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Cloning and Characterization of Two Pyruvate Decarboxylase Genes from *Pichia stipitis* CBS 6054

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In *Pichia stipitis*, fermentative and pyruvate decarboxylase (PDC) activities increase with diminished oxygen rather than in response to fermentable sugars. To better characterize *PDC* expression and regulation, two genes for PDC (*PsPDC1* and *PsPDC2*) were cloned and sequenced from *P. stipitis* CBS 6054. Aside from *Saccharomyces cerevisiae*, from which three PDC genes have been characterized, *P. stipitis* is the only organism from which multiple genes for PDC have been identified and characterized. *PsPDC1* and *PsPDC2* have diverged almost as far from one another as they have from the next most closely related known yeast gene. *PsPDC1* contains an open reading frame of 1,791 nucleotides encoding 597 amino acids. *PsPDC2* contains a reading frame of 1,710 nucleotides encoding 570 amino acids. An 81-nucleotide segment in the middle of the β domain of *PsPDC1* codes for a unique segment of 27 amino acids, which may play a role in allosteric regulation. The 5′ regions of both *P. stipitis* genes include two putative TATA elements that make them similar to the PDC genes from *S. cerevisiae*, *Kluyveromyces marxianus*, and *Hanseniaspora uvarum*.

Pichia stipitis is one of the best-known xylose-fermenting yeasts (5). Besides metabolizing all common monosaccharides, it uses xylan (21) and spent sulfite waste liquors (3) as carbon sources for ethanol production. Wild-type strains of *P. stipitis*, however, will not ferment xylose at rates or with yields that enable commercial ethanol production from hemicellulosic sugars (17, 24). Xylose fermentation is essential for the economic production of ethanol from angiosperm residue (9). Thus, to better understand xylose fermentation, identify rate-limiting steps, and improve overall ethanol production, we are isolating and altering expression of key genes involved in xylose metabolism (31).

Pyruvate decarboxylase (PDC; EC 4.1.1.1.) is one of the key enzymes involved in the fermentative process. It converts pyruvate to acetaldehyde, which is then reduced to ethanol by alcohol dehydrogenase (for a recent review, see reference 22). PDC is found in microorganisms whose predominant fermentation product is ethanol. Classically, pyruvate is viewed as partitioning between acetyl coenzyme A (leading to respiration) and acetaldehyde (leading to fermentation) through the activities of pyruvate dehydrogenase and PDC, respectively (16). Genetic and allosteric regulation of PDC activity seem to be instrumental in directing metabolite flow. During continuous cultivation of *Saccharomyces cerevisiae*, fermentative activity is induced in response to glucose, whereas activities of alcohol dehydrogenase, acetaldehyde dehydrogenase, and acetyl coenzyme A synthetase remain unchanged (30). In *S. cerevisiae*, PDC activity is induced by growth on glucose (28), and its appearance coincides with ethanol production (19). *PDC1* appears to be essential for fermentative growth of *S. cerevisiae* on glucose. Beyond fermentation, however, disruption of all three known PDC genes renders *S. cerevisiae* unable to grow on glucose in a defined minimal medium even though it can grow on a complex medium (7). This finding indicates that PDC may also serve some essential role for growth on glucose.

PDC genes have been cloned and sequenced from the yeasts *S. cerevisiae* (10, 12, 13, 19), *Kluyveromyces marxianus* (14), and *Hanseniaspora uvarum* (15). In *S. cerevisiae*, three PDC structural genes, *PDC1* (12, 19), *PDC5* (13, 29), and *PDC6*, have been characterized (10, 11). These genes are differently expressed at the transcriptional level (7, 11), and they appear to be under autoregulation because *PDC5* is expressed only in *PDC1* deletion mutants (13). The PDC gene of *K. marxianus* (*YskPDC1a*) and the gene from *H. uvarum* are very similar to those of *S. cerevisiae* (14, 15). In *P. stipitis*, PDC activity is induced as oxygen is restricted (23).

Our objective in this study was to determine the number and nature of the PDC genes in *P. stipitis*. The results show that *P. stipitis PDC1* (*PsPDC1*) is substantially different in structure from other yeast PDC genes. The two *P. stipitis* genes have also diverged significantly from one another.

MATERIALS AND METHODS

Strains and plasmids. *P. stipitis* CBS 6054 (NRRL Y-11545, ATCC 58785) was the source of all DNA. *Escherichia coli* DH5α (F$^-$ *recA1 endA1 hsdR17* [r_K^- m_K^+] *supE44 thi-1 gyrA relA1*) (Gibco BRL, Gaithersburg, Md.) was used for routine recombinant DNA experiments that required a bacterial host. *E. coli* XL-1 Blue MRF′ (*recA mcrA mcrB mrr*) and SOLR (Stratagene, La Jolla, Calif.) were used in conjunction with the λ-ZAP genomic DNA library. The *PDC1* and *PDC5* genes from *S. cerevisiae* (*ScPDC1* and *ScPDC5*) were kindly provided by S. Hohmann.

Media. *E. coli* was routinely cultivated in Luria broth. Yeast strains were routinely cultivated in YPD medium (1% yeast extract, 2% peptone, 2% glucose). Selective medium contained 0.17% yeast nitrogen base without amino acids, with 0.5% ammonium sulfate. Fermentation medium consisted of 0.17% yeast nitrogen base without amino acids and without ammonium sulfate (Difco, Detroit, Mich.), 0.23% urea, 0.66% peptone, and 8% glucose or xylose.

DNA and RNA isolation. Plasmid DNA was isolated and purified by using a QIAprep Spin Plasmid kit (Qiagen Inc., Chatsworth, Calif.). Yeast genomic DNA was isolated and purified as described previously (25).

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Genomic DNA library. Genomic DNA was purified from *P. stipitis* CBS 6054 (wild type), partially digested with *Tsp*509I, and fractionated by electrophoresis. The 5- to 10-kb DNA fragments were ligated into λ-ZAP (Stratagene) digested with *Eco*RI. The resultant library has approximately 10^6 individual, recombinant phages with an average insert size of 5 kb. Assuming that *P. stipitis* has a genome equivalent to that of *S. cerevisiae* (14,000 kb/haploid genome), this library has a complexity of 23 genome equivalents.

DNA sequencing. Nucleotide sequences of *PsPDC1* and *PsPDC2* were determined by the dideoxy method of Sanger et al. (27), using a Sequenase kit (United States Biochemicals, Cleveland, Ohio).

Enzymes and chemicals. Restriction enzymes and other DNA modification enzymes were obtained from New England Biolabs (Beverly, Mass.), Stratagene, or Promega Corp. (Madison, Wis.). Reaction conditions were those recommended by the suppliers. SeaKem GTG and SeaPlaque GTG agarose were obtained from FMC BioProducts (Rockville, Md.). Gelase (Epicenter Technology, Madison, Wis.) was used to purify DNA from low-melting-point agarose gels. RNase inhibitor (RNasin) was obtained from Promega.

Southern blot analysis. Southern transfer by capillary blotting was performed as described by Sambrook et al. (26). Both radioactive (^{32}P) and nonradioactive (Genius nonradioactive system; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) probes were used for hybridizations under moderate conditions (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–25% formamide at 37°C) and washes (2× SSC at 25°C and 0.5× SSC at 37 to 50°C).

RT-PCR. Reverse transcription (RT) and subsequent PCR amplifications were performed as described by Kawasaki (18), with the following modifications. Fifty units of reverse transcriptase in 100 μM $MgCl_2$, 20 μM each deoxynucleotide, 50 mM KCl, 10 mM Tris (pH 9.0), 0.1% Triton, 21.8 U of RNasin, 20 mM deoxynucleoside triphosphate, 21 pmol of oligo (dT), and 1 μg of total RNA (DNase treated) in a 20-μl final reaction volume were incubated at 23°C for 10 min and then at 42°C for 45 min. Reaction tubes were heated at 95°C for 10 min and then kept on ice for further use. To start the PCR, 10 μl of 10× reaction buffer, 2 U of *Taq* DNA polymerase, and 21 pmol of each primer were added to each tube in a final reaction volume of 100 μl, and then reaction mixtures were carried through a standard thermal cycle profile. The first cycle consisted of 94°C for 6 min, 54°C for 2 min, and 72°C for 40 s. This cycle was followed by 35 cycles of 94°C for 1 min, 54°C for 2 min, 72°C for 5 min, and finally 72°C for 15 min.

Sequence analysis and deposition. BLAST searches (1) were performed on the National Center for Biotechnology Information server. All sequence assembly, alignment, and analysis were performed with the Genetics Computer Group sequence analysis software package (4). Distances were calculated as substitutions per 100 amino acids by using the Kimura method (20) following deletion of gapped regions. The phylogenetic tree was drawn by using the neighbor-joining method.

Nucleotide sequence accession numbers. The sequences of *PsPDC1* and *PsPDC2* were deposited in GenBank, and the accession numbers are U75310 and U75311, respectively.

RESULTS

***PsPDC1* and *PsPDC2* clones.** Since other yeast genes for PDC had proven similar to *ScPDC1* and *ScPDC5* (14, 15), we reasoned that the *P. stipitis* PDC genes could be cloned through cross-hybridization with the coding sequences of homologous genes from *S. cerevisiae*. Southern hybridizations with either *ScPDC1* or *ScPDC5* resulted in the same banding patterns in blots of *Bam*HI-digested genomic DNA of *P. stipitis*. Only two bands were apparent (data not shown), which suggested that no more than two genes for PDC were closely homologous to the *S. cerevisiae* genes. *ScPDC1* was used to screen a total of 200,000 phage plaques from a λ library of *P. stipitis* CBS 6054. We identified five individual plaques that strongly cross-hybridized to *ScPDC1*. Restriction enzyme digestion and Southern hybridization showed that these plaques belonged to two distinct classes. Clones 17 and 18 overlapped to form *PsPDC1*, while clones 4, 5, and 20 overlapped to form *PsPDC2* (Fig. 1). These results likewise suggest that only two close PDC homologs were present.

Sequences of *PsPDC1* and *PsPDC2*. *PsPDC1* was sequenced by primer walking from the universal primer T3 located next to the multiple cloning site of clone 17. The 2,534-bp nucleotide sequence of *PsPDC1* contains an open reading frame of 1,791 nucleotides (nt) encoding a polypeptide of 597 amino acids. Two putative TATA elements, upstream of the putative AUG start codon, are located at −64 (TAAATATA) and −228 (TATATAAA). The 4.0-kb *Xho*I fragment containing the 3′-flanking region and partial coding sequence of *PsPDC2* was deleted from clone 5, and the religated plasmid was sequenced by primer walking from the universal primer T7. The 2,305-bp sequence of *PsPDC2* contains an open reading frame of 1,710 nt encoding a polypeptide of 570 amino acids. Two putative TATA elements are located at −126 (TATAAAT) and at −177 (TATAAT) relative to the start of translation. No obvious *cis*-acting sequences are apparently shared between the *PsPDC* genes or with the *ScPDC* genes. The thiamine binding structural motif which is characteristic of thiamine pyrophosphate binding proteins (8) was present in both of the predicted *PsPDC* structures.

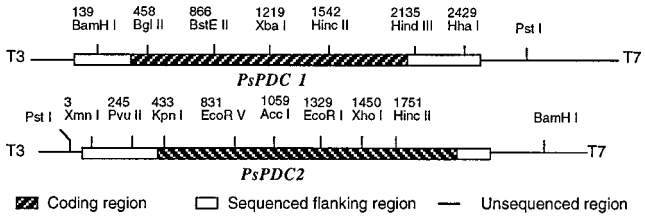

FIG. 1. Restriction map of PDC clones from *P. stipitis*. Restriction sites and approximate lengths of each clone (in kilobases) are indicated. T3 and T7 are adjacent regions of pBluescript KS II. Numbers indicate positions of restriction sites in the sequenced region.

Characteristics of *PsPDC1*. *PsPDC1* has an 81-nt segment (coding for 27 amino acids) that is not found in other known PDC genes (Fig. 2). This sequence in *P. stipitis* corresponds to and replaces amino acid residues 255 to 257 in *S. cerevisiae*. To determine whether this sequence is actually encoded within the mature mRNA—and is not an intron or cloning artifact—we amplified this segment from mRNA and DNA by using RT-PCR primers specific for each of the two *PsPDC* genes. We observed bands corresponding to *PsPDC1* and *PsPDC2* in mRNA from cells grown on both glucose and xylose, and these bands matched those observed from genomic DNA (Fig. 3). These results indicate that the 81-nt segment is indeed transcribed within the *PsPDC1* mRNA. BLAST searches (1) against all published sequences in all data banks provided no good matches or clues to its function.

At the amino acid and nucleotide levels, the *PsPDC1* gene is 70 and 63%, respectively, identical to the *ScPDC1* gene; the *PsPDC2* gene is slightly less conserved (68 and 62% identity to *ScPDC1*), and the *PsPDC* genes are 72.5 and 70% identical to each other. A phylogenetic analysis of *PsPDC1* and *PsPDC2* with other yeast PDC genes showed that the two *Pichia* genes have diverged farther from one another than have the three PDC genes in *S. cerevisiae* (Fig. 4).

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261                                        300
KGSIDE.... .......... .......... ...KHPRFGG K. lactis
KGSIDE.... .......... .......... ...QHPRFGG K. marxianus
KGSISE.... .......... .......... ...QHPRYGG S. cerevisiae PDC1
KGAIDE.... .......... .......... ...QHPRYGG S. cerevisiae PDC5
KGSIDE.... .......... .......... ...QHPRYGG S. cerevisiae PDC6
KGSIDE.... .......... .......... ...KHPRFGG H. uvarum
KSGISE.... .......... .......... ...SHPRLGG P. stipitis PDC1
KGTVDEGGVD GELLEDDPHL IAKVAARLSA GKNAASRFGG P. stipitis PDC2
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FIG. 2. Alignment of predicted protein sequences of yeast PDC genes where *PsPDC1* contains an additional segment. Accession numbers (from top to bottom): X85968, L09727, X04675, X15668, X55905, U13635, U75301, U75311. Position numbering is based on *PsPDC2*.

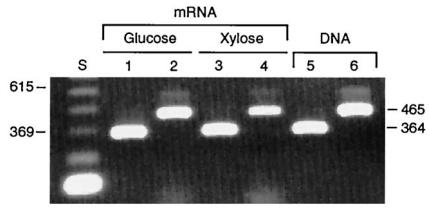

FIG. 3. Amplification of *PsPDC1* mRNA (lanes 1, 3, and 5) and *PsPDC2* mRNA (lanes 2, 4, and 6) from cells grown in glucose (lanes 1 and 2) or xylose (lanes 3 and 4) under aerobic conditions and of genomic DNA (lanes 5 and 6) yielded bands of the same size. Lane S is a standard DNA ladder with 123-bp increments.

DISCUSSION

Southern blot analysis indicated that at least two genes in *P. stipitis* were homologous to the *PDC1* and *PDC5* genes of *S. cerevisiae*. Plaque hybridization against a λ library of *P. stipitis* genomic DNA identified five clones that strongly hybridized to the *S. cerevisiae* sequence. Restriction analysis and southern blot hybridizations showed that these clones belonged to two overlapping groups, which we designated *PsPDC1* and *PsPDC2*. Examination of the 5′ flanking region revealed no obvious *cis*-acting sequences that could indicate *trans*-acting factors or environmental conditions that control their expression. We found that the predicted protein of *PsPDC1* contains a unique 27-amino-acid insertion relative to other known PDC proteins.

The fact that the two *P. stipitis* proteins diverged almost as far from one another as they did from other known yeast PDC sequences suggests that they may play different roles in *P. stipitis* metabolism. Aside from the three structural PDC genes from *S. cerevisiae*, this is the only organism from which multiple genes for PDC have been identified and characterized. The 5′ regions of *PsPDC1* and *PsPDC2* include two putative TATA elements, which make them similar to the PDC genes from *S. cerevisiae* (19), *K. marxianus*, and *H. uvarum* (15). Determination of the significance of potential *cis*-acting sequences in the 5′ region must await further sequence analysis and regulatory studies.

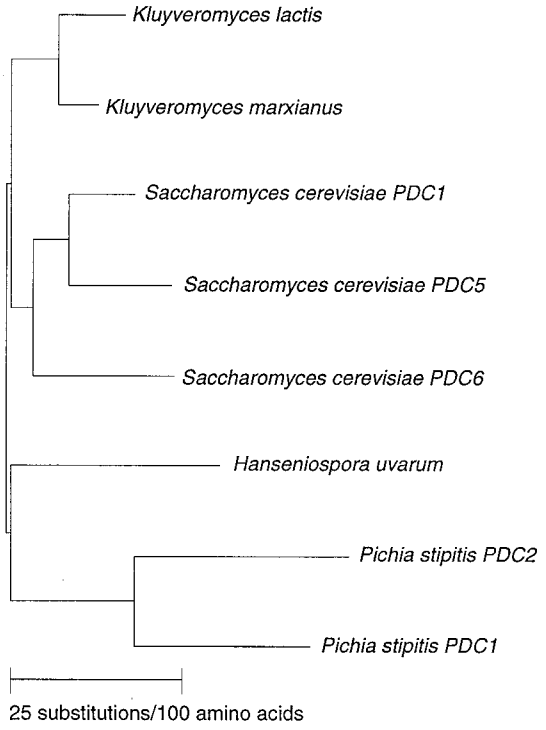

FIG. 4. Phylogenetic tree showing similarity among yeast PDC genes. Sequences of eight different PDC genes available from GenBank and EMBL data banks were compared by using Genetics Computer Group PileUp, Distances, and GrowTree programs. Source names are shown. Accession numbers are the same as those for Fig. 2.

The predicted primary amino acid sequence of PsPDC1 differs substantially from ScPDC1 in only three places. The most intriguing change is in the β domain with the previously mentioned 27-amino-acid insertion (amino acids 264 to 290) at amino acid residue 255 in ScPDC1p. Based on the three-dimensional structure of PDC from *S. cerevisiae* (2) and *Saccharomyces uvarum* (6), this loop could form an amphipathic α helix, based on hydrophobicity and probability, on the surface of the molecule. The new structure is very close in space to Cys221, which is known to be important for substrate activation in the tertiary structure (32). It is reasonable to suspect that the 27-amino-acid extension may be important for allosteric regulation of the molecule, especially in light of the different kinetic regulation of *P. stipitis* and *S. cerevisiae* PDC activities (23). This hypothesis could be directly tested by deletion of the 27-amino-acid insert by site-specific mutagenesis and expression, by either targeted gene replacement in *P. stipitis* or heterologous expression in PDC mutants of *S. cerevisiae.*

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