JOURNAL OF BACTERIOLOGY, Feb. 1990, p. 678–685
0021-9193/90/020678-08$02.00/0

Vol. 172, No. 2

pdc1^0 Mutants of *Saccharomyces cerevisiae* Give Evidence for an Additional Structural *PDC* Gene: Cloning of *PDC5*, a Gene Homologous to *PDC1*

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Received 8 May 1989/Accepted 20 September 1989

The *PDC1* gene coding for a pyruvate decarboxylase (PDC; EC 4.1.1.1) was deleted from the *Saccharomyces cerevisiae* genome. The resulting *pdc1*^{0} mutants were able to grow on glucose and still contained 60 to 70% of the wild-type PDC activity. Two DNA fragments with sequences homologous to that of the *PDC1* gene were cloned from the yeast genome. One of the cloned genes (*PDC5*) was expressed at high rates predominantly in *pdc1*^{0} strains and probably encodes the remaining PDC activity in these strains. Expression from the *PDC1* promoter in *PDC1* wild-type and *pdc1*^{0} strains was examined by the use of two reporter genes. Deletion of *PDC1* led to increased expression of the two reporter genes regardless of whether the fusions were integrated into the genome or present on autonomously replicating plasmids. The results suggested that this effect was due to feedback regulation of the *PDC1* promoter-driven expression in *S. cerevisiae pdc1*^{0} strains. The yeast *PDC1* gene was expressed in *Escherichia coli*, leading to an active PDC. This result shows that the *PDC1*-encoded subunit alone can form an active tetramer without yeast-specific processing steps.

In the yeast *Saccharomyces cerevisiae*, fermentable sugars can be converted to ethanol and carbon dioxide. One of the key enzymes, pyruvate decarboxylase (PDC), catalyzes the decarboxylation of pyruvate to acetaldehyde, which is further reduced to ethanol by an alcohol dehydrogenase. The PDC enzyme is a tetramer, the subunit structure of which is still a matter of discussion. Either one (8, 18) or two (10, 27, 31) types of subunits and two different isozymes (15) have been described, depending on the yeast strain and the purification procedure. Schmitt et al. (23) isolated a Pdc$^-$ mutant (*pdc1-8*) by selection for yeast mutants that were not able to grow under fermentative conditions on glucose-antimycin medium (Glu$^-$ phenotype). The *pdc1-8* mutant is mutated in a single gene, and it is not able to grow on glucose medium (YPD) even under respiratory conditions. Crude extracts prepared from *pdc1-8* mutant cells contain no PDC activity. Schmitt et al. (23) cloned a DNA fragment (*PDC1*) coding for PDC activity by complementation of the *pdc1-8* mutant.

The primary structure (14), expression (14, 23), and regulation (6, 13) of the *PDC1* gene have been intensively studied. Southern analysis of total yeast DNA probed with the *PDC1* gene showed two hybridizing loci, *PDC1* and a second sequence that was classified as a pseudogene (23, 25).

Several other DNA fragments involved in *PDC1* expression were cloned by complementation of Pdc$^-$ mutants. The regulatory genes *PDC2* (23, 24) and *PDC4* (T. Seehaus, Ph.D. thesis, Technische Hochschule Darmstadt, Darmstadt, Federal Republic of Germany, 1986), and a gene possibly involved in posttranslational modification, *PDC3* (28), have been described.

To analyze the function of *PDC1*, we performed a gene replacement experiment in which the *PDC1* gene was replaced with the β-lactamase gene. Surprisingly, this *pdc1*^{0} mutant still exhibits a Glu$^+$ phenotype and contains 60 to 70% of the wild-type PDC activity. While this report was in preparation, we learned that Schaaff et al. (22) had also constructed a *pdc1* deletion strain. Their results concerning the different behaviors of the *pdc1-8* mutant and the deletion mutant agree with the data presented here. Furthermore, we describe the cloning of a sequence homologous to *PDC1* and show that this gene, *PDC5*, is expressed in *pdc1*^{0} mutants and is probably responsible for the remaining PDC activity. In addition, we have cloned a second sequence with less homology to *PDC1*, the function of which is still under investigation.

(This work is part of the doctoral thesis submitted by P. G. Seeboth to Heinrich-Heine-Universität Düsseldorf, Dusseldorf, Federal Republic of Germany, 1990.)

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* JM107 *endA1 gyrA96 thi hsdR17 supE44 relA1* λ$^-$ Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q}Z Δ*M15*) (29) and DH5αF′ F′ *endA1 hsdR17* (r_K^- m_K^-) *supE44 thi-1 recA1 gyrA* Δ(*lacZYA-argF*)U196 (φ80d*lacZ* Δ*M15*) were used for plasmid amplification and PDC expression. The following published plasmids were used: pBR322 (4), ptac12 (1), pUC19 (29), pPA2 (2, 21), and pPS090 (14). pBM272 was kindly provided by M. Johnston and is directly derived from pBM150 (12), with the alteration that an additional *Hin*dIII site is created adjacent to the *Bam*HI site (M. Johnston, personal communication). Plasmids ptac-PDC1 and pPS891αCEN were constructed in this study.

Yeast strains. The strains used are described in Table 1.

Media. YPD medium contained 1% yeast extract (Difco Laboratories), 2% Bacto-Peptone (Difco), and either 2 or 5% glucose. YPE medium contained 1% yeast extract (Difco), 2% Bacto-Peptone (Difco), and 2% ethanol. Minimal medium (YNB) contained a 0.67% yeast nitrogen base without amino acids and 2% glucose.

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TABLE 1. *S. cerevisiae* strains

Strain	Genotype
Haploids	
PS2	a *PDC1 his4-519 leu2-3 leu2-112 pep4-3 ura3-52 cir*^{0}
PS43	α *PDC1 pep4-3 ura3-52*
PS2-T2	a *PDC1*::pPS090 *his4-519 leu2-3 leu2-112 pep4-3 ura3-52 cir*^{0}
PS3-1-5b	a *pdc1*^{0}::*bla' leu2-3 leu2-112 pep4-3 ura3-52*
PS3-2-3b	a *pdc1*^{0}::*bla' pep4-3 ura3-52*
Diploids	
PS3	*PDC1/PDC1*::pPS090 *pep4-3/pep4-3 ura3-52/ura3-52*
PS3-1	*PDC1/pdc1*^{0}::*bla' pep4-3/pep4-3 ura3-52/ura3-52*
PS3-2	*PDC1/pdc1*^{0}::*bla' pep4-3/pep4-3 ura3-52/ura3-52*

Recombinant DNA techniques and *E. coli* colony hybridization. Procedures were performed as described by Maniatis et al. (19).

DNA and RNA isolation from yeast cells. Total DNA was isolated as described by Sherman et al. (26). For RNA isolation, cells were grown in YPD containing 5% glucose and harvested at an optical density at 600 nm of 1.8 to 2.0. The yeast cells were broken with glass beads in 50 mM Tris hydrochloride (pH 7.6)–10 mM NaCl–5 mM $MgCl_2$–0.2% sodium dodecyl sulfate (SDS)–100 μg of cycloheximide per ml–200 μg of heparin per ml. Nucleic acids were purified by five phenol extractions and one chloroform extraction, followed by ethanol precipitation and RNA precipitation with 2.7 M LiCl.

Assay methods. Crude extracts were prepared from logarithmically growing cells. Detection of β-lactamase-positive yeast colonies and assays of β-lactamase activity were performed as described by Reipen et al. (20). PDC activity in *E. coli* crude extracts was assayed by the method of Bräu and Sahm (5), using sodium citrate buffer (0.1 M, pH 6.0). PDC activity in yeast crude extracts was detected by the method of Boiteux and Hess (3), using sodium citrate buffer (0.1 M, pH 6.0), a pyruvate concentration of 33 mM, and a thiamine PP_i concentration of 4 mM. α-Amylase activity was detected at 37°C, using the Merckotest 14358 (E. Merck AG) as described by the supplier. Protein concentrations were determined by the method of Lowry et al. (17).

Native gels for detection of PDC activity were prepared as described by Zehender et al. (30).

SDS-polyacrylamide gel electrophoresis. Proteins were separated in 10% SDS-polyacrylamide gels, using the buffer system described by Laemmli (16). Gels were stained with Coomassie brilliant blue.

Southern and Northern (RNA) blots. Blotting methods are described by Maniatis et al. (19). DNA was separated in agarose gels, using the Tris acetate buffer system. Southern blots were hybridized in 6× SSC (SSC is 0.15 M NaCl plus 0.015 sodium citrate)–0.5% SDS–1 mM EDTA–5× Denhardt solution–100 μg of salmon sperm DNA per ml at 65°C and washed at 65°C in 0.1× SSC–0.5% SDS. Total RNA was separated in agarose-formaldehyde gels and transferred to nylon membranes (Hybond N; Amersham Corp.). Northern blots were hybridized at 42°C in 5× SSC–50 mM sodium phosphate buffer (pH 6.5)–1× Denhardt solution–250 μg of salmon sperm DNA per ml–50% formamide and washed in 0.1× SSC–0.1% SDS at 50°C.

RESULTS

Construction of *S. cerevisiae pdc1*^{0} strains. The *PDC1* gene was deleted by replacing it with a β-lactamase gene (*bla*) that lacks the bacterial signal sequence (*bla'*). This was performed by a two-step gene replacement procedure that has been described in detail elsewhere (14). The replacing recombination event was carried out in the diploid strain PS3, resulting in the heterozygous identical *PDC1/pdc1*^{0}::*bla'* strains PS3-1 and PS3-2. These strains are homozygous for *pep4-3* and therefore not able to sporulate (32). For sporulation, strains PS3-1 and PS3-2 were transformed with plasmid pPA2, which carries the *PEP4* gene. Spores were grown on ethanol medium (YPE) and then transferred to YPD containing antimycin to find the *pdc1*^{0} segregants, which should not be able to grow under fermentative conditions. Surprisingly, all segregants could ferment glucose. Segregants harboring the *pdc1*^{0}::*bla'* arrangement were found by testing all segregants for β-lactamase expression, which indicates the segregants containing the replacement chromosome. To prevent interfering β-lactamase activity from the *bla* gene carried on plasmid pPA2, segregants were grown unselectively, and only clones without plasmid pPA2 were used.

Two *pdc1*^{0} segregants (PS3-1-5b and PS3-2-3b) were chosen for further investigations. All steps of the gene replacement and the haploidization were checked by Southern analysis (Fig. 1). The integration of plasmid pPS090 was accompanied by the presence of the 2.4-kilobase-pair (kb) fragment in strains PS2-T2 and PS3 (Fig. 1A and B, lanes c and d). The diploid strain PS3 showed in addition the 6.2-kb band of the untransformed chromosome. The replacing second step was detectable by the disappearance of the 2.4-kb band and the appearance of a 1.5-kb band, visible in the diploid strains PS3-1 and PS3-2 (Fig. 1A and B, lanes e and g) and the haploid segregants PS3-1-5b and PS3-2-3b (Fig. 1A and B, lanes f, h, and i). Haploidization was proved by the loss of the 6.2-kb band.

During the genetic analysis, the following phenotype of the *pdc1*^{0} strains was observed. Although *pdc*^{0} strains PS3-1-5b and PS3-2-3b are able to grow on glucose like wild-type cells, they cannot complement the Glu^- phenotype of the *pdc1-8* mutation. Diploids obtained by crossing of *pdc1-8* strains with *PDC1* wild-type strains have a slightly decreased PDC activity but exhibit a Glu^+ phenotype (24). To check whether the *pdc1-8* strain is able to mate with our *pdc1*^{0} strains, *pdc1-8* revertants were isolated. This revertant mated perfectly with our strains, and the complementation deficiency must be a direct effect of the *pdc1-8/pdc1*^{0} genotype.

PDC activity in *pdc1*^{0} strains. Comparison of the PDC activities in the haploid *PDC1* wild-type strains (PS2 and PS43) and the haploid *pdc1*^{0} mutants (PS3-1-5b and PS3-2-3b) demonstrated that the mutant strains contained about 30 to 40% less PDC activity (Fig. 2A). The initial integration event leading to strain PS2-T2 and diploidization resulting in PS3 did not alter the PDC activity. A 20% decrease was seen in the diploid strain PS3-1, which was due to the deletion of the *PDC1* gene in one chromosome of strain PS3. Haploidization of PS3-1 resulted in *pdc1*^{0} segregants that showed an additional 20% decrease in PDC activity.

***PDC1* promoter-driven expression of β-lactamase and α-amylase genes in *PDC1* and *pdc1*^{0} strains.** *pdc1*^{0} strains PS3-1, P3-1-5b, and PS3-2-3b (Fig. 1C, line d) carry, because of the replacement event, the *bla'* gene under the control of the *PDC1* promoter and linked to its original more upstream sequences. Strains PS2-T2 and PS3 (Fig. 1C, line b) express the *bla'* gene from the *PDC1* promoter as well, but its integration site is the 3′ end of the *PDC1* gene. Comparing the β-lactamase activity of the haploid *PDC1* strain PS2-T2

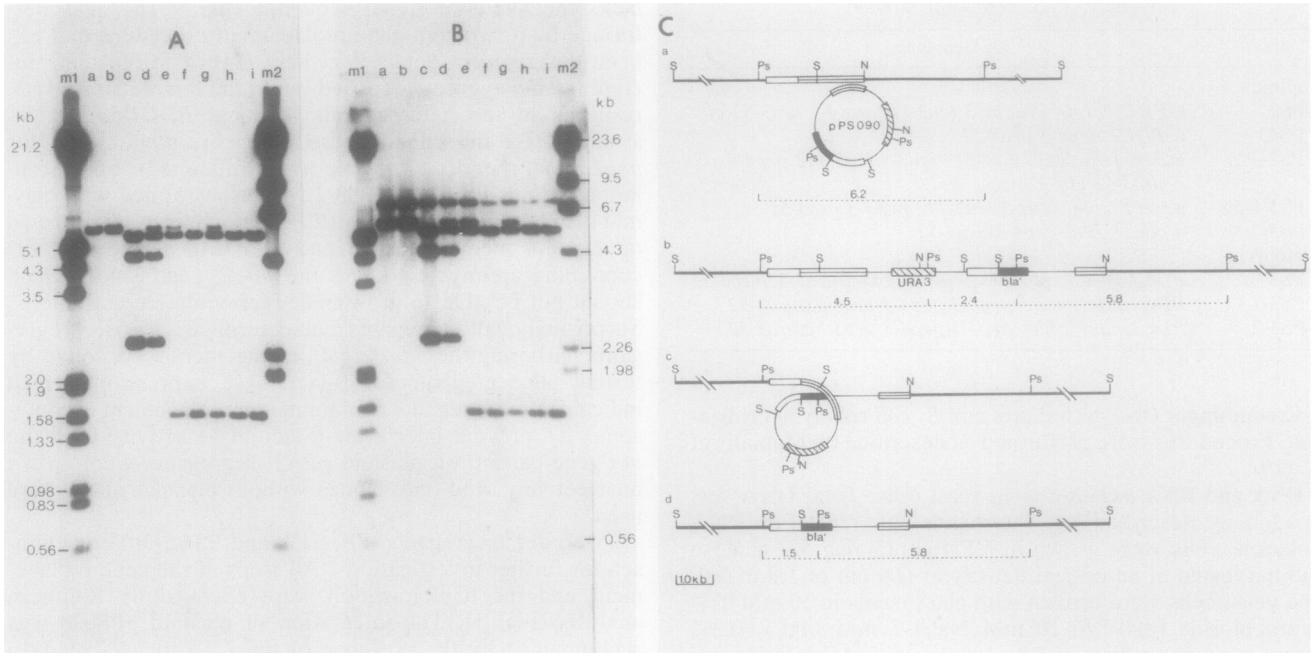

FIG. 1. (A and B) Southern blot analysis of the *pdc1*^{0}::*bla'* replacement. For fragment sizes, see panel C. Genomic DNA was isolated from the following strains and digested with *Pst*I: PS2 (lanes a) and PS43 (lanes b) (panel C, line a); haploid transformant PS2-T2 (lanes c) (panel C, line b); diploid transformants before replacement step PS3 (lanes d) (panel C, line b); diploid transformants after replacement step PS3-1 (lanes e) and PS3-2 (lanes g) (panel C, line d); and haploid *pdc1*^{0}::*bla'* replacement strains derived by haploidization of PS3-1 and PS3-2, called PS3-1-5b (lanes f and i) and PS3-2-3b (lanes h) (panel C, line d). Blot A was probed with a plasmid consisting of pBR322 with a 900-base-pair *PDC1* promoter fragment (▭ in panel C) inserted. Blot B was probed with a plasmid containing the 500-base-pair *Eco*RI-*Sal*I *PDC1* fragment (Fig. 4D, line c) inserted into the same sites of pBR322. Lane m1, Size marker, bacteriophage lambda digested with *Eco*RI and *Hin*dIII; lane m2, size marker, phage lambda digested with *Hin*dIII. (C) Schematic representation of the integration and *pdc1*^{0}::*bla'* replacement event. Line a, *PDC1* region of strains PS2 and PS43 and integrating plasmid pPS090; line b, *PDC1* region after integration of plasmid pPS090, strains PS2-T2 and PS3; line c, *pdc1*^{0}::*bla'* replacement event; line d, *pdc1*^{0}::*bla'* region in strains PS3-1, PS3-2, PS3-1-5b, and PS3-2-3b. Symbols: —, pBR322 sequences; ▬, genomic sequences flanking the *PDC1* gene; ▭, *PDC1* promoter; ▤, coding region of the *PDC1* gene. *bla'*, *bla* gene lacking the signal sequence. Restriction sites: N, *Nco*I; Ps, *Pst*I; S, *Sal*I. The dotted lines indicate the *Pst*I fragments. Sizes are given in kilobase pairs.

with those of the haploid *pdc1*^{0} strains PS3-1-5b and PS3-2-3b showed a 100% increase coupled to the replacement event (Fig. 2B). The *PDC1* replacement event itself carried out in the diploid strain PS3 leading to strain PS3-1 did not result in such increased β-lactamase expression. Only strains without an intact *PDC1* gene as produced by haploidization of PS3-1 exhibit this increase in β-lactamase expression driven by the *PDC1* promoter. It therefore seems likely that the low PDC activity in the cells without an intact PDC gene leads by feedback control to an increase of expression from the *PDC1* promoter.

We cannot completely exclude the possibility that the higher β-lactamase activity in *pdc1*^{0} strains is a direct effect of coupling the *PDC1* promoter to its original more upstream sequences. However, the position effect should have been visible also in the diploid background of strain PS3-1 when compared with the diploid strain PS3. Furthermore, it seems possible that the presence of an additional *PDC1* promoter copy in strain PS2-T2 compared with the *pdc1*^{0} strains PS3-1-5b and PS3-2-3b could titrate a positive regulator required for optimal transcription and expression.

In an another approach to distinguish between these possibilities, the *PDC1*-driven expression of a second reporter gene located on an autonomous replicating centromere plasmid was examined. The reporter gene chosen was the *Schwanniomyces occidentalis* α-amylase gene *AMY1*. This gene has been expressed in *S. cerevisiae* under the control of the *PDC1* promoter, and it was demonstrated that more than 95% of the synthesized α-amylase is secreted into the culture medium as an active enzyme (P. Seeboth et al., manuscript in preparation). To have a stable low-copy-number expression plasmid for *S. cerevisiae*, the *PDC1-AMY1* fusion was cloned on a centromere vector (Fig. 3A). This plasmid (pPS891αCEN) was used to analyze *PDC1* promoter activity in the *PDC1* strains PS2 and PS43 and in the *pdc1*^{0} strains PS3-1-5b and PS3-2-3b by measuring the α-amylase activity in the culture medium. Again, the *pdc1*^{0} strains showed at least a twofold-higher expression of the reporter gene than did the *PDC1* strains (Fig. 2C). pPS891αCen transformants of the diploid *PDC1*/*pdc1*^{0}::bla' strain PS3-1 secreted as much α-amylase activity as did the haploid *PDC1* strains PS2 and PS43. As already shown for β-lactamase, the increase in *PDC1* promoter-driven expression was coupled to haploidization of PS3-1, leading to haploid cells without an intact *PDC1* gene. These findings can be explained by feedback regulation of expression from the *PDC1* promoter caused by the *pdc1*^{0} genotype. The arguments against this explanation, such as position effects and titration of positive *PDC1* promoter regulation factors, were excluded by the experiments using the autonomously replicating centromere plasmid. All transformants were selected for uracil prototrophy and should contain the same plasmid copy number per genome. On this assumption, the haploid *PDC1* strains PS2 and PS43 and the haploid *pdc1*^{0}

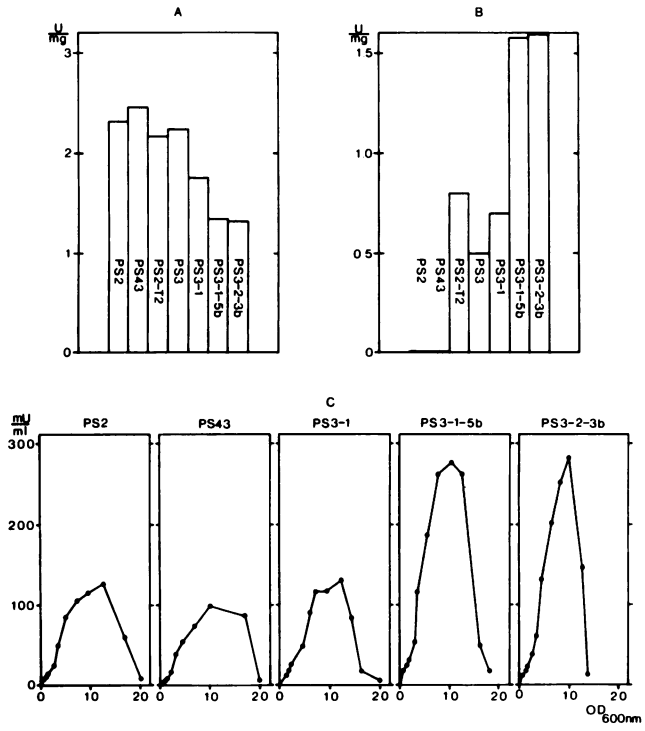

FIG. 2. (A and B) PDC (A) and β-lactamase (B) activities in yeast crude extracts. Cells were grown in YPD and harvested in the logarithmic phase (optical density at 600 nm of 0.8). Activity is indicated in units per milligram of protein. (C) α-Amylase activity in the culture medium of the given strains transformed with pPS891αCEN. α-Amylase activity and growth were monitored during batch culture growth in YPD containing 5% glucose. Activity was calculated per milliliter of culture medium. For enzyme measurements, see Materials and Methods.

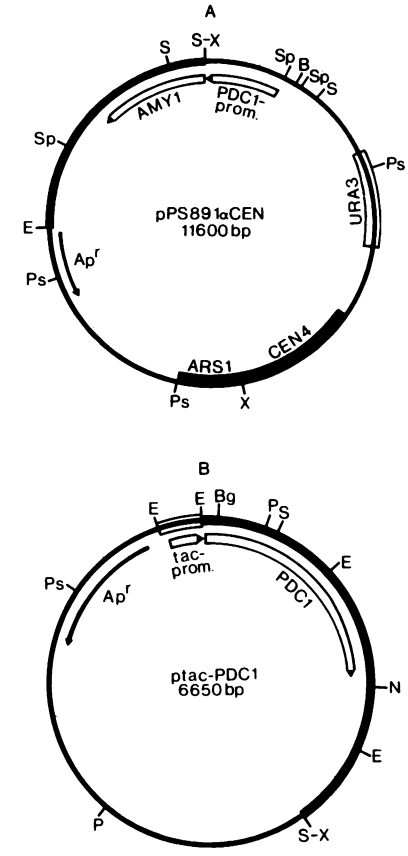

FIG. 3. (A) Yeast α-amylase expression plasmid. Plasmid pPS891αCEN is derived from pBM272. The *PDC1-AMY1* fusion was inserted as a *Bam*HI-*Eco*RI fragment into the same sites of pBM272. Symbols: —, pBR322 sequences except for the *PDC1* promoter; ■, *Schwanniomyces occidentalis* sequences. *ARS1*, *CEN4*, and *PDC1* promoter are *S. cerevisiae* sequences. (B) *E. coli* PDC expression plasmid ptac-PDC1. The plasmid is derived from pBR322 by insertion of the *tac* promoter from ptac12, which was fused to the *PDC1* gene. Symbols: —, pBR322 sequences; ■, *S. cerevisiae* sequences. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; N, *Nco*I; P, *Pvu*II; Ps, *Pst*I; S, *Sal*I; Sp, *Sph*I; X, *Xho*I. S-X, Fusion point of a *Sal*I/*Xho*I ligation.

strains PS3-1-5b and PS3-2-3b contain the same number of *PDC1* promoters.

Cloning of two sequences homologous to the *PDC1* sequence. During Southern blot analysis of the integrants, a *Pst*I fragment of 7 kb was detected that hybridized to *PDC1* sequences but was not affected by replacement of the *PDC1* gene (Fig. 1B). Further Southern analysis with different fragments of the *PDC1* gene showed that the homology covered the whole *PDC1* coding region but not the promoter region. The Southern blot shown in Fig. 1A, which was probed with the *PDC1* promoter, is missing the hybridizing 7-kb fragment visible in Fig. 1B. To clone this fragment, genomic DNA of the *pdc1*^{0} strain PS3-1-5b was digested with *Pst*I, and fragments of sizes ranging from 6 to 8 kb were ligated into plasmid pUC19. Of 2,500 transformants, 24 clones hybridizing with the *Eco*RI-*Sal*I *PDC1* fragment (Fig. 4D, line c) were identified. Twelve positive clones were further analyzed, and it appeared that two different 7-kb *Pst*I fragments were cloned, each five times. Both fragments hybridized with the *PDC1* 5′ (*Eco*RI-*Sal*I fragment) and 3′ (*Sal*I-*Nco*I fragment) coding regions but with different intensities (Fig. 4A to C). The strong hybridizing gene was called *PDC5* (Fig. 4A to C, lanes 6, 8, 9, 11, and 12). The weaker hybridizing sequence (Fig. 4A to C, lanes 1, 2, 4, 7, and 10) was hardly detectable on a yeast genomic Southern blot with *PDC1* as a probe. Restriction and hybridization analysis of the *Pst*I fragment encoding *PDC5* proved that the entire hybridizing sequence is contained in the cloned fragment and that this gene is homologous but not identical to *PDC1*. Of 11 mapped restriction sites, 3 corresponded to the sites mapped in the *PDC1* gene (Fig. 4D, lines b and c).

Expression of the cloned fragments in *PDC1* and *pdc1*^{0} strains. To determine the potential transcripts from the two *Pst*I fragments, we hybridized both fragments to RNA from *PDC1* wild-type and *pdc1*^{0} strains by Northern blotting. The fragment encoding *PDC5* hybridized to an RNA of the same length as *PDC1*, whereas the other cloned *Pst*I fragment hybridized to two smaller RNAs (Fig. 5). This finding strongly suggests that the *PDC5* fragment is transcribed into an mRNA similar in size to *PDC1*. Interestingly, the RNA signal that hybridized to *PDC5* was stronger in *pdc1*^{0} strains than in *PDC1* wild-type cells (Fig. 5B). Taking into account the cross-hybridization between *PDC1* and *PDC5*, which can be estimated from the extent of *PDC1* hybridization to the RNA from the *pdc1*^{0} strains (Fig. 5A, lanes 3 and 4), it appeared that in *PDC1* wild-type cells, the *PDC5* gene was transcribed little or not at all. For the *PDC5* probe, the cross-hybridization could cause the entire signal in the *PDC1* strains (Fig. 5B, lanes 1 and 2), representing mostly or entirely the *PDC1* mRNA.

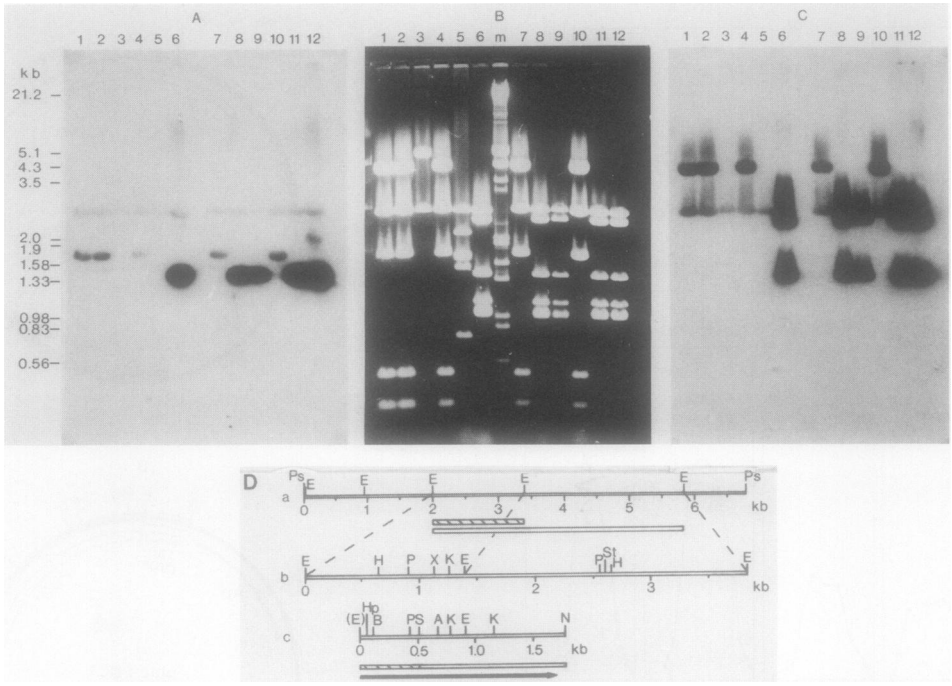

FIG. 4. (A to C) Southern analysis of 12 positive clones identified with the *PDC1 Eco*RI-*Sal*I fragment (panel D, line c). In panel B, plasmid DNA was digested with *Pst*I and *Eco*RI. The DNA was blotted onto two filters. Filter A was probed with the *PDC1 Eco*RI-*Sal*I fragment, and filter C was probed with the *PDC1 Sal*I-*Nco*I fragment (panel D, line c). All 12 clones showed a weak hybridization signal at the size of 2.6 kb, which is the vector (pUC19) band, due to contamination of the probes with vector sequences. (D) Schematic representation of the cloned *Pst* fragment encoding *PDC5* and comparison of its restriction map with that of the *PDC1* gene. Line a, *Eco*RI fragments of the cloned *Pst*I fragment. Symbols: ▨, *Eco*RI fragment hybridizing with the *PDC1 Eco*RI-*Sal*I fragment; ▭, *Eco*RI fragments hybridizing with the *PDC1 Sal*I-*Nco*I fragment. These *PDC1* fragments used as probes are shown in line c. Line b, Enlarged restriction map of the PDC5-encoding *Eco*RI fragments; line c, restriction map of the *PDC1* gene. (E), An *Eco*RI site created by insertion of a linker and not present in the genomic fragment. The hatched and open boxes represent the fragments used as probes in panels A and C. The arrow indicates the *PDC1* coding region. Restriction sites A, *Aat*II; B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; N, *Nco*I; P, *Pvu*II; Ps, *Pst*I; S, *Sal*I; St, *Stu*I; X, *Xba*I.

From these results, we conclude that the PDC5-encoding sequence is likely the gene responsible for PDC activity in the *pdc1*^{0} strains. The other cloned *Pst*I fragment is expressed independently of the *PDC1* genotype of the tested yeast strains (Fig. 5C).

The *PDC1*-encoded subunit can form an active homotetramer. PDC is a tetrameric enzyme, the subunit structure of which is not yet clear. To determine whether the *PDC1*-encoded subunit alone can form an active homotetramer, we expressed *PDC1* in *E. coli*, a bacterium that is devoid of PDC activity. For this purpose, the yeast *PDC1* gene was fused to the inducible *E. coli tac* promoter (Fig. 3B). Crude extracts prepared from *E. coli* JM107 transformants bearing the expression plasmid ptac-PDC1 (Fig. 3B) contain an isopropyl-β-D-galactopyranoside (IPTG)-inducible PDC activity. After subtracting the background activity contained in control crude extracts of *E. coli* JM107 and *E. coli* ptac12 transformants (0.7 U/mg of protein) that is caused by lactate dehydrogenase, the remaining PDC activities were 1.3 U/mg of protein for cells grown without IPTG and 4.5 U/mg of protein for cells grown in presence of 1 mM IPTG. The plasmid-encoded protein was detectable on Coomassie brilliant blue-stained SDS-polyacrylamide gels and showed the expected PDC subunit size (Fig. 6A). We also analyzed the holoenzyme on activity-stained native gels and could detect no differences between the yeast PDC protein and the PDC protein synthesized in *E. coli* (Fig. 6B).

These results demonstrate that the PDC1 subunit encoded by the *PDC1* gene is able to form an active homotetramer and does not need a second subunit or yeast-specific processing to form an active enzyme.

DISCUSSION

Properties of *pdc1*^{0} mutants. The reported Pdc$^-$ mutant *pdc1-8*, which was used to clone the *PDC1* gene from *S. cerevisiae* (23), is not able to grow on glucose medium even under respiratory conditions and contains no detectable PDC activity (24). The *pdc1*^{0} mutant described here behaves totally differently. It is able to grow on glucose medium even when respiration is blocked with antimycin, and it contains 60 to 70% of the wild-type PDC activity. Similar findings were described recently by Schaaff et al. (22). Although the *pdc1*^{0} strain is able to grow on glucose like the wild type, it cannot complement the Glu$^-$ phenotype of the *pdc1-8* mutant, whereas *PDC1* wild-type strains complement the Glu$^-$ phenotype (24). Obviously, the dominant effect of the *pdc1-8* mutation is much more pronounced in *pdc1-8*/*pdc1*^{0} cells than in *pdc1-8*/*PDC1* strains. As we reported, the *PDC5* gene is weakly expressed in *PDC1* wild-type cells, whereas in *pdc1*^{0} strains the *PDC5* mRNA level is increased drastically. Possibly, this *PDC5* expression is also low or absent in *pdc1-8* strains or the *pdc1-8* subunit has particular properties, as discussed below.

Cloning of the *PDC5* gene. Schmitt et al. (23) reported a second DNA fragment of the *S. cerevisiae* genome that hybridizes with the *S. cerevisiae PDC1* gene, but no further analysis was described. The sequence was designated a

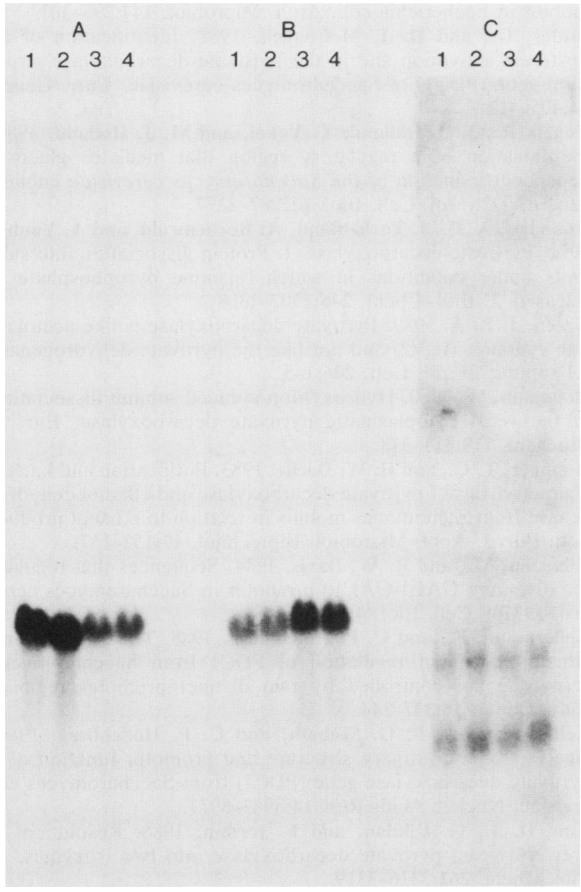

FIG. 5. Northern analysis. Samples (15 μg) of total RNA from the *PDC1* strains PS2 (lanes 1) and PS43 (lanes 2) and from the *pdc1*^{0} strains PS3-1-5b (lanes 3) and PS3-2-3b (lanes 4) were separated in a formaldehyde gel. After transfer of the RNA, the membrane was cut into three identical parts. Membrane A was probed with the *PDC1* gene, a mixture of the *Eco*RI-*Sal*I fragment and the *Sal*I-*Nco*I fragment shown in Fig. 4D, line c. Membrane B was probed with the cloned *PDC5* *Pst*I fragment shown in Fig. 4D, line a. Membrane C was probed with the second cloned *Pst*I fragment.

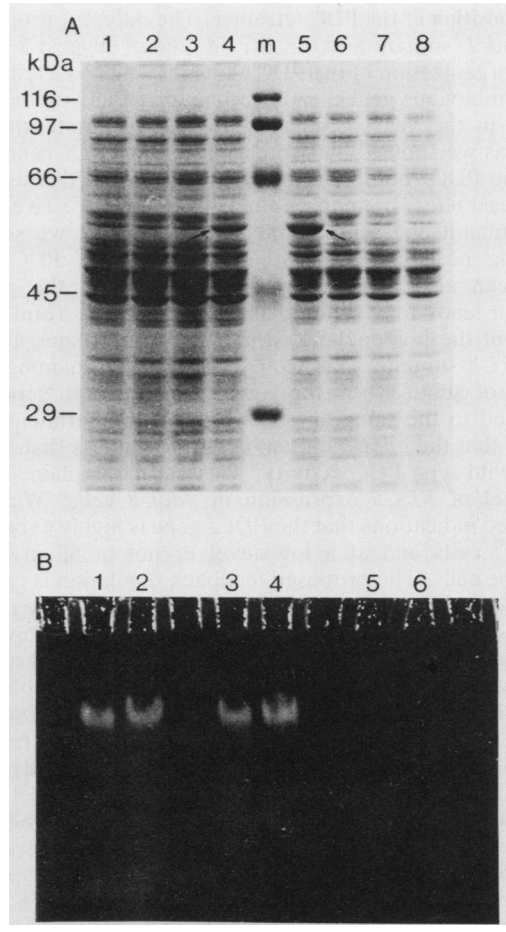

FIG. 6. (A) SDS-polyacrylamide gel stained with Coomassie brilliant blue. Crude extracts (25 μg) of *E. coli* cells untransformed (lanes 1 and 8), transformed with ptac12 (lanes 2 and 7), transformed with ptac-PDC1 (Fig. 3B) (lanes 4 and 5), and transformed with a plasmid derived from ptac-PDC1 in which the *PDC1* *Sal*I-*Sal*I/*Xho*I fragment is deleted (lanes 3 and 6) were separated. Lanes 1 to 4, Not induced; lanes 5 to 8, induced with 1 mM IPTG; lane m, molecular mass marker. Arrows indicate the additional band appearing in ptac-PDC1 transformants. (B) Native gel for detection of PDC activity. Lanes: 1 and 2, crude extract from yeast strain PS43; 3 and 4, crude extract from *E. coli* transformed with ptac-PDC1; 5 and 6, untransformed *E. coli*. *E. coli* cells were grown in the presence of 1 mM IPTG. Lanes 1, 3, and 5 contained 450 μg and lanes 2, 4, and 6 contained 900 μg of crude extract.

pseudogene because the *pdc1-8* mutant is mutated in a single gene and no PDC activity was detectable (24). We have cloned this sequence and provided strong evidence that it encodes a PDC. The gene, called *PDC5*, is expressed weakly or not at all in *PDC1* wild-type cells, but its expression is strongly increased in *pdc1*^{0} cells. *PDC5* shows strong homology to *PDC1*; 3 of 11 mapped restriction sites in the coding region of *PDC1* show the same distance from each other in *PDC5*. The homology covers only the coding region. Hybridization experiments with a *PDC1* promoter fragment of 900 base pairs showed no signal even under less stringent conditions. A comparison of the protein sequences derived from *PDC1* with those in sequence data bases revealed extensive homology between the *PDC1* gene product and the acetolactate synthase encoded by the yeast *ILV2* gene (9). However, no homology between *PDC1* and *ILV2* could be detected at the DNA level. We therefore conclude that none of the cloned fragments result from the *ILV2* gene.

Expression of the PDC5 gene. In *PDC1* wild-type cells, the *PDC5* gene is transcribed only in low amounts or not at all. In *pdc1*^{0} cells, the gene is transcribed in approximately the same amount as is *PDC1* in *PDC1* wild-type strains. It seems that there is a feedback regulation for expression of the PDC-encoding genes, controlling PDC activity in the cells. Interestingly, *PDC1* promoter-driven expression is also higher in *pdc1*^{0} cells than in wild-type cells, as we could show with two different reporter genes, either integrated into the genome or encoded on an autonomously replicating plasmid. The feedback regulation caused by low PDC activity in the cells seems to affect both the *PDC1* and *PDC5* genes. The reported regulation of *PDC1* expression depending on the carbon source (14, 23) does not hold for *PDC5*. In cells that are wild type for both genes, the *PDC1* gene is strongly expressed on glucose medium, whereas the *PDC5* gene is very weakly expressed. Schaaff et al. (22) described the appearance of an abundant truncated *PDC1* transcript in a *pdc1* deletion mutant. This observed increase in *PDC1* promoter-driven transcription probably reflects the same regulatory effect that resulted in the increase in expression from the *PDC1* promoter described here.

Composition of the PDC tetramer. The different properties of the *pdc1*^{0} mutant and the *pdc1-8* mutant suggest a model for the organization of the PDC enzyme, which is a tetramer. Two homologous genes, each coding for a PDC subunit, are present in the yeast genome. The PDC1 homotetramer is active, as we showed by expression of the *PDC1* gene in *E. coli*. The PDC5 homotetramer most likely is active as well, since yeast *pdc1*^{0} mutants contain an active PDC. To explain the dominant character of the *pdc1-8* mutant, we see the following possibilities. First, the subunits of PDC1 and PDC5 can also form active heterotetramers; the *pdc1-8* mutation leads to a subunit that is still able to form tetramers, but the holoenzyme is inactive if it contains at least one *pdc1-8* subunit. This would result in a remaining PDC activity of about 6% in the *pdc1-8* strain if both genes are expressed to the same amount. Reported experiments (24) showed that the *pdc1-8* mutant cells contain less than 0.15% of the wild-type PDC activity. This can be explained by a low level of *PDC5* expression in *pdc1-8* cells. We have presented indications that the *PDC5* gene is highly expressed in *pdc1*^{0} cells and at a low level or not at all in *PDC1* wild-type cells. The proposed feedback regulation is exerted by the wild-type gene as well as by expression of the *pdc1-8* subunit. Second, it might be possible that *PDC5* expression is repressed in *PDC1* and *pdc1-8* cells and induced only in the absence of *PDC1*-encoded subunits. The available data do not imply a clear function for *PDC5*. As observed for other yeast genes present in two or more alleles per genome (7), the expression of *PDC5* is regulated differently from *PDC1*. The fact that *PDC5* can yield a fully active tetramer and that its expression seems to be regulated by the PDC level in the cell indicates that we certainly are not working with a pseudogene.

Expression of the *S. cerevisiae PDC1* gene in *E. coli*. Expression of the yeast *PDC1* gene in *E. coli* leads to an active PDC tetramer. This result was also reported for a PDC-encoding gene from *Zymomonas mobilis* (5). *E. coli* cells expressing the *Z. mobilis pdc* gene produce a higher level of ethanol than do plasmid-free strains (5). This was also observed by us for *E. coli* cells expressing the *PDC1* gene from yeast cells (data not shown). The PDC enzymes of *Z. mobilis* and *S. cerevisiae* are both composed of four subunits and need thiamine pyrophosphate (11, 31).

ACKNOWLEDGMENTS

We are grateful to M. Ciriacy for useful discussions and for help in performing yeast tetrad analysis.

This work was supported in part by the Deutsche Forschungsgemeinschaft.

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