and 3, Cell lysate (20 µg) of *E.coli* BL21-CodonPlus-RIL transformed with pJAM419/pSJS1240 uninduced and IPTG induced, respectively. Lanes 5 and 6, Cell lysate (20 µg) of *B. megaterium* WH320 transformed with pJAM420 xylose induced and uninduced, respectively.

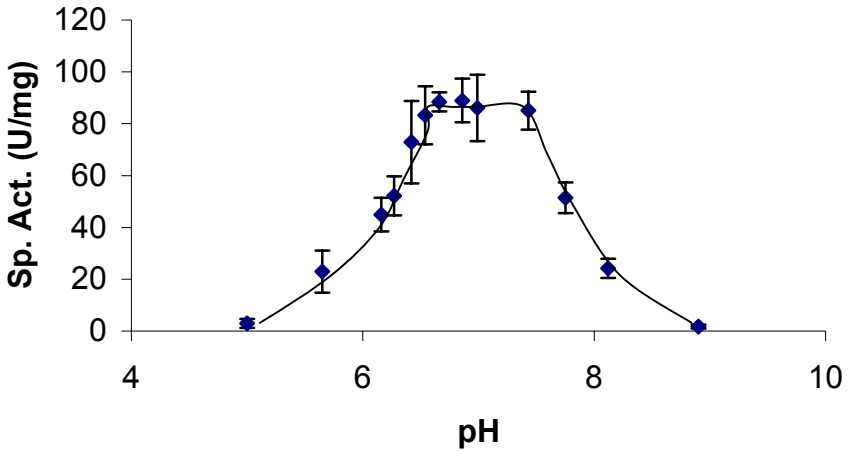

Figure 3-2. pH profile for *S. ventriculi* PDC activity.

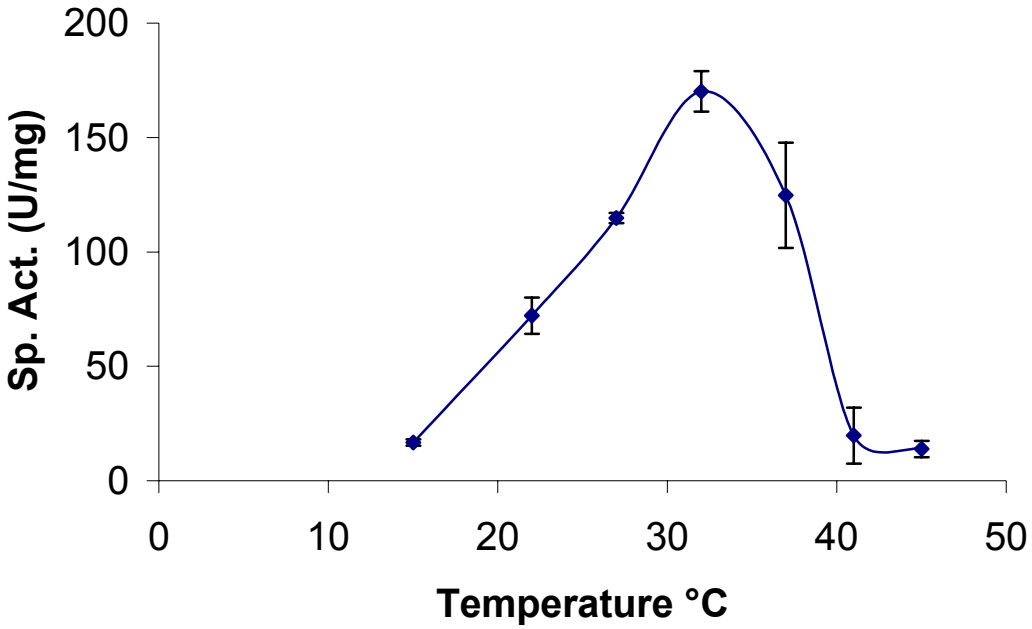

Figure 3-3. Effect of temperature on *S. ventriculi* PDC activity.

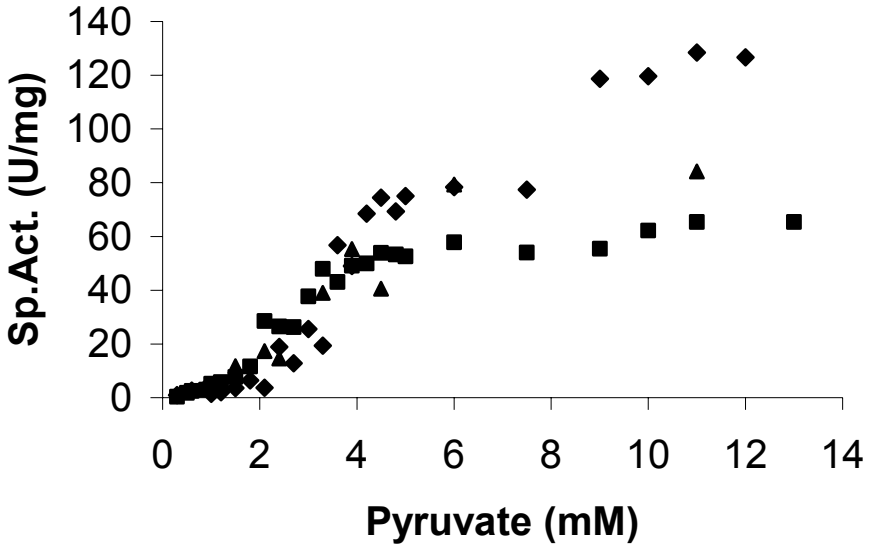

Figure 3-4. Effect of pyruvate concentration on *S. ventriculi* PDC synthesized in recombinant *E. coli* (■), and *B. megaterium* (▲) at 25°C and pH 6.5. *S. ventriculi* PDC at 32°C and pH 6.72 from recombinant *B. megaterium* (♦). The data represent mean results from triplicate determinations of PDC activity.

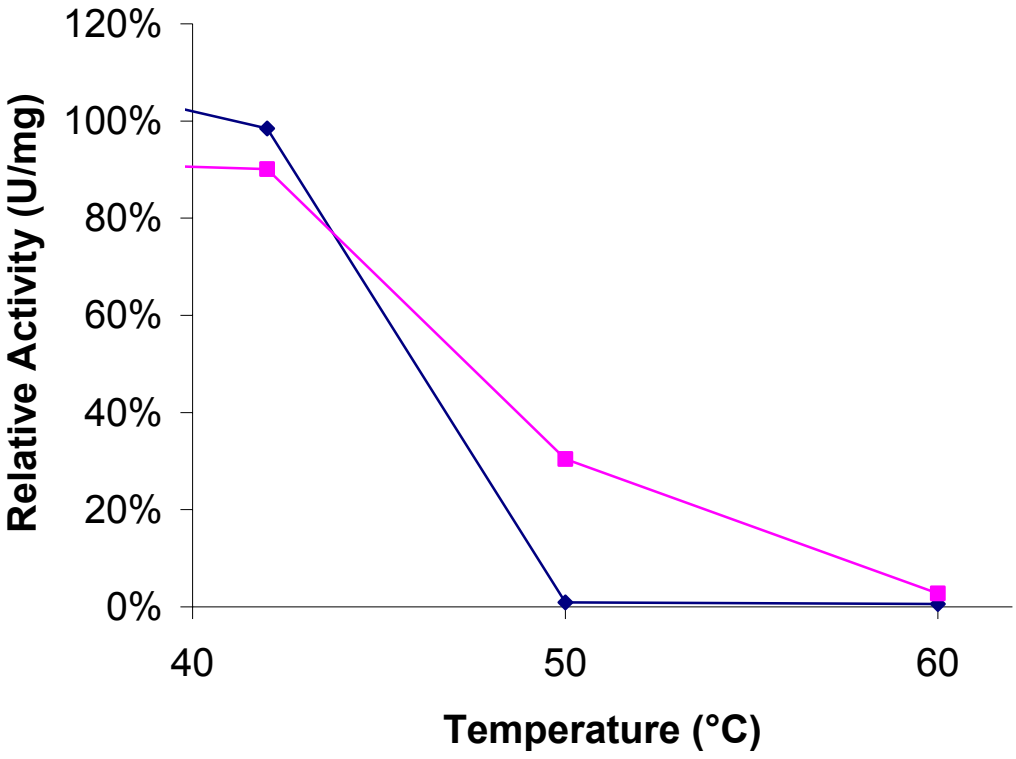

Figure 3-5. Thermostability of recombinant *S. ventriculi* PDC produced in *B. megaterium* (♦) and *E. coli* CodonPlus with plasmid pSJS1240 (■).

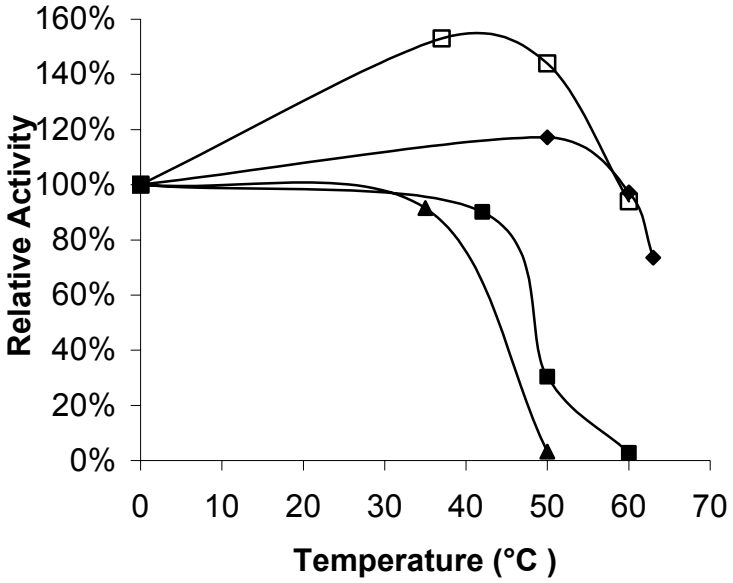

Figure 3-6. Effect of pH on the thermostability of the *S. ventriculi* PDC produced in *B. megaterium*. Thermostability was tested at a pH 5.0 (□), pH 5.5 (♦), pH 6.5 (■), and pH 7.5 (▲).

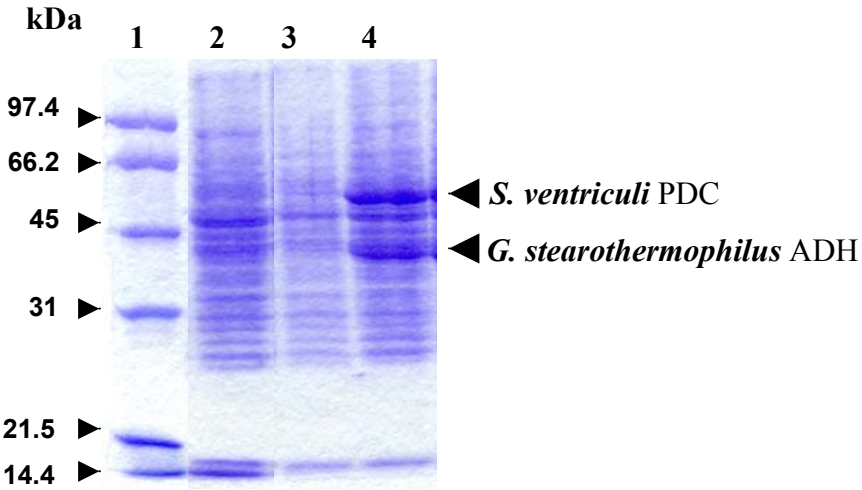

Figure 3-7. Induction of *S. ventriculi* PDC and *G. stearothermophilus* ADH in *B. megaterium*. Proteins were seperated by reducing SDS-PAGE using 12% polyacrylamide gels and stained with Coomassie blue R-250. Lanes 1, Molecular mass standard (5 µg). Lanes 2, Cell lysate (20 µg) of *B. megaterium* transformed with pWH1520 induced with xylose. Lanes 3 and 4, Cell lysate (20 µg) of *B. megaterium* WH320 transformed with pJAM423 uninduced and xylose induced, respectively.

CHAPTER 4
EXPRESSION OF PDCs IN THE GRAM-POSITIVE BACTERIAL HOST, *B. megaterium*

Introduction

PDC (PDC, EC 4.1.1.1) is a central enzyme in ethanol fermentation and catalyzes the non-oxidative decarboxylation of pyruvate to acetaldehyde with release of carbon dioxide. The acetaldehyde generated from this reaction is then converted to ethanol by alcohol dehydrogenase (ADH, EC1.1.1.1). The recombinant production of these two enzymes (PDC and ADH) converts intracellular pools of pyruvate to ethanol. The current portable production of ethanol (PET) operon used to engineer this conversion consists of the *pdc* and *adh* genes from *Zymomonas mobilis*, a Gram-negative organism (24, 25, 129, 227). While this strategy has been highly successful in the modification of Gram-negative bacteria for ethanol production, improvements in host strains are necessary (4, 129).

To enhance the commercial competitiveness of biocatalysts for the large-scale production of ethanol, the hosts must withstand low pH, high temperature, high salt, high sugar, high ethanol, and various other harsh conditions. Many of these qualities are not found in Gram-negative bacteria and must be introduced through metabolic engineering. In contrast, Gram-positive bacteria naturally possess many desirable traits for the industrial production of ethanol (228); however, modifying them for ethanol production has met with only limited success. Several attempts to engineer the PET operon into

Gram-positive organisms have resulted in low levels of PDC activity and only small elevations in ethanol production (33-35, 228).

Prior to this work, construction of PET operons for engineering high-level synthesis of ethanol in recombinant Gram-positive bacteria has been limited by the availability of bacterial *pdc* genes. Recently, however, the cloning and DNA sequence of a *pdc* gene from the Gram-positive bacterium, *S. ventriculi* (*Sv*), was described (133). Synthesis of the *Sv*PDC protein in recombinant *Escherichia coli* was low but enhanced by augmentation with accessory tRNAs (133). Based on these results, it is hypothesized that reduced translation due to differences in codon usage can be a major factor in limiting PDC production in recombinant bacterial hosts.

In this study, *pdc* genes from diverse organisms (*i.e.*, *S. ventriculi*, *Z. mobilis*, *Acetobacter pasteurianus* and *Saccharomyces cerevisiae*) with differing GC content were expressed in recombinant *Bacillus megaterium.* Superior levels of active *Sv*PDC were produced in this host. Assessment of the mRNA transcript levels and rates of protein degradation in these recombinant strains revealed that the differences in PDC were at the level of protein synthesis. This is the first report of high level PDC production in a recombinant Gram-positive host and reveals that *Sv*PDC is an ideal candidate for the metabolic engineering of ethanol production in this desirable group of organisms.

Materials and Methods

Materials

Biochemicals were purchased from Sigma (St. Louis, Mo.). Other organic and inorganic analytical-grade chemicals were from Fisher Scientific (Atlanta, Ga.). Restriction enzymes were from New England Biolabs (Beverly, Mass.).

Oligonucleotides were from QIAgen Operon (Valencia, Ca.) and Integrated DNA Technologies (Coralville, Ind.). *Bacillus megaterium* Protein Expression System was from MoBiTec (Marco Islands, Fla.). Rnase-free water and solutions were from Ambion (Austin, Tx.).

Bacterial Strains and Media

Strains and plasmids used in this study are listed in Table 4-1. *E. coli* DH5α was used for routine recombinant DNA experiments. *B. megaterium* WH320 was used for PDC production, pulse-chase, and transcript analysis. Strains were grown in Luria-Bertani (LB) medium unless otherwise indicated. Medium was supplemented with 2% (wt/vol) glucose and antibiotics (ampicillin 100 mg per liter, kanamycin 30 mg per liter, or tetracycline 15 mg per liter) as needed. All strains were grown at 37ºC and 200 rpm. Isolated colonies of *B. megaterium* were grown overnight in liquid medium and used as a 1.0% (vol/vol) inoculum into fresh medium unless otherwise indicated.

Protoplast Formation and Transformation of *B. megaterium*.

B. megaterium WH320 was grown to an $O.D._{600nm}$ of 0.6 units (early-log phase). Protoplasts were generated according to Puyet *et al.* (223) with the following modifications. Cells were treated with lysozyme (10 μg per ml) 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% (wt/vol) SeaKem GTG agarose (Cambrex Corp., East Rutherford, NJ) gels using the QIAquick gel elution kit (QIAgen). To generate the *B. megaterium* expression plasmids (pJAM420, pJAM430, pJAM432,

and pJAM435), similar strategies were used (Figure 4-1) (Table 4-1). For example, plasmid pJAM420 was constructed as follows. A *Bsp*HI-to-*Xho*I DNA fragment with the complete *S. ventriculi pdc* gene was generated by PCR amplification and cloned into the *Nco*I and *Xho*I sites of plasmid pET21d (133). The 1.9-kb *XbaI*-to-*Bsp*EI DNA fragment of the resulting plasmid (pJAM419) was ligated into the *Spe*I and *Xma*I sites of plasmid pWH1520. This resulted in generation of a pWH1520-based expression plasmid (pJAM420) that carried the *S. ventriculi pdc* gene, along with the Shine-Dalgrano site and T7 transcriptional terminator of the original pET21d vector. The *pdc* gene was positioned to interrupt the *B. megaterium xylA* gene (*xylA'*) of plasmid pWH1520 and to generate a stop codon within *xylA'*. The Shine-Dalgrano site originally from pET21d of upstream of the inserted *pdc* gene was positioned directly downstream of the *xylA'* stop codon to allow for translational coupling in which the ribosomes would terminate at the stop codon for *xylA'* and then reinitiate at the *pdc* start codon.

Production of PDC Proteins In Recombinant *B. megaterium*.

PDC proteins were independently synthesized in *B. megaterium* WH320 cells using the expression plasmids described above. Cells were grown to an $O.D._{600\ nm}$ of 0.3 units (early-log phase). Transcription from the *xylA'* promoter was induced by addition of xylose (0.5% [wt/vol]). Cells were harvested after 3 h by centrifugation (5,000 × *g*, 10 min, 4°C) and stored at -80°C. Cell pellets (0.5 g) were thawed in 6 volumes (wt/vol) of 50 mM Na_2HPO_4 buffer at pH 6.5 containing 1 mM $MgSO_4$ and 1 mM TPP. Cells were passed through a French pressure cell at 20,000 lb per in^2. Debris was removed by centrifugation (16,000 × *g*, 20 min, 4°C). Cell lysate was immediately assayed for activity.

Activity Assays and Protein Electrophoresis Techniques

PDC activity was assayed by monitoring the pyruvic acid-dependant reduction of NAD^+ with alcohol dehydrogenase (ADH) as a coupling enzyme at pH 6.5, as previously described (115). Cell lysate (10 μl) was added to a final volume of 1 ml containing 0.15 mM NADH, 0.1 mM thiamine pyrophosphate, 50.0 mM pyruvate, and 10 U ADH in 50 mM K-MES buffer at pH 6.5 with 5 mM $MgCl_2$. The reduction of NAD^+ was monitored in a 1 cm path length cuvette at 340 nm over a 5 min period using a BioRad SmartSpec 300 (BioRad). Protein concentration was determined using BioRad Protein assay dye with bovine serum albumin as the standard according to supplier (BioRad).

Protein molecular masses were analyzed by reducing and denaturing SDS-PAGE using 12% polyacrylamide gels that were stained by heating with Coomassie blue R-250 (225). 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

Cultures were grown in triplicate to an $O.D._{600\ nm}$ of 0.3 units (early-log phase). Transcription of *pdc* was induced for 15 min with 0.5% (wt/vol) xylose. Total RNA was isolated using the RNeasy miniprep kit. Samples were treated with lysozyme and On-column DNase as recommended by supplier (QIAgen). The removal of DNA from RNA samples was confirmed by performing PCR using Jumpstart *Taq* Readymix in the absence of reverse transcriptase (Sigma). Quality and quantity of RNA were determined by 0.8% agarose gel electrophoresis and absorbance at 260 nm, respectively.

RNA Quantifications

The MAXIscript T7 In vitro transcription kit (Ambion) was used to generate transcript from the *E. coli* expression vectors (pJAM419, pJAM429, pJAM431, and pScPDC1). Nuc-Away spin columns (Ambion) were used to remove unincorporated nucleotides. RNA products expressed *in vitro* were used to generate standard curves of absolute copy number for each experiment. Transcript levels were analyzed using quantitative real time reverse transcriptase PCR with an Icycler (BioRad). Total RNA (100 pg) was used as a template with the primers listed in Table 4-1. RNA Quantification reactions were performed using the QuantiTect SYBR Green 1-step RT-PCR kit according to supplier (QIAgen). All data had PCR efficiency of 90 to 100% and were analyzed using the Icycler software version 3.0.6070 (BioRad) and Microsoft Excel.

Pulse Chase

Recombinant *B. megaterium* strains 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 dH_2O at pH 7.0) supplemented with tetracycline (MM Tet) using a 1% (vol/vol) inoculum. Cells were grown to an $O.D._{600nm}$ of 0.3 units (early-log phase) and recombinant gene transcription was induced for 15 min with 0.5% (wt/vol) xylose. Cells were harvested by centrifugation (5000 × *g*, 10 min, 25°C) and resuspended in 2 ml of MM Tet supplemented with 0.5% xylose and 50 μCi per ml L-[^{35}S]-methionine (DuPont-NEN). Cells were incubated for 15 min (37°C, 200 rpm) and harvested as above. Cell pellets were resuspended in MM Tet supplemented with 0.5% xylose and 5mM L-methionine with or without chloramphenicol (15 mg per L) and incubated (37°C, 200 rpm). Aliquots (0.5 ml) were withdrawn after 5, 10, 15, 30, 60,

90, 120, 150, and 180 min of 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 per ml). Cells were incubated on ice (5 min), harvested at 16,000 × *g* (10 min, 25ºC), and stored at –80°C.

Cell pellets were subjected to 3 cycles of freeze-thaw (–80°C and 0°C) to weaken the cell membrane. Pellets were resuspended to an $O.D._{600nm}$ of 0.0134 units per µl Lysis solution (75 mM NaCl, 25 mM EDTA, 20 mM Tris pH 7.5, and 0.2 mg lysozyme per ml) and incubated (25ºC, 15 min). Samples ($O.D._{600nm}$ of 0.02 units per lane) were boiled (20 min) in SDS-PAGE loading dye (BioRad) and separated by SDS-PAGE. Gels were dried and exposed to X-ray film. A VersaDoc Model 1000 with Quantity One Software (BioRad) was used for densitometric readings.

Results

Construction of Gram-positive PDC Expression Plasmids

Previous work suggested that codon usage effects the synthesis of PDCs in Gram-negative bacteria (133). 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 pdc* gene (*Svpdc*) that is poorly expressed in *E. coli* and is the only known PDC from a Gram-positive bacterium. In addition, the *Saccharomyces cerevisiae* PDC1 (*ScPDC1*) was chosen because the encoded protein is closely related to *Sv*PDC (130, 133) and is currently used in corn-to-ethanol production (2). The *Acetobacter pasteurianus* (130) and *Zymomonas mobilis* (111-115) *pdc* genes (*Appdc* and *Zmpdc*) were also used. These

latter two genes are from Gram-negative bacteria and have high levels of expression and activity in Gram-negative hosts (130, 131, 133).

To construct the expression plasmids, the *pdc* genes were initially cloned into pET vectors (Figure 4-1)(Table 4-1). DNA fragments containing the *pdc* gene of interest and the Shine-Dalgrano and T7-terminator from the pET plasmid were cloned into the *B. megaterium* expression plasmid pWH1520. This generated a truncation of the *xylA* gene, which encodes xylose isomerase, and allowed for induction of *pdc* expression by xylose in *B. megaterium*.

Expression of PDC In Recombinant *B. megaterium*

After 3 h induction of recombinant *pdc* gene expression, the levels of PDC protein produced in the *B. megaterium* strains were estimated by SDS-PAGE (Figure 4-2). High-levels of *Sv*PDC protein were evident and estimated to account for 5% of soluble protein based on Coomassie blue R-250 stained gels. In contrast, only low-level synthesis of *Zm*PDC, *Ap*PDC, and *Sc*PDC1 were apparent. To determine if the PDC proteins were produced in an active form, cell lysate of the recombinant *B. megaterium* strains was assayed for PDC activity (Table 4-2). The *Sv*PDC had the highest specific activity in cell lysate, with 5.29 U per mg protein. Thus, approximately 5% of the total soluble protein was active *Sv*PDC, consistent with the levels of *Sv*PDC protein estimated by SDS-PAGE. In contrast, the specific activity of the *Zm*PDC and *Sc*PDC was 5-fold and 10-fold lower than *Sv*PDC, respectively. Previous studies have determined the specific activity of *Zm*PDC to be 6.2 to 8 U per mg protein (113, 131) when produced in recombinant *E. coli*, in contrast with 1.1 U per mg in this study. There was no detectable activity for the *Ap*PDC protein.

It was previously reported that purified *Sv*PDC from recombinant *E. coli* and reported specific activities in cell lysate of 0.16 U per mg from BL21-CodonPlus-RIL augmented with accessory *t*RNAs for the AUA and AGA codons (133). No tRNA augmentation was necessary in recombinant *B. megaterium* and yet there was a 33-fold increase in the specific activity in cell lysate. These results demonstrate that *Sv*PDC is not only produced in very high quantity, but is produced in an active form within the *B. megaterium* host cell. This is quite remarkable because it is the first report of high levels of PDC production in a recombinant Gram-positive bacterium. These results indicate that *B. megaterium* is a better host for production of the *Sv*PDC while it is sub optimal for the production of the Gram-negative PDCs, *Zm*PDC and *Ap*PDC, which were expressed more efficiently in *E. coli.*

Analysis of PDC Transcript Levels

The factors responsible for low-level production of PDC protein in recombinant Gram-positive bacteria are unknown (33-35, 228). In order to determine if transcription and/or mRNA degradation were limiting production of PDC in Gram-positive hosts, we analyzed *pdc* transcript levels for the various recombinant *B. megaterium* strains. Total RNA was isolated and quantitative reverse transcriptase PCR was performed to determine if transcript levels correlated with PDC production (Figure 4-3). 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 and *Scpdc1* the highest. There was not an abundance of *Svpdc* transcript compared to the other *pdc* gene 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.

PDC Protein Stability In Recombinant *B. megaterium*

Gram-positive bacteria, particularly the bacilli, are well known for an abundance of proteases (229). This is often a problem when producing heterologous proteins in these hosts (229-231). To determine if protein degradation was responsible for limiting PDC production in *B. megaterium*, pulse-chase analysis was performed. The *Sv*PDC and *Zm*PDC were chosen for analysis based on the availability of antibodies. After induction of *pdc* transcription (15 min), protein was labeled with L-[^{35}S]-methionine (15 min) and chased with excess unlabeled L-methionine. This enabled the rate of protein degradation after induction of *pdc* gene transcription to be monitored over a period of several hours (Figure 4-4).

During the initial half-hour, the rate of degradation of recombinant PDC protein ranged from 1.3 to 3% of labeled PDC protein per min. The degradation of *Sv*PDC was at a higher rate than that of *Zm*PDC. After these elevated initial rates, however, degradation of both *Sv*PDC and *Zm*PDC were similar at 0.48% and 0.44% labeled PDC protein per minute, respectively. In contrast, samples that had chloramphenicol, a protein synthesis inhibitor, present during the entire chase exhibited no degradation of the PDC proteins. It is, therefore, interesting to note that the protease or proteases responsible for the degradation of the PDC proteins are induced during the induction of the recombinant proteins.

This data proves that degradation of recombinant PDC proteins occurs at very similar rates, yet the amounts of the SvPDC present after 3 h induction is dramatically different when visualized on SDS-PAGE gel (Figure 4-1). Protein degradation is, therefore, not a factor influencing the levels of active PDC protein in *B. megaterium*.

Discussion

For production of ethanol in Gram-positive bacteria to become a viable fuel alternative it will be necessary to find a PDC that can be expressed at high enough levels to rapidly funnel pyruvate to acetaldehyde. Until now, there has not been a PDC that has been expressed well in a recombinant Gram-positive bacterium (33-35, 228).

In this study, *B. megaterium* expression vectors were designed in such a way to transcribe all four *pdc* genes at similar rates by using the same x*ylA* promoter, Shine-Dalgrano sequence, and T7 terminator. Using this approach, the *S. ventriculi* PDC was expressed at high levels in the recombinant Gram-positive host. The *Sv*PDC protein levels and activity were at least 5-fold higher than when the *Z. mobilis*, *A. pasteurianus*, or *S. cerevisiae* PDC proteins were expressed. To assess the biological reason for these differences, quantitative reverse transcriptase PCR and pulse-chase experiments were performed. Similar levels of *pdc*-specific transcript and similar rates of PDC protein degradation were determined. 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 addition of accessory tRNAs is necessary for enhancement of protein levels of *Sv*PDC in *E. coli* by ten-fold (133). This is not the case when *Ap*PDC and *Zm*PDC 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, *Sv*PDC is expressed at very high levels, while expression of *Ap*PDC and *Zm*PDC is poor. The results of the expression of the PDC proteins in *E. coli* and *B. megaterium* indicate that codon usage of the *pdc* genes is one of the primary factors influencing expression of these proteins in Gram-positive hosts (131, 133) (Table 4-3). The contrasting codon usage of the *pdc* genes used in this study becomes evident when analyzing the % G+C in the wobble position. *B. megaterium* has a wobble position % G+C of 30.8%. The *S. ventriculi pdc* gene has the lowest % G+C in the wobble position at 12.3%, the *A. pasteurianus pdc* gene has the highest at 74.2%, and the *Z. mobilis* and *S. cerevisiae pdc* genes have similar percentages at 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 these results. Previous 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* was improved (232) and production of antigen 85A from *Mycobacterium tuberculosis* in *E. coli* was increased 54-fold (233).

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, *Sv*PDC. Alternatively, a *pdc* gene with optimized codon usage could be synthesized for high-level production of alternative PDCs.

Table 4-1. Strains, plasmids, and primers used in Chapter 4.

Strain, Plasmid or PCR primer	Phenotype, genotype or primer sequence	Source
Strain:		
E. coli DH5α	F^- *recA1 endA1 hsdR17* (r_k^- m_k^+) *supE44 thi-1 gyrA relA1*	GibcoBRL (Gathersburg, Md.)
B. megaterium WH320	*lac*$^-$ *xyl*$^+$	MoBiTec
Plasmid:		
pET21d	Ap^r; *E. coli* expression vector	Novagen
pET24b	Kan^r; *E. coli* expression vector	Novagen
pWH1520	Ap^r Tc^r; *E. coli* and *B. megaterium* shuttle vector for expression in *B. megaterium*	(220)
pJAM419	Ap^r; pET21d derivative encoding SvPDC	(133)
pJAM420	Ap^r Tc^r; 1.9-kb *Bsp*EI-to-*Xba*I fragment of pJAM419 ligated with the *Spe*I-to-*Xma*I fragment of pWH1520; used for synthesis of SvPDC in *B. megaterium*	This study
pJAM429	Kan^r; pET24b derivative encoding ApPDC; 1.9-kb PCR product ligated into *Nde*I and *Xho*I sites of pET24b; ApPDC For 5'-GGCC**CATATG**TATCTTGCAGAACG-3', ApPDC Rev 5'-ATTAT**CTCGAG**TCAGGCCAGAGTGG-3'(*Nde*I and *Xho*I sites in bold)	This study
pJAM430	Ap^r Tc^r; 1.9-kb *Bsp*EI-to-*Xba*I fragment of pJAM429 ligated into *Spe*I and *Xma*I sites of pWH1520; used for synthesis of ApPDC in *B. megaterium*	This study
pJAM431	Ap^r; pET21d derivative encoding ZmPDC; 2.1-kb PCR product ligated into *Nco*I and *Xho*I sites of pET21d; ZmPDC For 5'-GGCC**TCATGA**GTTATAGTGTCGG-3', and ZmPDC Rev 5'GATTT**CTCGAG**CTAGAGGAGCTTG-3' (*Bsp*HI and *Xho*I sites in bold)	This study
pJAM432	Ap^r Tc^r; 2.1-kb *Xba*I-to-*Ngo*MIV fragment of pJAM429 ligated into *Spe*I and *Xma*I sites of pWH1520; used for synthesis of ZmPDC in *B. megaterium*	This study

Table 4-1. Continued.

Strain, Plasmid or PCR primer	Phenotype, genotype or primer sequence	Source
pScPDC1	Ap^r; pET22b derivative encoding ScPDC1 with a 6xHIS tag	(90)
pJAM435	Ap^r Tc^r; 1.9-kb *Bsp*EI-to-*Xba*I fragment of pScPDC1 ligated into *Spe*I and *Xma*I sites of pWH1520; used for synthesis of ScPDC1 in *B. megaterium*	This study
RT Primers[a]:		
Svpdc For	5'-AATCGAAATGAAACCGCTAA-3'	This study
Svpdc Rev	5'-TGAGCTTGCAACCATTTCTTTTA-3'	This study
Appdc For	5'-CGCGCCCAACAGCAATGATCA-3'	This study
Appdc Rev	5'-GGGCGGAGTGAGCGTCGGTAAT-3'	This study
Zmpdc For	5'-TGGCGAACTGGCAGAAGCTATCA-3'	This study
Zmpdc Rev	5'-CGCGCTTACCCCATTTGACCA-3'	This study
Scpdc1 For	5'-CACGGTCCAAAGGCTCAATACAA-3'	This study
Scpdc1 Rev	5'-CCGGTGGTAGCGACTCTGTGG-3'	This study

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

Table 4-2. PDC activity of *B. megaterium* strains transformed with *pdc* expression plasmids.

Expression Plasmid	Recombinant Protein[a]	Sp. Act. (U · mg $^{-1}$protein)[b]	Std. Dev.	Purified Sp. Act. (U · mg $^{-1}$protein)
pWH1520	None	0.29	0.05	NA
pJAM420	SvPDC	5.29	0.23	65[c] – 103[d]
pJAM430	ApPDC	0.14	0.06	120[e] – 134.2[f]
pJAM432	ZmPDC	1.11	0.03	51.9[g]
pJAM435	ScPDC1	0.53	0.04	92[h]

[a]Abbrevations: Sv, *S. ventriculi*; Ap, *A. pasteurianus*; Zm, *Z. mobilis*; Sc*, S. cerevisiae*.
[b]PDC specific activity, determined for cell lysate using the ADH coupled assay.
References: c, (133); d, (132); e, (113); f, (111); g, (50); h, (131).

Table 4-3. Codon usage of PDC genes and *B. megaterium* genome.

Amino Acid	Codon	Frequency per Thousand				
		B. megaterium genome	*S. ventriculi pdc*	*A. pasteurianus pdc*	*Z. mobilis pdc*	*S. cerevisiae pdc*
A	GCT	28.1	34.4	9.1	73.8	75.8
	GCC	7.5	0	74.3	33.4	14.1
	GCA	29.2	32.6	18.1	31.6	0
	GCG	11.5	1.8	30.8	10.5	1.8
C	TGT	3.4	7.2	3.6	1.8	8.8
	TGC	2.3	1.8	16.3	10.5	0
D	GAT	36.5	47.1	16.3	19.3	15.9
	GAC	15.4	3.6	32.6	22.8	33.5
E	GAA	60.2	85.1	43.5	63.3	51.1
	GAG	17.1	5.4	10.9	5.3	1.8
F	TTT	29.1	29.0	5.4	5.3	0
	TTC	10.6	18.1	19.9	26.4	40.6
G	GGT	20.6	19.9	7.2	58.0	72.3
	GGC	12.5	0	63.4	21.1	1.8
	GGA	26.8	50.7	3.6	1.8	0
	GGG	8.4	0	3.6	0	0
H	CAT	13.7	12.7	14.5	8.8	0
	CAC	6.3	3.6	10.9	12.3	21.1
I	ATT	43.5	21.7	18.1	14.1	35.3
	ATC	14.9	9.1	34.4	35.1	30.0
	ATA	8.9	39.8	0	0	0
K	AAA	56.2	63.4	12.7	35.1	3.5
	AAG	17.7	5.4	23.6	28.1	58.2
L	TTA	37.3	59.8	0	1.8	7.1
	TTG	10.6	0	5.4	17.6	86.4
	CTT	21.6	14.5	10.9	15.8	0
	CTC	4.5	0	9.1	19.3	0
	CTA	10.4	7.2	0	0	1.8
	CTG	8.0	0	70.7	33.4	0
M	ATG	25.7	27.2	27.2	21.1	22.9
N	AAT	27.6	27.2	16.3	10.5	1.8
	AAC	19.9	19.9	32.6	49.2	51.1
P	CCU	13.9	7.2	5.4	10.5	0
	CCC	2.2	0	21.7	3.5	0
	CCA	12.0	18.1	1.8	3.5	45.9
	CCG	6.7	1.8	14.5	29.9	0
Q	CAA	28.0	29.0	3.6	1.8	38.8
	CAG	11.7	0	29.0	15.8	0
R	CGT	12.8	0	9.1	12.3	3.5
	CGC	7.1	0	29.0	15.8	0
	CGA	6.1	0	0	0	0
	CGG	2.1	0	5.4	1.8	0
	AGA	8.5	39.8	0	0	24.7

Table 4-3. Continued.

		Frequency per Thousand				
Amino Acid	Codon	*B. megaterium genome*	*S. ventriculi pdc*	*A. pasteurianus pdc*	*Z. mobilis pdc*	*S. cerevisiae pdc*
	AGG	2.4	0	1.8	0	0
S	TCT	16.7	7.2	1.8	5.3	35.3
	TCC	4.4	0	19.9	14.1	19.4
	TCA	15.7	32.6	7.2	1.8	0
	TCG	5.0	0	7.2	0	0
	AGT	9.9	12.7	0	5.3	0
	AGC	11.2	12.7	19.9	15.8	1.8
T	ACT	10.5	30.8	1.8	7.0	26.5
	ACC	6.2	0	29.0	28.1	49.4
	ACA	24.1	34.4	10.9	0	0
	ACG	14.2	0	23.6	10.5	0
V	GTT	25.0	43.5	12.7	38.7	31.7
	GTC	8.2	0	23.6	33.4	40.6
	GTA	27.3	36.2	7.2	0	0
	GTG	12.5	0	25.4	5.3	0
W	TGG	10.4	5.4	12.7	12.3	12.3
Y	TAT	23.9	32.6	14.5	26.4	1.8
	TAC	11.0	7.2	14.5	12.3	28.2
Stop	TAA	2.4	1.8	0	0	3.5
	TAG	0.4	0	0	1.8	0
	TGA	0.5	0	1.8	0	0

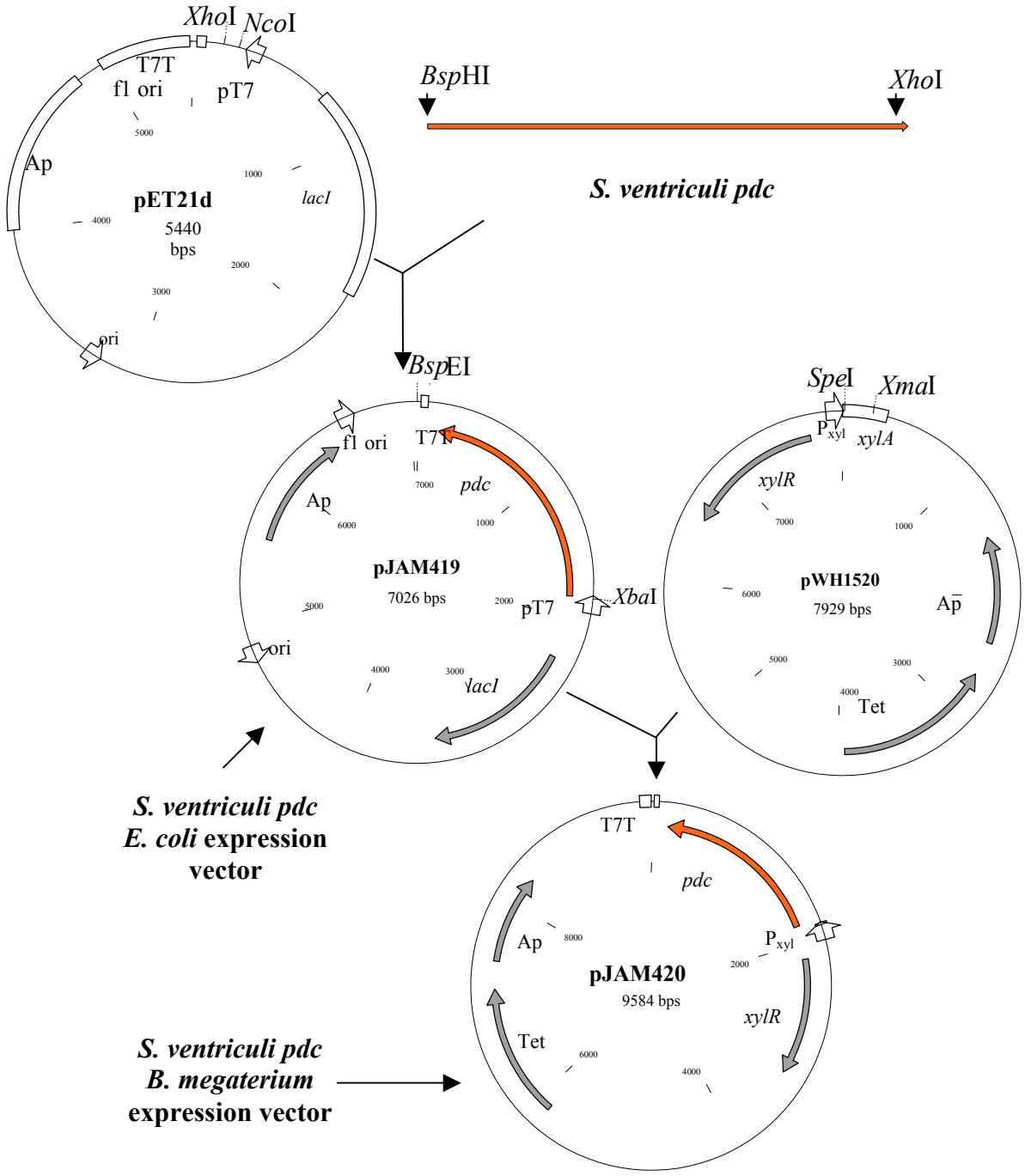

Figure 4-1. Strategy used to construct plasmids for expression of *S. ventriculi pdc* in recombinant *B. megaterium*. A similar approach was used to generate plasmids for expression of *Z. mobilis*, *A. pasteurianus*, and *S. cerevisiae pdc* genes in *B. megaterium*. Abbreviations: Ap = Ampicillin Resistance, pT = T7 polymerase promoter, T7T = T7 polymerase promoter, lacI = lactose operon repressor, f1 = f1 origin of replication, ori = origin of replication, Te = tetracycline resistance, P_{xyl} = *xylA* promoter, and *xylR* = xylose repressor.

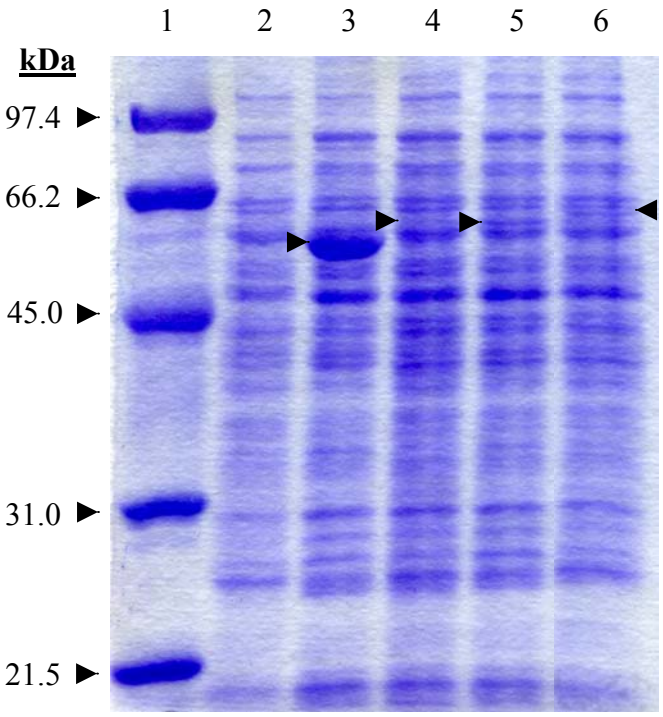

Figure 4-2. PDC proteins synthesized in recombinant *B. megaterium*. After 3 h induction with 0.5% xylose, cell lysate (6 µg) was separated by reducing SDS-PAGE and stained with Coomassie blue R-250. Lane 1) Molecular mass standards (5 µg). Lanes 2-6 Cell lysate of *B. megaterium* WH320 transformed with plasmid vector pWH1520, pJAM420, pJAM430, pJAM432, and pJAM435, respectively. *Sv*PDC (lane 3), *Ap*PDC (lane 4), *Zm*PDC (lane 5), and *Sc*PDC1 (lane 6) are indicated by arrowheads.

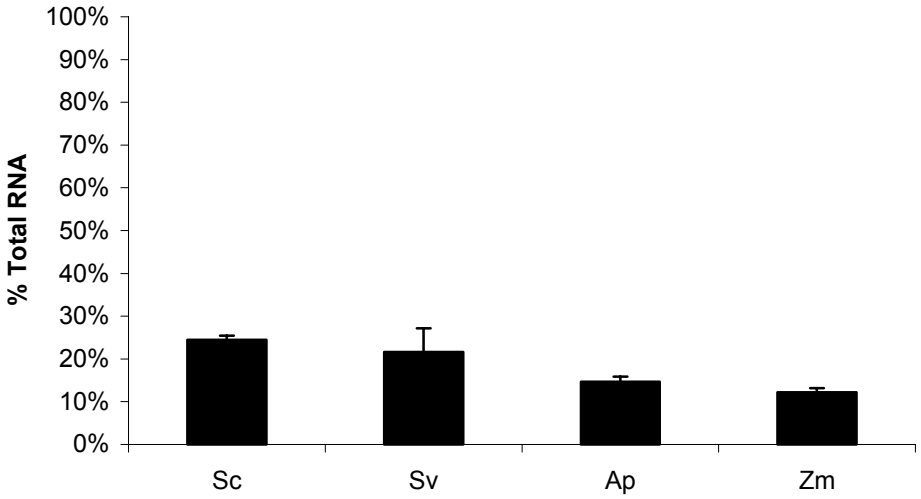

Figure 4-3. Levels of *pdc*-specific transcripts in recombinant *B. megaterium.* Transcript levels were measured in triplicate using real time quantitative reverse transcription. Abbreviations for genes expressed in recombinant *B. megaterium* are Sc (*S. cerevisiae pdc1*), Sv (*S. ventriculi pdc*), Ap (*A. pasteurianus pdc*), and Zm (*Z. mobilis pdc*).

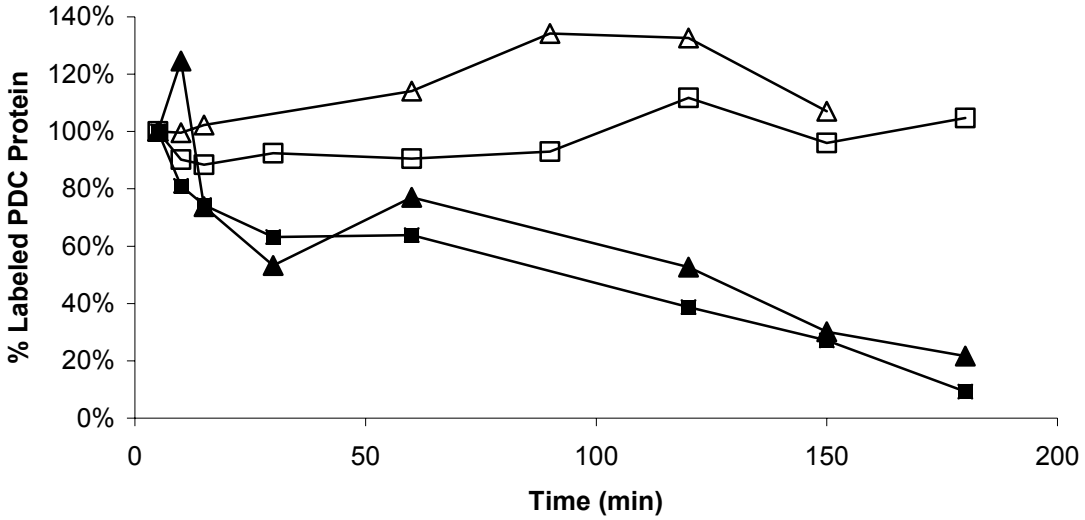

Figure 4-4. PDC protein stability in recombinant *B. megaterium*. Labeled PDC protein levels of *B. megaterium* strains grown without (*Zm*PDC, ■; *Sv*PDC, ▲) and with (*Zm*PDC, □; *Sv*PDC, Δ) addition of chloramphenicol to the chase medium.

CHAPTER 5
GENERAL DISCUSSION AND CONCLUSIONS

The *S. ventriculi pdc* is the first pyruvate decarboxylase to be cloned from a Gram-positive bacterium. *S. ventriculi* PDC protein appears to share similar primary sequence structure to TPP-dependent enzymes and is highly related to the fungal PDC and eubacterial IPD enzymes. The close relationship of the *S. ventriculi* and fungal PDC structures is consistent with the similar biochemical properties of these enzymes. Both types of enzymes display substrate cooperativity with similar affinities for pyruvate. The structure and biochemistry of the *S. ventriculi* PDC, however, dramatically contrast with the only other bacterial PDC (*Z. mobilis*) that has been characterized. The *Z. mobilis* PDC is closely related to plants in primary structure; however, it is the only PDC enzyme known to display Michaelis-Menten kinetics.

This study also demonstrates the synthesis of active, soluble *S. ventriculi* PDC protein in recombinant *E. coli*. Only two other genes, the *Z. mobilis pdc* and *S. cerevisiae PDC1* genes, have been reported to synthesize PDC protein in recombinant bacteria (114, 115, 218). Of these, at least 50% of the *S. cerevisiae* PDC1 forms insoluble inclusions in *E. coli* and thus has not been useful in engineering bacteria for high-level ethanol production (218). Due to codon bias, accessory tRNA is essential for efficient production of *S. ventriculi* PDC in recombinant *E. coli*. However, the low G+C codon usage of the *S. ventriculi pdc* gene should broaden the spectrum of bacteria that can be engineered as hosts for high-level production of PDC protein and the engineering of homo-ethanol

pathways (4). The *S. ventriculi* PDC is unique among previously characterized bacterial PDCs. This has enabled the identification of a new subfamily of PDC-like proteins from Gram-positive bacteria that will broaden the host range of future endeavors utilizing Gram-positive bacterial hosts.

The *Sv*PDC protein is poorly expressed in recombinant *E. coli* (133). Therefore, we reasoned that a host more similar to *S. ventriculi* might express this PDC at higher levels. *B. megaterium* was chosen as a host because it has several benefits over other Gram-positive expression systems. These include a xylose inducible expression vector and absence of alkaline proteases that are often responsible for degradation of foreign proteins (220, 221). Augmentation of the host, *B. megaterium*, with accessory *t*RNAs was not necessary for high-level *Sv*PDC production. The *Sv*PDC protein was more active when produced in *B. megaterium* compared to *E. coli*.

The *Sv*PDC protein produced in *B. megaterium* has a higher V_{max} (98 U per mg protein) at RT than when produced by *E. coli* (66 U per mg protein). The *Sv*PDC produced in *B. megaterium* is also more thermostable than the *E. coli* produced protein. Choosing the correct host appears to have affected the quality of *Sv*PDC protein that was recovered. These results indicate that differences can occur in the biochemical properties of recombinant protein based on host.

In this study, we discovered that the pH of the incubation buffer has an effect on the thermostability of *Sv*PDC. Low pH stabilized *Sv*PDC at higher temperatures. These results suggest that residues of *Sv*PDC gain a charge between pH 5.0–5.5 that allows the tetramer conformation to remain stable at higher temperatures. This is an important

discovery because it gives insight into residues that can be altered in future experiments in order to engineer *Sv*PDC to be more thermostable at cytosolic pH.

The portable production of ethanol (PET) operon used in *E. coli* consists of the *pdc* and *adh* genes from *Zymomonas mobilis*, a Gram-negative organism (24, 25, 129, 227). Past research to engineer a Gram-positive host for ethanol production has focused on using this PET operon, but these attempts have met with limited success (33-35, 228) primarily due to poor expression of the PDC. We have shown that *Sv*PDC is expressed at high levels in *B. megaterium*, a Gram-positive host. Our construction and expression of the Gram-positive ethanol production operon using the *Sv*PDC and *G. stearothermophilus* ADH has demonstrated that recombinant PDC and ADH production no longer limit ethanol production in Gram-positive biocatalysts.

Our research shows that selection of host for recombinant production of proteins can affect the quality and stability of the recombinant protein. We have also demonstrated that *Sv*PDC has qualities that make it unique among bacterial PDCs, including its substrate activation and elevated pH optimum. *Sv*PDC is the only bacterial PDC that is not thermostable, but our results indicate that alteration of charged residues may facilitate the engineering of thermostable *Sv*PDC variants. Lastly, we have created a Gram-positive ethanol production operon that will be useful in engineering future Gram-positive hosts for ethanol production.

B. megaterium expression vectors were designed in such a way to transcribe all four *pdc* genes at similar rates by using the same x*ylA* promoter, Shine-Dalgrano sequence, and T7 terminator. Using this approach, the *S. ventriculi* PDC was expressed at high levels in the recombinant Gram-positive host. The *Sv*PDC protein levels and

activity were at least 5-fold higher than when the *Z. mobilis*, *A. pasteurianus*, or *S. cerevisiae* PDC proteins were expressed. To assess the biological reason for these differences, quantitative reverse transcriptase PCR and pulse-chase experiments were performed. Similar levels of *pdc*-specific transcript and similar rates of PDC protein degradation were determined. 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 addition of accessory tRNAs is necessary for enhancement of protein levels of *Sv*PDC in *E. coli* by ten-fold (133). This is not the case when *Ap*PDC and *Zm*PDC 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, *Sv*PDC is expressed at very high levels, while expression of *Ap*PDC and *Zm*PDC is low. The results of the expression of the PDC proteins in *E. coli* and *B. megaterium* indicate that codon usage of the *pdc* genes is one of the primary factors influencing expression of these proteins in Gram-positive hosts (131, 133).

Thus, this research has now identified a PDC that is expressed at high levels within a Gram-positive bacterial host. Codon usage has also been identified as a major factor to consider when attempting to produce recombinant PDC. Future efforts to engineer Gram-positive hosts for ethanol production now have a PDC available that has been proven to be expressed at high levels or alternatively to synthesize a *pdc* with codon usage that will lead to optimal expression in the host.

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BIOGRAPHICAL SKETCH

LeeAnn Talarico Blalock was born on April 12, 1977, in Charleston, South Carolina. She grew up in Hanahan, South Carolina, with her mother, Sandra Lee. Upon graduation from Stratford High School in Goose Creek, South Carolina, she attended Charleston Southern University on a Board of Trustees Academic Scholarship. She graduated *cum laude* with a major in biology and a minor in chemistry. She is a member of Beta Beta Beta biological honor society and Alpha Chi academic honor society. While attending college, LeeAnn gained experience working in microbiology, chemistry, and biotechnology laboratories, as well as tutoring college students in biology and chemistry.

In August 1999, LeeAnn was accepted as a graduate student in the Department of Microbiology and Cell Science at the University of Florida. She worked in the laboratory of Dr. Julie Maupin-Furlow on the expression of pyruvate decarboxylase in Gram-positive bacteria. In November 2002, she received the President's Award for Graduate Student Oral Presentation at the Southeastern Branch of the American Society for Microbiology annual meeting. In December 2003, LeeAnn will be conferred the degree of Doctor of Philosophy. Upon graduation, LeeAnn will move with her husband, Timothy Blalock, to Boston, Massachusetts, where she will be a Postdoctoral Fellow with Dr. Dennis Kasper in the Department of Microbiology and Molecular Genetics at Harvard Medical School.