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Characterization of *PDC6*, a Third Structural Gene for Pyruvate Decarboxylase in *Saccharomyces cerevisiae*

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Pyruvate decarboxylase is the key enzyme in alcoholic fermentation in yeast. Two structural genes, *PDC1* and *PDC5* have been characterized. Deletion of either of these genes has little or no effect on the specific pyruvate decarboxylase activity, but enzyme activity is undetectable in mutants lacking both *PDC1* and *PDC5* (S. Hohmann and H. Cederberg, Eur. J. Biochem. 188:615–621, 1990). Here I describe *PDC6*, a gene structurally closely related to *PDC1* and *PDC5*. The product of *PDC6* does not seem to be required for wild-type pyruvate decarboxylase activity in glucose medium; Δ*pdc6* mutants have no reduced specific enzyme activity, and the *PDC6* deletion did not change the phenotype or the specific enzyme activity of mutants lacking either or both of the other two structural genes. However, in cells grown in ethanol medium the *PDC6* deletion caused a reduction of pyruvate decarboxylase activity. Northern (RNA) blot analysis showed that *PDC6* is weakly expressed, and expression seemed to be higher during growth in ethanol medium. This behavior remained obscure since pyruvate decarboxylase catalyzes an irreversible reaction. Characterization of all combinations of *PDC* structural gene deletion mutants, which produce different amounts of pyruvate decarboxylase activity, showed that the enzyme is also needed for normal growth in galactose and ethanol medium and in particular for proper growth initiation of spores germinating on ethanol medium.

Pyruvate is the end product of glycolysis. It can be further degraded either by the pyruvate dehydrogenase (PDH; EC 1.2.4.1) complex to acetyl coenzyme A which enters the tricarboxylic acid cycle or via pyruvate decarboxylase (PDC; EC 4.1.1.1) to acetaldehyde and subsequently to ethanol. In the yeast *Saccharomyces cerevisiae*, however, most of the pyruvate is channeled through the PDC reaction (9); synthesis of mitochondrial functions, in particular of respiratory enzymes, is reduced in the presence of glucose (glucose repression [6, 9]), while expression of several genes for glycolytic enzymes and for PDC is induced (glucose induction [14, 20]). Although the degradation of pyruvate is a key point in sugar catabolism, the genetics of the reactions involved have not been fully deciphered yet, while all the other glycolytic reactions are genetically well characterized (9).

The PDH complex consists of three catalytic activities and at least four or five different polypeptides (34). One of the three activities is PDH itself, which is composed of two different subunits, E1α and E1β (34). The gene for the E1α subunit from yeast, *PDA1*, has been cloned. Deletion mutants of *PDA1* have a largely reduced PDH activity but no obvious growth deficiencies, indicating the PDH is not essential for growth on glucose (28). Small amounts of acetyl coenzyme A can probably also be synthesized via acetaldehyde and acetate, thus bypassing the PDH reaction (9).

Mutants lacking PDC or showing reduced PDC activity are severely impaired for growth on glucose. Schmitt and Zimmermann (21) isolated mutants in the gene *PDC1* which showed reduced or almost undetectable PDC activity in crude extracts. This gene was cloned (20), sequenced (13), and long thought to be the only structural gene for PDC. In contrast to the *pdc1* point mutants, however, deletion mutants turned out to grow well on glucose and they had only slightly reduced PDC activity (19, 23). The effect was shown to be due to enhanced expression of *PDC5*, a second closely related and normally only weakly expressed structural gene for PDC (12, 19, 23). Thus, in addition to being induced by glucose (12, 20), the expression of the *PDC* genes seems to be under autoregulation at the transcriptional level (11, 12). The products of the *pdc1* point mutant alleles, although catalytically inactive, are apparently still recognized by the autoregulatory mechanism, and therefore expression of *PDC5* is not enhanced in such mutants (11). Deletion of both *PDC1* and *PDC5* in the same strain results in complete loss of *PDC* activity and in failure to ferment glucose and to grow normally in glucose medium (12).

The genes *PDC2* (21), *PDC3* (32), and *PDC4* (24) have been identified in different screens. All these mutants have between 20 and 40% of the wild-type PDC activity, and they grow slowly on glucose. These genes may code for regulatory functions, but none of them has been characterized yet. Interestingly, the *pdc2* mutation just reduces expression of *PDC1* while it completely abolishes *PDC5* expression, even in the Δ*pdc1* mutant in which expression of *PDC5* is normally enhanced (12). *PDC2* may function posttranscriptionally (20). In this work I further characterize the properties of mutants with reduced or no PDC activity. Moreover, by low-stringency Southern blot analysis a third structural gene for PDC has been identified, and the molecular characterization of this gene is presented.

MATERIALS AND METHODS

Yeast strains. The yeast strains used are summarized in Table 1. For the determination of enzyme activities additional strains with identical markers derived from the same cross as the descendants listed in Table 1 (the YSH 5.135 series) were used.

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TABLE 1. Yeast strains used

Designation	Genotype	Source
YSH 4.125.-1c	*MATa leu2-3/112 trp1-92 ura3-52*	This work
YSH 56-1-4B	*MATa leu2-3/112 trp1-92 ura3-52 Δpdc1::LEU2*	19
M5 (diploid)	*MATa leu2-3/112 trp1-92 ura3-52 his4* *MATα leu2-3/112 trp1-92 ura3-52 HIS4*	19
YSH 4.136.-4D	*MATa leu2-3/112 trp1-92 ura3-52 Δpdc1::LEU2 Δpdc5::URA3*	12
YSH 5.103.-2b	*MATα leu2-3/112 trp1-92 ura3-52 Δpdc6::TRP1*	This work
YSH 149	*MATa leu2-3/112 trp1-92 ura3-52 PDC1-PDC6 Δpdc1::leu2 Δpdc5::URA3*	11
YSH 5.135.-2A	*MATa leu2-3/112 trp1-92 ura3-52*	This work
YSH 5.135.-1B	*MATa leu2-3/112 trp1-92 ura3-52 Δpdc1::LEU2*	This work
YSH 5.135.-5A	*MATα leu2-3/112 trp1-92 ura3-52 Δpdc5::URA3*	This work
YSH 5.135.-1C	*MATa leu2-3/112 trp1-92 ura3-52 Δpdc6::TRP1*	This work
YSH 5.135.-4C	*MATa leu2-3/112 trp1-92 ura3-52 Δpdc1::LEU2 Δpdc5::URA3*	This work
YSH 5.135.-3B	*MATa leu2-3/112 trp1-92 ura3-52 Δpdc5::URA3 Δpdc6::TRP1*	This work
YSH 5.135.-9C	*MATα leu2-3/112 trp1-92 ura3-52 Δpdc1::LEU2 Δpdc6::TRP1*	This work
YSH 5.135.-4D	*MATa leu2-3/112 trp1-92 ura3-52 Δpdc1::LEU2 Δpdc5::URA3 Δpdc6::TRP1*	This work

Bacterial strains and plasmids. *Escherichia coli* JM101 and JM109 were used for the propagation of plasmids and M13 phages. pUC18 and pUC19 served as cloning and subcloning vectors, and M13mp18 and M13mp19 were used as subcloning vectors for sequencing (17, 33).

Media. Yeast cells were grown in standard media (26). Oxoid (Basingstoke, United Kingdom) products were used, in particular for solid yeast extract-peptone (YEP) media, where certain other products gave problems with the germination of *pdc* mutant spores. Bacteria were propagated as described by Sambrook et al. (17).

DNA manipulation. For all work with recombinant DNA, standard procedures were applied (17).

DNA sequencing. Appropriate fragments were subcloned into M13mp18 or M13mp19 (33). Some of these fragments were shortened by nested deletions, or synthetic oligonucleotides were used as primers (17). The entire gene was sequenced on both strands by the method of Sanger et al. (18) by using the T7 DNA polymerase-based sequencing kit from Pharmacia-LKB (Bromma, Sweden). Sequences were analyzed by the DNASIS/PROSIS software package from Hitachi (Brisbane, Calif.).

Cloning of *PDC6*. Southern blots with genomic yeast DNA were hybridized under reduced stringency with the *Eco*RI-*Bgl*II fragment of the *PDC1* coding region (19) as probe. Reduced stringency was achieved by slowly decreasing the temperature during hybridization from 68 to 45°C (8 h at 68°C, 2 h to decrease to 45°C, and 4 h at 45°C), omitting the final high-stringency wash and otherwise following the instructions in the Boehringer (Mannheim, Germany) dioxigenin labeling and detection kit. In addition to the expected signals for *PDC1* and *PDC5* a third sequence was identified (Fig. 1). This sequence was cloned on a *Bam*HI fragment from strain YSH 149 (Table 1) after preparation of partial genomic libraries and colony hybridization as described previously (19).

Deletion of *PDC6*. The strategy followed the procedure of Rothstein (16). The 5.5-kb *Kpn*I-*Xba*I fragment carrying *PDC6* (Fig. 2) was subcloned into pUC18. The resulting plasmid was digested with *Cla*I and *Bgl*II, cutting out 71 bp of the *PDC6* coding region. The *TRP1* gene from plasmid YRp7 (29) was inserted as a *Cla*I-*Bam*HI fragment of 0.85 kb. The plasmid with this *PDC6* deletion allele was digested with *Nco*I and *Kpn*I and transformed into the diploid strain M5. Several stable tryptophan prototrophic transformants were subjected to Southern blot analysis (not shown); one transformant showed the expected bands and was sporulated, and the spores were analyzed for further investigation.

PDC assays. Cells were pregrown for 2 days in YEP–3% ethanol to stationary phase. Aliquots of 0.5 or 1 ml from these cultures were inoculated into 5 ml of YEP with 8% glucose or 3% ethanol and shaken for 6 h at 30°C. PDC activity was determined as described by Schmitt and Zimmermann (21), and protein was measured by the method of Zamenhoff (35). For each genotype three different strains were tested in duplicate.

Spore germination. The dissection of tetrads was performed directly on YEP–3% ethanol plates and spores were allowed to germinate at 30°C (26). For further analysis the

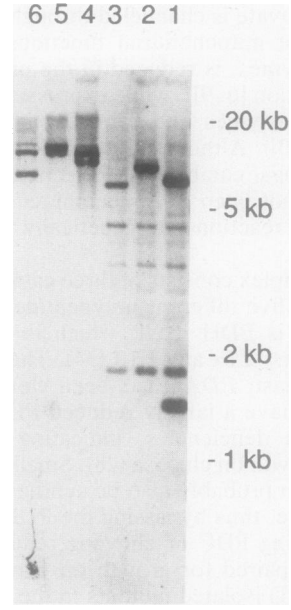

FIG. 1. Reduced-stringency Southern blot analysis of genomic yeast DNA. Probe: 815-bp *Eco*RI-*Bgl*II fragments from *PDC1* (19). Lanes: 1 to 3, *Eco*RI digest; 1, wild type (expected fragments for *PDC1*, 6.5 kb; for *PDC5*, 1.4 kb); 2, Δ*pdc1* Δ*pdc5* mutant (Δ*pdc1*, 7.5 kb; Δ*pdc5*, no signal); 3, YSH 149 (Δ*pdc1* rearranged, 6.5 kb; Δ*pdc5*, no signal); 4 to 6, *Bam*HI digest; 4, wild type (*PDC1*, 9 kb; *PDC5*, 8 kb); 5, Δ*pdc1* Δ*pdc5* mutant (Δ*pdc1*, 10 kb; Δ*pdc5*, no signal); 6, YSH 149 (Δ*pdc1* rearranged, 6.8 kb; Δ*pdc5*, no signal).

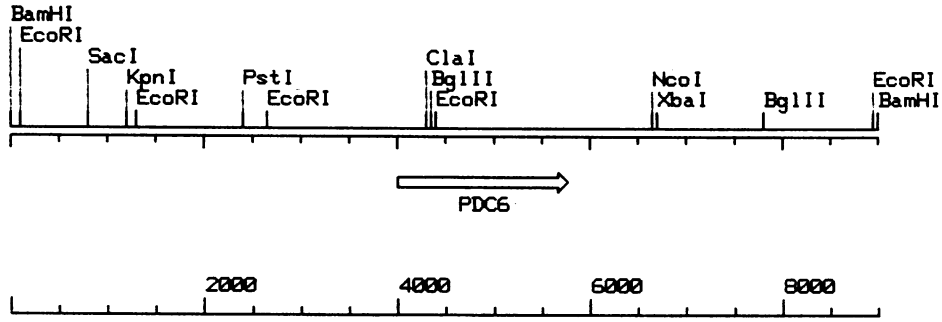

FIG. 2. Restriction map of the cloned 9-kb *Bam*HI fragment carrying *PDC6*. No sites were found for *Hin*dIII, *Sal*I, *Sma*I, *Sph*I, and *Xho*I.

colonies derived from the spores were picked up and transferred to a fresh plate in the order of their size (the biggest one first and the smallest one last). The size of the colony was correlated with the genotype by counting the number of spores in each position in 97 tetrads and triads. The biggest colony was given 1 point, and the smallest colony or a spore not grown up to a colony (the genotype of which was predicted from the genotype of the other three spores) was given 4 points. The average position in the tetrad was calculated by summing up the points and dividing them by the total number of spores of the corresponding genotype following the example for wild-type spores as follows: biggest colony in a tetrad, 21 times (21 points); second biggest, 16 times (32 points); third biggest, 8 times (24 points); smallest or not grown up, 1 time (4 points); total points, 81; total number of wild-type spores, 46; average position, 81/46 = 1.76.

Growth curves. Yeast cells were grown for 3 days in YEP–3% ethanol so that they were entirely in stationary growth phase. These cultures were diluted 1:100 (to give an optical density at 600 nm of about 0.1) into fresh YEP medium with different carbon sources and shaken at 30°C. Cell density was measured every 30 or 60 min at 600 nm.

Northern (RNA) blot analysis. Cells were pregrown in 100 ml of YEP–3% ethanol overnight to an optical density at 600 nm of about 2. The culture was then sedimented and divided into 100 ml of YEP–3% ethanol and 100 ml of YEP–8% glucose and shaken for 2 h. Crude RNA was prepared by breaking the cells with glass beads in the presence of phenol, four extractions with phenol, and precipitation with ethanol (4). mRNA was enriched by using the Pharmacia-LKB mRNA purification kit [oligo(dT)-cellulose-spun columns]. After electrophoretical separation, the mRNA was transferred to a Hybond N nylon membrane by vacuum blotting and hybridized with radioactive probes essentially as described in the Hybond N blotting membrane product information from Amersham (Amersham, United Kingdom).

Nucleotide sequence accession number. The nucleotide sequence of *PDC6* has been deposited with the EMBL and GenBank data bases under the accession number X55905. The sequences of *PDC1* (12, 13) (data base accession number X04675) and *PDC5* (12) data base accession number X15668) have been described previously.

RESULTS

Isolation of *PDC6*. Southern blot analysis of genomic yeast DNA at reduced hybridization stringency with a *PDC1* probe showed at least one additional sequence with significant homology to *PDC1* and *PDC5* (Fig. 1). The sequence showed up as 1.7- and 4.3-kb *Eco*RI fragments and as a 9-kb *Bam*HI fragment (Fig. 1 and 2). Additional weak signals (e.g., 3.7-kb *Eco*RI or 10-kb *Bam*HI) could correspond to *ILV2* (coding for acetolactate synthase [7]) or to further as yet unidentified thiamine PP_i-dependent enzymes. The 9-kb *Bam*HI fragment was cloned from strain YSH149 (Table 1). This strain carries the Δ*pdc5* allele (12) and a rearranged Δ*pdc1* allele (11). Therefore, *PDC1* or *PDC5* sequences could not interfere in the detection of the 9-kb *Bam*HI fragment (Fig. 2). The exact location of the sequence homologous to *PDC1* was determined by Southern analysis and by DNA sequencing (Fig. 2).

Sequence analysis of *PDC6*. Figure 3 shows an alignment of the predicted amino acid sequences of the three PDC isoenzymes. All three genes code for primary translation products of 563 amino acids. The overall identity of all three protein

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      1        10        20        30        40        50        60
PDC1 MSEITLGKYLFERLKQVNVNTVFGLPGDFNLSLLDKIYEVEGMRWAGNANELNARYAADG
PDC5 MSEITLGKYLFERLSQVNCNTVFGLPGDFNLSLLDKLYEVKGMRWAGNANELNAAYAADG
PDC6 MSEITLGKYLFERLKQVNVNTIFGLPGDFNLSLLDKIYEVDGLRWAGNANELNAAYAADG

             70        80        90       100       110       120
PDC1 YARIKGMSCIITTFGVGELSALNGIAGSYAEHVGVLHVVGVPSISSQAKQLLLHHTLGNG
PDC5 YARIKGMSCIITTFGVGELSALNGIAGSYAEHVGVLHVVGVPSISSQAKQLLLHHTLGNG
PDC6 YARIKGLSVLVTTFGVGELSALNGIAGSYAEHVGVLHVVGVPSISAQAKQLLLHHTLGNG

             130       140       150       160       170       180
PDC1 DFTVFHRMSANISETTAMITDIATAPAEIDRCIRTTYVTQRPVYLGLPANLVDLNVPAKL
PDC5 DFTVFHRMSANISETTAMITDIRNAPAEIDRCIRTTYTTQRPVYLGLPANLVDLNVPAKL
PDC6 DFTVFHRMSANISETTSMITDIATAPSEIDRLIRTTFITQRPSYLGLPANLVDLKVPGSL

             190       200       210       220       230       240
PDC1 LQTPIDMSLKPNDAESEKEVIDTILALVKDAKNPVILADACCSRHDVKAETKKLIDLTQF
PDC5 LETPIDLSLKPNDAEAEAEVVRTVVELIKDAKNPVILADACASRHDVKAETKKLMDLTQF
PDC6 LEKPIDLSLKPNDPEAEKEVIDTVLELIQNSKNPVILSDACASRHNVKKETQKLIDLTQF

             250       260       270       280       290       300
PDC1 PAFVTPMGKGSISEQHPRYGGVYVGTLSKPEVKEAVESADLILSVGALLSDFNTGSFSYS
PDC5 PVYVTPMGKGAIDEQHPRYGGVYVGTLSRPEVKKAVESADLILSIGALLSDFNTGSFSYS
PDC6 PAFVTPLGKGSIDEQHPRYGGVYVGTLSKQDVKQAVESADLILSVGALLSDFNTGSVSYS

             310       320       330       340       350       360
PDC1 YKTKNIVEFHSDHMKIRNATFPGVQMKFVLQKLLTNIADAAKGYKPVAVPARTPANAAVP
PDC5 YKTKNIVEFHSDHIKIRNATFPGVQMKFALQKLLDAIPDVVKDYKPVAVPARVPITKSTP
PDC6 YKTKNVVEFHSDYVKVKNATFLGVQMKFALQNLLKVIPDVVKGYKSVPVPTKTPANKGVP

             370       380       390       400       410       420
PDC1 ASTPLKQEWMWNQLGNFLQEGDVVIAETGTSAFGINQTTFPNNTYGISQVLWGSIGFTTG
PDC5 ANTPMKQEWMWNQLGNFLREGDIVIAETGTSAFGINQTTFPTDVYAIVQVLWGSIGFTVG
PDC6 ASTPLKQEWLWNELSKFLQEGDVIISETGTSAFGINQTIFPKDAYGISQVLWGSIGFTTG

             430       440       450       460       470       480
PDC1 ATLGAAFAAEEIDPKKRVILFIGDGSLQLTVQEISTMIRWGLKPYLFVLNNDGYTIEKLI
PDC5 ALLGATMAAEELDPKKRVILFIGDGSLQLTVQEISTMIRWGLKPYIFVLNNNGYTIEKLI
PDC6 ATLGAAFAAEEIDPNKRVILFIGDGSLQLTVQEISTMIRWGLKPYLFVLNNDGYTIEKLI

             490       500       510       520       530       540
PDC1 HGPKAQYNEIQGWDHLSLLPTFGAKDYETHRVATTGEWDKLTQDKSFNDNSKIRMIEVML
PDC5 HGPHAEYNEIQGWDHLALLPTFGARNYETHRVATTGEWEKLTQDKDFQDNSKIRMIEVML
PDC6 HGPHAEYNEIQTWDHLALLPAFGAKKYENHKIATTGEWDALTTDSEFQKNSVIRLIELKL

             550       560       570
PDC1 PVFDAPQNLVKQAKLTAATNAKQ
PDC5 PVFDAPQNLVKQAQLTAATNAKQ
PDC6 PVFDAPESLIKQAQLTAATNAKQ
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FIG. 3. Alignment of the predicted protein sequences of the three PDC isoenzymes. Positions with differences are marked with a dash.

TABLE 2. PDC activities of wild-type and *pdc* mutant strains grown with different carbon sources

Genotype	Glucose		Ethanol	
	Sp act (mU/mg of protein)	% of wild-type activity	Sp act (mU/mg of protein)	% of wild-type activity
PDC1 PDC5 PDC6	1,500	100	370	100
Δpdc1 PDC5 PDC6	1,150	77	40	11
PDC1 Δpdc5 PDC6	1,550	103	510	138
PDC1 PDC5 Δpdc6	1,500	100	280	76
Δpdc1 Δpdc5 PDC6	<1	<1	<5	<1.5
Δpdc1 PDC5 Δpdc6	1,100	73	40	11
PDC1 Δpdc5 Δpdc6	1,550	103	540	146
Δpdc1 Δpdc5 Δpdc6	<1	<1	<5	<1.5

TABLE 3. Growth characteristics of wild-type and *pdc* mutant strains[a]

Genotype	Lag phase (h)				Generation time (h)			
	E	Gal	D	DA	E	Gal	D	DA
PDC1 PDC5 PDC6	4.0	6.0	4.2	4.3	3.4	1.7	1.3	1.5
Δpdc1 PDC5 PDC6	3.0	8.1	4.8	8.0	3.3	2.1	1.45	3.05
PDC1 Δpdc5 PDC6	3.1	7.2	3.3	4.2	3.3	1.8	1.2	1.45
PDC1 PDC5 Δpdc6	4.0	7.2	3.6	4.4	3.4	1.75	1.3	1.6
Δpdc1 Δpdc5 PDC6	4.0	7.0	4.5	NG	4.1	9.0	7.0	NG
Δpdc1 PDC5 Δpdc6	4.4	7.6	4.7	9.3	3.2	2.15	1.4	3.15
PDC1 Δpdc5 Δpdc6	4.3	8.6	3.6	3.9	3.3	1.9	1.25	1.55
Δpdc1 Δpdc5 Δpdc6	4.1	7.2	4.0	NG	4.5	8.5	6.5	NG

[a] Cells were pregrown to stationary phase in YEP–ethanol and then inoculated into YEP medium with ethanol (E), galactose (Gal), glucose (D), and glucose with antimycin A (DA). NG, no growth.

sequences is 78%, which means that in 114 positions of the 563 amino acids the three sequences are not identical. About half of these amino acid exchanges represent conservative substitutions. In just 11 positions all three sequences have a different amino acid. The pairwise comparisons revealed 88% identity for *PDC1* and *PDC5* (DNA level, 88% [12]), 88% identity for *PDC1* and *PDC6* (DNA level, 74%), and 80% identity for *PDC5* and *PDC6* (DNA level, 73%).

A tryptophan residue was suggested to be involved in binding of the cofactor thiamine PP_i to PDC (36). All seven tryptophan residues are conserved, allowing no conclusion which could be important. The sequence of *PDC6* contains only a single cysteine, while *PDC1* and *PDC5* both have four cysteine residues.

As pointed out previously, the differences in the amino acid sequence of *PDC1* and *PDC5* are unevenly distributed (12). *PDC6* perfectly follows this pattern. This means that in regions where *PDC1* and *PDC5* are almost identical the sequence of *PDC6* is also very similar to these two proteins. In clusters of relatively high dissimilarity between *PDC1* and *PDC5*, *PDC6* also exhibits the most pronounced divergence in comparison to the other two isoenzymes (Fig. 3). *PDC1* and also *PDC5* have very high codon bias indices of 0.95 and 0.84, respectively. This index gives a measurement for the biased use of the preferred codons in *S. cerevisiae* which correspond to the most abundant tRNA species (3). The codon bias index (maximum value, 1.0) correlates well with the level of expression; highly expressed genes have a high value, while the value for weakly expressed genes is low (1, 25). *PDC6* has a codon bias index of only 0.29, which classifies this gene clearly with the weakly expressed genes, in contrast to *PDC1* and *PDC5*, which belong to the class of the highly expressed genes.

Genetic analysis of deletion mutants. A deletion mutant of *PDC6* was constructed as described in Materials and Methods in a diploid recipient strain. After sporulation of this transformant the *Δpdc6* descendants were identified by the inserted *TRP1* gene. No difference with the wild-type descendants was observed, either in growth on plates containing glucose as carbon source or in specific PDC activity. One of the *Δpdc6* mutants was crossed with strain YSH 113 (*Δpdc1 Δpdc5*) to obtain all possible combinations of PDC structural gene mutants for further analysis.

Specific PDC activities. According to the specific PDC activities the different genotypes can be grouped into three categories (Table 2). The first group comprises the wild type, the *Δpdc5* and the *Δpdc6* mutants and the *Δpdc5 Δpdc6* double mutant, which all have about 100% activity. The *Δpdc5* and *Δpdc5 Δpdc6* mutants seem to have higher activities than the wild type after growth in ethanol medium. Group 2 consists of the *Δpdc1* mutant and the *Δpdc1 Δpdc6* double mutant, with about 75% residual activity in glucose and about 10% in ethanol medium. The double mutant *Δpdc1 Δpdc5* and the triple mutant *Δpdc1 Δpdc5 Δpdc6* compose group 3, and they did not show detectable PDC activity either in glucose or in ethanol medium.

Thus, the presence of just *PDC1* is sufficient for the cells to achieve full PDC activity, and as observed before (12, 19), deletion of *PDC1* can be partially compensated for by *PDC5* in glucose but not in ethanol medium. The *Δpdc6* deletion did not change the PDC activity in any of the strains after growth with glucose. In ethanol medium, however, the *Δpdc6* allele affected PDC activity slightly, but only in a *PDC1* wild-type background.

Growth characteristics. To characterize the growth behavior of the *pdc* mutant strains on different carbon sources, growth curves were measured (Table 3). The mutants grouped according to PDC activity also behaved similarly for growth with glucose and glucose with the respiration inhibitor antimycin A. The mutants lacking PDC activity (*Δpdc1 Δpdc5* and *Δpdc1 Δpdc5 Δpdc6*) grew slowly in glucose and not at all when antimycin A was added. The mutants lacking *PDC1* but retaining the compensatory *PDC5* (*Δpdc1* and *Δpdc1 Δpdc6*) had lag phases and generation times in glucose with antimycin A twice as long as those of the wild type, but growth in glucose without antimycin A was only slightly reduced. All strains which still possessed *PDC1* grew as fast as the wild type with all carbon sources investigated.

The two mutants lacking PDC activity grew very slowly with galactose, a sugar which does not cause full catabolite repression and which is at least partially degraded via the tricarboxylic acid cycle (9). Strikingly, these two strains (*Δpdc1 Δpdc5* and *Δpdc1 Δpdc5 Δpdc6*) did also grow more slowly in ethanol medium. For metabolism of ethanol, PDC activity is not expected to be required (9). Reduced growth in galactose medium was also observed for the strains lacking *PDC1* and therefore exhibiting reduced PDC activity.

Spore germination. During tetrad analysis it was observed that the colonies growing up directly on the dissection plates (YEP–3% ethanol) differed greatly in size (between 0.5 and 3 mm in diameter). The spores were ordered according to their size in each tetrad, and an average position for the different genotypes in the tetrads was calculated as described in Materials and Methods. Wild type and the *Δpdc5* mutant gave rise to the biggest colonies (average positions, 1.76 and 1.72, respectively). The *Δpdc5* mutant and the *Δpdc5 Δpdc6*

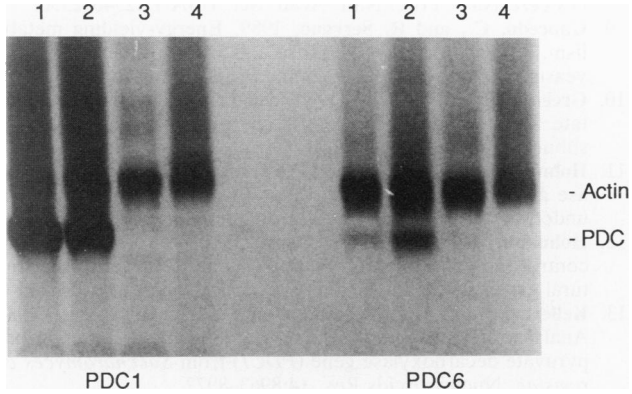

FIG. 4. Northern blot analysis of mRNA from wild-type and Δ*pdc1* Δ*pdc5* mutant cells isolated after growth in YEP–8% glucose medium or YEP–3% ethanol medium. Lanes: 1, wild type-glucose; 2, wild type-ethanol; 3, Δ*pdc1* Δ*pdc5* mutant-glucose; 4, Δ*pdc1* Δ*pdc5* mutant-ethanol. *PDC1* probe, 1.2-kb *Eco*RI fragment (19); *PDC6* probe, 2.4-kb *Eco*RI-*Xba*I fragment (Fig. 2). Actin (8) was used in both hybridizations as control.

double mutant grew to slightly smaller colonies (average positions, 2.08 and 2.02, respectively). The mutants lacking *PDC1* but retaining *PDC5* (Δ*pdc1* and Δ*pdc1* Δ*pdc6*) produced significantly smaller colonies (average positions, 2.64 and 2.88, respectively), and the mutants without detectable PDC activity were usually found as the smallest colony in the respective tetrad (average positions, 3.52 and 3.80). These results differ from the simple growth behavior of these strains in ethanol medium in which only the mutants devoid of PDC activity showed a small difference from the wild type. Moreover, spore germination was the only condition in which deletion of *PDC5* led to a visible effect even in a *PDC1* wild-type background. Spores without PDC activity did not grow up on galactose or glucose plates, and spores lacking *PDC1* showed reduced spore viability on these media (data not shown).

Northern blot analysis. So far no clear indication for expression of *PDC6* had been obtained. To rule out the unlikely possibilities that the gene product of *PDC6* is not a PDC but a different enzyme and that its expression has therefore not been observed with the genetic and physiological assays performed, I tried to identify a mRNA hybridizing to a *PDC6* probe (Fig. 4). Although high-stringency hybridization conditions were applied, a very weak cross-hybridization with the *PDC1* or *PDC5* mRNAs could not be excluded (controls not shown). Therefore, the strain most useful for looking for a *PDC6* mRNA is the Δ*pdc1* Δ*pdc5* double deletion mutant. However, only on an overexposed autoradiogram could a very weak and rather doubtful signal for *PDC6* be seen. In the wild-type strain the signals were clearly stronger, certainly also because of cross-hybridization with *PDC1* or *PDC5* mRNA. Strikingly, the signal was stronger with mRNA isolated from ethanol-grown cells. Since there is less *PDC* mRNA in ethanol-grown cells than in cells incubated with glucose (12, 20) (Fig. 4), this cannot be simply explained by cross-hybridization with *PDC1* and *PDC5*. Thus, *PDC6* may be expressed in the wild type grown in ethanol medium but only weakly in glucose medium and even more weakly in the Δ*pdc1* Δ*pdc5* mutant.

DISCUSSION

In this work I have described the analysis of *PDC6*, a yeast gene isolated as a homolog of *PDC1*, the major structural gene for PDC of *S. cerevisiae* (12, 19, 20, 21, 23). *PDC6* does not seem to contribute to the total PDC activity of the cell during growth in glucose; the Δ*pdc1* Δ*pdc5* double deletion mutant, which still possesses *PDC6*, did not show detectable PDC activity, and no *PDC6* mRNA was found in such cells. Moreover, the Δ*pdc6* mutant did not exhibit decreased PDC activity in a wild-type background or in any of the combinations with other *PDC* mutations. Surprisingly, the signal for *PDC6* mRNA was clearly stronger in wild-type cells after growth in ethanol, and in accordance with this finding PDC activity is slightly reduced in Δ*pdc6* mutants grown in ethanol. Since no PDC activity or mRNA was found in the Δ*pdc1* Δ*pdc5* double mutant either after growth in glucose or in ethanol medium, this suggests that expression of *PDC6* is regulated in a way opposite from expression of the other two structural genes (11, 12): activation only in the presence of *PDC1* and preferentially with a nonfermentable carbon source. Since PDC catalyzes an irreversible step in alcoholic fermentation (9) and, following the established schemes for gluconeogenic metabolism (9), is not required for growth in ethanol, this behavior is difficult to interpret.

One possible explanation, that the *PDC6* product is a component of PDH, which virtually catalyzes the same reaction but within an enzyme complex (34), is unlikely since the triple deletion mutant Δ*pdc1* Δ*pdc5* Δ*pdc6* still grows slowly in the presence of glucose, as does the Δ*pdc1* Δ*pdc5* double deletion strain. Moreover, the amino acid sequences of the PDC isoenzymes do not exhibit mitochondrial targeting sequences (2, 5).

The *PDC6* product could also have a completely different function. However, *PDC6* is certainly different from *ILV2*, the gene for acetolactate synthase, an enzyme related to PDC (7, 10). The Δ*pdc6* mutant does not show any obvious growth requirements, and the strain grows as well as the wild type on different media. Although distinct from *PDC1* and *PDC5*, the sequence of the *PDC6* protein has the same length and, if conservative substitutions are considered, a similarity of more than 90% to the other two isoenzymes. There are no regions in the sequence which are well conserved between *PDC1* and *PDC5* but diverged in *PDC6* which could have indicated distinct functions.

Finally, *PDC6* can function as a structural gene for PDC in mutants derived from the Δ*pdc1* Δ*pdc5* strain in which *PDC6* has been duplicated, the second copy being under the control of the *PDC1* promoter. These mutants ferment glucose and have PDC activity (11). Taken together, I conclude that *PDC6* is indeed a structural gene for PDC.

PDC activity is certainly required for normal growth with a fermentable carbon source like glucose (9, 12, 21). However, strains lacking PDC, such as the Δ*pdc1* Δ*pdc5* or the Δ*pdc1* Δ*pdc5* Δ*pdc6* mutants, are still able to grow slowly in glucose medium. Since this growth is sensitive to the respiration inhibitor antimycin A, it is likely that glucose is degraded via the tricarboxylic acid cycle. Since synthesis of mitochondrial functions and of respiratory enzymes is largely reduced on fermentable carbon sources (6, 9), glucose degradation via this pathway seems to be very ineffective (9). Growth in galactose medium requires PDC activity in very much the same way as glucose. This is remarkable since the degradation of galactose is regulated differently from glucose catabolism. Galactose represses synthesis of mitochondrial enzymes only partially (9), and induction of

PDC synthesis is weak and slow and cells do not grow in galactose medium containing antimycin A (not shown). Most surprisingly, the mutants lacking PDC activity are even impaired for growth in ethanol. Which function PDC could serve during growth with ethanol as a carbon source is not obvious (9). A more sensitive assay for the influence of PDC activity as growth seems to be spore germination. Even on YEP medium with 3% ethanol the colony size clearly correlated with the PDC genotype and with the PDC activity. Actually, this was the only condition under which an effect of the *PDC5* deletion, which does not result in reduced specific activity, could be observed. PDC activity may be required for efficient mobilization of trehalose during spore germination (30, 31), and therefore PDC mutants achieve normal growth later after germination than the wild type.

Taken together, *PDC1* codes for the major PDC isoenzyme; *PDC5* may be required to achieve full PDC activity under certain conditions, probably during spore germination; and *PDC6* seems to contribute to the total PDC activity mainly during growth in nonfermentable carbon sources. However, conditions under which *PDC6* is important for growth or yeast metabolism have not been identified yet.

The expression of a *PDC* gene preferentially in ethanol medium, the reduced growth rate of mutants without PDC activity in ethanol medium, and the surprisingly complex genetics and regulation of the PDC reaction suggest that this enzyme plays a more complex role in yeast metabolism. One idea is that PDC could have a second catalytic activity which is required for normal growth and which could be in some way responsible also for autoregulation of *PDC1* and *PDC5*. On the other hand, it has been suggested that certain glycolytic enzymes are physically associated (27). PDC could be part of such a complex, and this association could be important for the flux through glycolysis and gluconeogenesis. Although speculative, formation of such an aggregate could provide a possible explanation for PDC autoregulation, a phenomenon which has been observed for components of multiprotein aggregates such as histones or ribosomal proteins (15, 22).

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