

## Genetic Analysis of the Pyruvate Decarboxylase Reaction in Yeast Glycolysis†

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Six different pyruvate decarboxylase mutants of *Saccharomyces cerevisiae* were isolated. They belong to two unlinked complementation groups. Evidence is presented that one group is affected in a structural gene. The fact that five of the six mutants had residual pyruvate decarboxylase activity provided the opportunity for an intensive physiological characterization. It was shown that the loss of enzyme activity in vitro is reflected in a lower fermentation rate, an increased pyruvate secretion, and slower growth on a 2% glucose medium. The different effects of antimycin A on leaky mutants grown on ethanol versus the same mutants grown on glucose support the view that glucose induces some of the glycolytic enzymes, especially pyruvate decarboxylase.

Carbon catabolite repression in the yeast *Saccharomyces cerevisiae* has been intensively studied, and many attempts have been made to elucidate its genetic basis (4, 7, 20, 22, 27, 29). Repression of enzymes involved in oxidative metabolism and also proteolytic inactivation of gluconeogenic enzymes (11) are induced by fermentable sugars or their metabolites. Besides this, fermentable sugars can bring about other changes in the metabolism of yeasts: they activate enzymes of the glycolytic pathway, increase the glycolytic flux, and induce alcoholic fermentation. The data on the extent of this activation vary with different enzymes, species, strains, culture and testing conditions, and sugar species (7, 16). We investigated this phenomenon, hoping that the genetic basis of this activation would be easier to elucidate than that of carbon catabolite repression.

The strains used in our laboratory show the highest activities of pyruvate kinase and cytoplasmic pyruvate decarboxylase only after the addition of glucose to the medium (7). The synthesis of these two enzymes is likely to be regulated for two reasons. First, uncontrolled pyruvate kinase and decarboxylase activities would compete with gluconeogenesis by converting the phosphoenolpyruvate formed by the phosphoenolpyruvate carboxykinase to pyruvate. Second, allosteric control of their activity may not be sufficient to prevent this leakage. Maitra and Lobo (17) obtained a mutant whose

pyruvate kinase did not require fructose-1,6-bisphosphate, which is an allosteric activator of that enzyme, for full activity. The mutant was still able to grow on gluconeogenic carbon sources. The only known allosteric effector for the cytoplasmic pyruvate decarboxylase is phosphate (3), but phosphate concentrations in yeast do not change significantly during the shift from glycolysis to gluconeogenesis (1). Therefore, additional regulation at the transcriptional or translational level by mRNA processing or protein maturation may be involved in controlling these two enzyme activities.

Yeast pyruvate kinase mutants have been isolated by several investigators (5, 6, 13, 18, 24). As far as tested, all mutants isolated fell into the same complementation group. Moreover, the mutations characterized by Clifton et al. and Cyriacy and Breitenbach probably affected the pyruvate kinase structural gene (5, 6). The failure to detect regulatory mutants for pyruvate kinase does not rule out that there are regulatory genes. However, such mutations may have pleiotropic effects and could interfere with other metabolic reactions. In the case of pyruvate decarboxylase, a search for regulatory mutants looked more promising since the mutants of Lam and Marmor (13), with a "pdc" defect, could not grow on glucose media. This cannot be explained by the enzymatic defect alone since mutants lacking all three alcohol dehydrogenases can still grow in the presence of the standard glucose media concentrations of 2% (5). On the other hand, Lancashire et al. (14) obtained one pdc mutant that could grow in the presence of glucose. This discrepancy could potentially

† Dedicated to H. Holzer on the occasion of his 60th birthday in appreciation of his outstanding contributions to the field of yeast physiology.

be explained as the difference between a regulatory gene and a structural gene mutation.

We are reporting here the isolation of a number of mutants with reduced pyruvate decarboxylase activity, which represent defective alleles of the two unlinked genes *PDC1* and *PDC2*. Defective but leaky *pdcl* mutant alleles could be shown to produce pyruvate decarboxylase with altered enzymatic properties.

(The present communication is part of a thesis project towards a doctoral degree of Hans Dieter Schmitt.)

## MATERIALS AND METHODS

**Yeast strains.** Strain SMC-1B (a *his4 MAL2-8<sup>c</sup> MAL3 SUC3*) was used for the isolation of mutants. For the construction of heterozygous diploids and further genetic analysis, strain SMC-1B and mutants were crossed to strain SMC-19A (*leu1 MAL2-8<sup>c</sup> MAL3 SUC3*). For the designations MAL and SUC, see Mortimer and Schild (21).

**Media and growth conditions.** YEP medium (2% peptone [Difco Laboratories], 1% yeast extract [Difco]) was used as a rich medium. It was supplemented with 3% ethanol (YEPE) or 2% glucose (YEPD). Antimycin A was added at a concentration of 1  $\mu\text{g/ml}$  to block respiration. Oxoid agar (10 g/liter) was used for solid media plates. Liquid cultures were incubated at 28°C under constant shaking. Synthetic medium contained 2% glucose and 0.67% Difco yeast nitrogen base. If necessary, the synthetic medium was buffered with 0.1 M potassium phosphate (pH 6.5) and supplemented with histidine and leucine (0.13 and 0.23 mM, respectively) to meet the growth requirements of strain SMC-1B and SMC-19A.

**Mutagenesis and isolation of mutants.** Cells grown on YEPE were mutagenized at 28°C at a titer of  $1 \times 10^7$  haploid cells per ml in 0.1 M potassium phosphate buffer (pH 7.0) containing 1% ethyl methane sulfonate. After 1 h, the cells were washed twice with buffer and incubated in YEPE for 13 h to allow for mutation fixation and expression (5). This was followed by incubation in YEPD plus antimycin A to prepare the cells for the nystatin mutant enrichment method of Snow (23). This method is based on the assumption that nystatin predominantly kills growing cells. In contrast to wild type, pyruvate decarboxylase mutants should be inhibited by the YEPD medium plus antimycin A. After 6 h, nystatin at a final concentration of 10  $\mu\text{g/ml}$  was added for 1 h. Nystatin was removed by washing twice with buffer. Cells were spread onto YEPE plates ( $4 \times 10^5$  cells per plate). From there, 1,660 colonies were replica plated onto YEPD plus antimycin plates. A total of 137 isolates showed reduced or no growth on this medium. Twenty-one stable clones, which showed good growth on a synthetic complete medium with 2% glycerol and 2% ethanol, were tested for pyruvate decarboxylase activity.

**Preparation of crude extracts and enzyme assays.** Cells were pregrown in 5 to 50 ml of YEPE medium to about  $5 \times 10^6$  cells per ml. It is important to use cells from an early-logarithmic-phase culture to get repro-

ducible results (J. Ullrich [Freiburg], personal communication). Glucose was added to this culture to give a concentration of 2%. Cells were harvested after 4 h and washed twice with 50 mM imidazole buffer (pH 6.8) containing 10 mM  $\text{Mg}^{2+}$ . Crude extracts were prepared as described by Ciriacy and Breitenbach (5), centrifuged at  $27,000 \times g$  and 4°C for 30 min, and immediately assayed for enzyme activities because pyruvate decarboxylase is not stable in crude extracts. When sodium citrate buffer (100 mM, pH 6.0) was used for the preparation of crude extracts, the samples could be stored at -20°C without loss of activity. Pyruvate kinase and pyruvate decarboxylase were assayed by the method of Maitra and Lobo (16), using imidazole buffer (pH 6.8) instead of triethanolamine hydrochloride (pH 7.4). Heat inactivation was performed by the method of Maitra and Lobo (17). One unit of enzyme activity is defined as 1  $\mu\text{mol}$  of substrate converted per min per mg of protein at 30°C. Protein concentrations of crude extracts were determined by the method of Lowry et al. (15), using bovine serum albumin as a standard.

**Assay for metabolites.** Cellular metabolites were extracted by the method of Ciriacy and Breitenbach (5). Their concentrations were determined spectrophotometrically by the methods described by Bergmeyer (2). Glycerol was measured in a glycerokinase reaction using phosphoenolpyruvate, NADH, pyruvate kinase, and lactic dehydrogenase. Pyruvate was determined in the same cuvette. The simultaneous estimation of pyruvate and glycerol was useful because their excretion by yeasts was followed during growth on glucose medium.  $\text{CO}_2$  production in intact cells was estimated manometrically by a standard Warburg respirometer. We used YEP medium adjusted to pH 5.85, the final glucose concentration was 2%, and the temperature was 30°C. The fermentation rate was calculated by subtracting the simultaneously determined  $\text{O}_2$  consumption rate. Both parameters were measured over a period of at least 6 h, beginning with the addition of glucose. Therefore, the values had to be corrected for the growth rate, which was determined spectrophotometrically at 578 nm in parallel cultures. Standard curves were used for the estimation of dry weights.

**Genetic methods.** Crosses between haploid cells of different mating types and complementary amino acid requirements had to be performed on YEPE medium. Diploids of known genotype were selected by repeated replica-plating onto a synthetic minimal medium, with ethanol instead of glucose as the sole carbon source since glucose would have been strongly selective for mitotic recombinants in noncomplementing diploids. Moreover, the direct determination of amino acid requirements of glycolytic mutants by replica-plating on synthetic minimal medium was not possible. Therefore, complementation tests had to be performed with wild-type strains for the two *PDC* loci (5). Sporulation was induced by replica-plating onto potassium acetate medium. The ascus walls were digested with a 1:50 dilution of  $\beta$ -glucuronidase-arylsulfatase (Boehringer-Mannheim) during a 12-min incubation at room temperature.

**Enzymes and chemicals.** Nystatin and antimycin A were obtained from Serva (Heidelberg, Federal Republic of Germany); ancillary enzymes, cofactors, and substrates were obtained from Boehringer (Mannheim, Federal Republic of Germany).

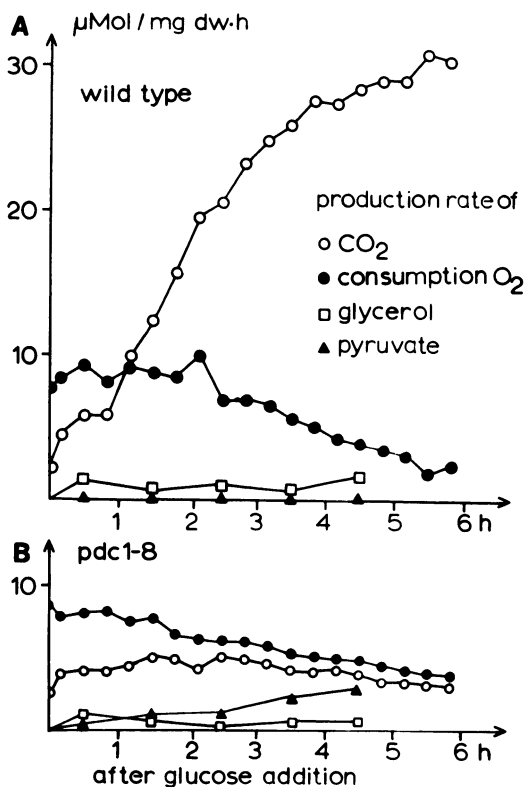


FIG. 1. CO<sub>2</sub>, pyruvate, and glycerol production rates and O<sub>2</sub> consumption rates of the wild type and mutant *pdc1-8* in a synthetic minimal medium with glucose as the sole carbon source. Glucose was added at zero time to give a concentration of 2%.

## RESULTS

When glucose was added to cells growing with ethanol as the sole carbon source, pyruvate decarboxylase activity increased from 0.2 to 3.6 U over 3 to 4 h. This documented again that pyruvate decarboxylase is not a constitutive enzyme. As shown in Fig. 1A, the fermentation rate, that is, the pyruvate decarboxylase activity *in vivo*, increased in a similar way. (Fermentation rate is the CO<sub>2</sub> production minus the O<sub>2</sub> consumption rate.)

We isolated 137 clones which could not grow in an indicator medium with glucose and antimycin A, a potent inhibitor of respiration (19). Only cells with a functional glycolytic system can grow on such a medium. Six clones showed reduced or no pyruvate decarboxylase activity on glucose medium (Table 1). Mutants 8, 14, and 30 were partially dominant in contrast to mutants 92, 122, and 137, which apparently were completely recessive. Tetrad analysis showed that these two groups represented two different complementation groups. Consequently, there

were two genes involved in the formation of pyruvate decarboxylase, now called *PDC1* and *PDC2*. Mutants with a defect in gene *PDC1* were called, e.g., *pdc1-8*; the ones with a defect in *PDC2* were called, e.g., *pdc2-92* (Table 1). Sporulation of diploids *pdc1/PDC1 PDC2/pdc2* gave 45 wild-type segregants out of 192, indicating that *PDC1* and *PDC2* are not closely linked. No pyruvate decarboxylase activity nor fermentation was detectable in double-mutant strains, even when both component alleles were quite leaky, as in the case of *pdc1-30* and *pdc2-137*. The distinctly different behavior of the two sets of mutants and the epistatic effects in double mutants suggest that the two genes may serve entirely different functions. Except for mutant *pdc1-8*, all mutants formed enough activity to investigate the properties of their pyruvate decarboxylase. Boiteux and Hess (3) had shown that the kinetic properties of pyruvate decarboxylase from yeast, determined with highly purified enzyme and in crude extracts, were identical. Therefore, without any further purification, mutant crude extracts were used to determine various enzyme parameters. Heat inactivation, determination of  $K_m$  values, and even the allosteric properties of mutant enzymes in phosphate buffer (3) did not reveal any differences from the wild type. However, full activity in the *pdc1-14* mutant extracts required much more of cofactor thiamine pyrophosphate than did the wild type (Fig. 2A). Wild-type and *pdc1-14* mutant extracts showed the same response to the substrate pyruvate over the entire range when tested in phosphate buffer. The activities in phosphate buffer tended to reach the levels in

TABLE 1. Pyruvate decarboxylase activities<sup>a</sup>

Strain designation	Enzyme activity (mU/mg of protein)
Wild type	3,610 <sup>b</sup>
<i>pdc1-8</i>	5 <sup>b</sup>
<i>pdc1-14</i>	340 <sup>b</sup>
<i>pdc1-30</i>	1,090 <sup>b</sup>
<i>pdc2-92</i>	590 <sup>b</sup>
<i>pdc2-122</i>	330 <sup>b</sup>
<i>pdc2-137</i>	440 <sup>b</sup>
Wild type (2n)	3,355 <sup>b</sup>
<i>pdc1-8</i> × wild type	890 <sup>b</sup>
<i>pdc1-14</i> × wild type	1,190 <sup>b</sup>
<i>pdc1-30</i> × wild type	2,210 <sup>b</sup>
<i>pdc2-92</i> × wild type	3,990
<i>pdc2-122</i> × wild type	3,280
<i>pdc2-137</i> × wild type	3,390

<sup>a</sup> Crude extracts were prepared from log-phase cells 4 h after the addition of glucose to a thin culture growing on YEPE.

<sup>b</sup> Strains included in the experiments shown in Fig. 3.

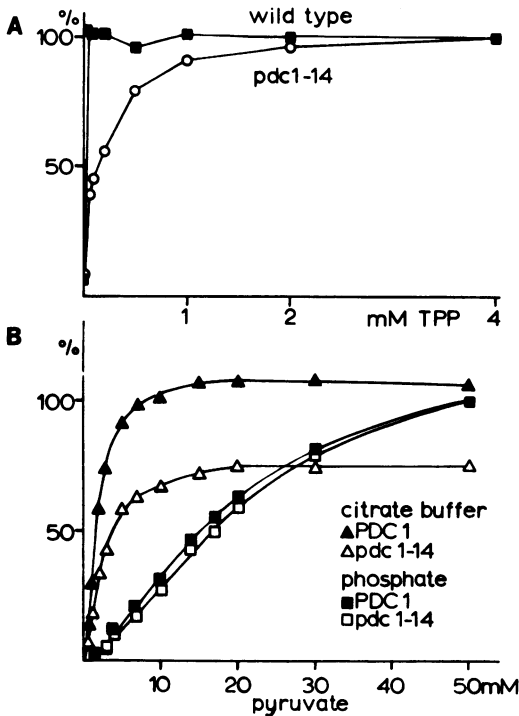


FIG. 2. Effect of thiamine pyrophosphate (A) and pyruvate (B) concentrations on the reaction velocity of mutant *pdc1-14* and wild-type pyruvate decarboxylase. Thiamine pyrophosphate dependence was followed in imidazole buffer (pH 7.0) with 33 mM pyruvate; pyruvate dependence was followed in 100 mM citrate and 100 mM phosphate buffer (pH 6.0) by the method of Boiteux and Hess (3), using 4 mM thiamine pyrophosphate. All tests were done with crude extracts. Activities at 4 mM thiamine pyrophosphate and 50 mM pyruvate-100 mM phosphate were taken as a 100% reference. Absolute values for the maximal rates were: (A) wild type, 3,610 mU; *pdc1-14*, 340 mU; (B) wild type (citrate), 2,880 mU; wild type (phosphate), 2,670 mU; *pdc1-14* (citrate), 300 mU; *pdc1-14* (phosphate), 402 mU.

citrate buffer (3; Fig. 2B). However, the final activities observed in *pdc1-14*, using citrate buffer, were only 74% of those in phosphate buffer (Fig. 2B). Allele *pdc1-30* caused the formation of yet another type of mutant enzyme. Whereas mutant enzyme from *pdc1-14* required more cofactors than did the wild type, *pdc1-30* had a reduced cofactor requirement. Wild-type crude extracts tested without the addition of cofactor had activities of less than 15% of the level observed at saturating levels of cofactor. In mutant *pdc1-30*, the residual activity was 63%. This increased residual activity did not change with different amounts of crude extracts in the enzyme assays. In contrast to these clear-cut effects with *pdc1* mutants, no differences be-

tween *pdc2* mutants and the wild type could be observed.

A mutant wild-type heterozygote *pdc1-8/PDC1*, with only a quarter of the normal wild-type activity (Table 1), was also investigated. But there was only enzyme activity of the wild-type kind. This suggested that all of the activity may derive from pure wild-type enzyme, with no contribution of mutant protein subunits to a potential oligomeric aggregate.

Our studies have yielded yeast strains with various activities of pyruvate decarboxylase. This allowed us to establish the correlation between various metabolic and growth parameters and the activity of this enzyme. The abscissae in Fig. 3A to C indicate the specific activities of cells after growth on glucose. Mutant *pdc1-8* (open circle) and the double mutants *pdc1-30 pdc2-122* and *pdc1-30 pdc2-137* (closed circles) showed no measurable enzyme activity. Fermentation, as determined from CO<sub>2</sub> production minus O<sub>2</sub> consumption, was apparently negative in the enzymeless mutant strains (Fig. 3A). This is an artifact, because cells respiring without any glycolytic activity have a respiration quotient of more than 1. It can be seen (Fig. 3A) that there is a strict correlation between the fermentation rate and pyruvate decarboxylase activity up to a specific activity of almost 2 U. Growth rates in a medium with glucose, yeast extract, and peptone, but without antimycin A, depended also on the pyruvate decarboxylase levels. On this medium, there was a basic doubling time of about 5 h under the conditions used. Any reduction in doubling time was due to a contribution by glycolytic activity. It can be seen that the enzymeless mutants could still grow in the presence of glucose, i.e., they were not inhibited by glucose as are most other glycolytic mutants (5).

A surprising observation was that, besides mutant *pdc1-8*, which had no measurable enzyme activity, mutants with considerable residual pyruvate decarboxylase activity were isolated. It was not quite clear why this residual activity should not enable the cells to grow on the selective medium with glucose and antimycin A. This question induced us to reexamine the selection procedure: mutagenized cells were plated on a glucose-free medium with ethanol. After colonies had grown up, they were replicated onto a glucose-antimycin A medium. On this selective medium, respiration was blocked by antimycin A (19), and energy could only be generated from a glycolytic breakdown of glucose. Wild-type cells continued to grow on such a medium, whereas all of the mutants stopped dividing. The reason for this became obvious when pyruvate decarboxylase activities were determined in cells after growth in the absence of glucose. The wild type had 0.2 U, the hetero-

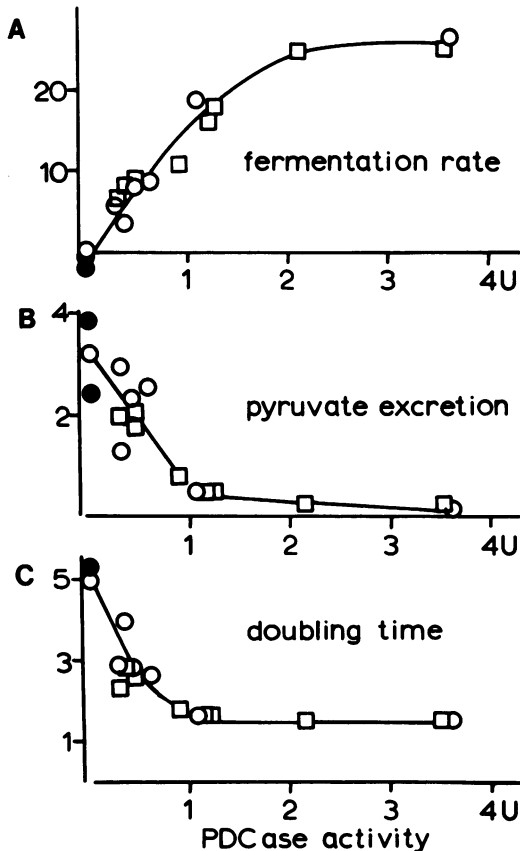


FIG. 3. Effect of in vitro pyruvate decarboxylase activity in mutants, mutant  $\times$  wild type crosses, and wild type on fermentation rate (A), pyruvate excretion rate (B), and growth rate (C). Fermentation rates and doubling times were determined 4 h after glucose addition after growth on YEPE to give a final concentration of 2%. Pyruvate excretion rates are mean values of the first 4 h after glucose addition. Symbols:  $\circ$ , haploid cells;  $\square$ , diploid cells; and  $\bullet$ , double mutants. Fermentation rates were calculated by subtracting the  $O_2$  consumption rate from the  $CO_2$  production rate. This led to negative values for *pdcl-8* and the double mutants. For the exact enzyme values of most strains, see Table 1. In addition to these strains, four heteroallelic diploids and two double mutants were included in this investigation.

zygote *pdcl-8/PDC1* had only 0.06 U, and activities in all of the other mutants were less than 0.04 U, actually too small to be accurately determined. Apparently, this small residual activity was not enough to support a glycolytic energy production sufficient for an adaptation to the new metabolic needs.

This assumption was tested by comparing cultures of various yeast strains, mutant and wild type, that were grown on ethanol and then incubated with glucose. Antimycin A was either added simultaneously or after a 3-h delay. Mu-

tants with alleles *pdcl-14*, *pdcl-30*, and *pdcl-92*, the heteroallelic diploid *pdcl-122/pdcl-137*, and the mutant/wild-type heterozygote *pdcl-8/PDC1* were not able to start fermentation and stopped growing when glucose and antimycin A were added at the same time. In contrast to this, the wild type was not much affected by this treatment, and after 2 h, the fermentation rate reached  $30 \mu\text{mol mg}^{-1} (\text{dry weight}) \text{h}^{-1}$ , a normal value (Fig. 3A). When antimycin A was added at 3 h after transfer into the glucose medium, all mutant strains except *pdcl-8* (with no pyruvate decarboxylase activity) continued fermenting even though some of them grew at reduced rates. Mutant strain *pdcl-14* had a doubling time of 7.8 h, and *pdcl-30* (with high residual enzyme activity) had a doubling time of 2.1 h. Consequently, it was the lack of adaptation to full glycolytic conditions on glucose medium in the presence of antimycin A which enabled us to isolate leaky pyruvate decarboxylase mutants by the protocol used. This may have been critical for the isolation of *pdcl-2* defective mutants.

We note two interesting questions about pyruvate decarboxylase-negative mutants: what happens with pyruvate and how NAD is regenerated since no acetaldehyde can be reduced at the expense of NADH in the alcohol dehydrogenase reaction. There is, indeed, considerable pyruvate excretion into the medium inversely related to the residual pyruvate decarboxylase activity (Fig. 3B). This excretion led to an acidification of the medium. When this was counteracted by an increase in the buffer capacity (additional 0.1 M potassium phosphate or morpholinoethanesulfonate [pH 7]), *pdcl* mutants grew faster than in the standard medium.

The stoichiometry of glycolytic products was studied in more detail with strains carrying either allele *pdcl-8* or the wild type *PDC1*. Oxygen consumption in mutant *pdcl-8* always exceeded  $CO_2$  production (Fig. 1B). This could have been due to a regeneration of NAD in an oxidative reaction. Another mechanism of NAD regeneration would be Neuberg's fermentation yielding glycerol when sulfite is added. Sulfite has been thought to trap acetaldehyde and, consequently, prevent regeneration of NAD in the alcohol dehydrogenase reaction. However, mutant *pdcl-8* produced less glycerol than did the wild type (Fig. 1). Moreover, when all of the mutants were tested for glycerol formation on a synthetic glucose medium, it was found that they excreted between 0.1 and  $0.3 \mu\text{mol mg}^{-1} (\text{dry weight}) \text{h}^{-1}$ , whereas the wild type generated  $0.6 \mu\text{mol}$ . When antimycin A was added to such cultures at 3 h after glucose addition, fermentation rates increased slightly, and pyruvate excretion was reduced by 58 to 86% com-

pared with samples without antimycin A. It was only then that the excretion of glycerol went up by factors of 2.7 to 3.7, both in leaky mutants and the wild type. As mentioned above, mutant *pdcl-8*, lacking residual enzyme activity, was completely inhibited by this treatment.

### DISCUSSION

The results of these physiological experiments suggest that all mutations affected genes specifically involved in the synthesis of cytoplasmic pyruvate decarboxylase. The loss of activity in vitro is reflected by a correspondingly lower fermenting rate, by a strong accumulation and secretion of pyruvate, and by a slower growth on glucose medium. But even the tight mutation *pdcl-8* did not prevent growth completely. The same phenomenon was observed by Lancashire et al. (14) in the case of their *pdc* mutant. This confirms the idea that mutants with defective pyruvate decarboxylase activity should show the same phenotype as alcohol dehydrogenase mutants, which are also blocked in alcoholic fermentation but show little inhibition by glucose (5). This is in contrast, however, to the results of Lam and Marmor (13), but it could be interpreted to mean that their mutations might be regulatory in nature (14).

The possibility of finding regulatory mutants, as well as mutants affected in a structural gene, was the main reason for a further search for *pdc* mutants. But it is still a matter of discussion whether the amount of glycolytic enzymes can be regulated (D. G. Fraenkel, *The Molecular Biology of the Yeast Saccharomyces*, vol. 2, *Metabolism and Gene Expression*, in press). The inducibility of the pyruvate decarboxylase activity of yeast by fermentable sugars (7) has been confirmed by experiments using leaky *pdc* mutants. These mutants are sensitive to antimycin A when it is added together with glucose to ethanol-grown cells. The addition of antimycin A 3 h after the glucose addition, only reduced the growth of the leaky mutants but did not prevent fermentation. This suggests that there is a threshold activity of pyruvate decarboxylase for the formation of the complete glycolytic system after a simultaneous addition of glucose and antimycin A to cells adapted to a gluconeogenic metabolism. Sufficient basal activity may be present in the wild type (0.2 U/mg of protein). None of the leaky mutants had more than 20% of this activity.

The growth of most mutants on glucose alone was reduced when compared with the wild type, and this reduction was proportional to the rate of pyruvate secretion. Pyruvate excretion could cause a growth retardation in two ways: (i) by a stronger acidification and (ii) by the simultaneous accumulation of NADH. The manometric

experiments using the tight mutant *pdcl-8* showed that in a medium with glucose as the sole carbon source, the O<sub>2</sub> consumption rate exceeded the CO<sub>2</sub> production rate. Therefore, respiration seems to be the main route of NAD<sup>+</sup> regeneration in this mutant. The dihydroxyacetone-phosphate/3-phosphoglycerin shuttle may be the rate-limiting step in the production of cytoplasmic NAD<sup>+</sup> (26). Wills and Phelps maintain that glycerol is only a byproduct of this conversion. Moreover, we did not succeed in simulating "Neuberg's second form of fermentation" with *pdc* mutants. None of the growth conditions used resulted in an increased glycerol formation by mutants compared with the wild type. The high pyruvate secretion, even by leaky mutants, leads to the conclusion that there is no high excess amount of pyruvate decarboxylase present in the yeast cells, as supposed by Lam and Marmor (13). This was also confirmed by the strong relationship between pyruvate decarboxylase activity in vitro and the fermentation rate, which directly reflects the pyruvate decarboxylase activity in living cells. The fact that this relationship is not completely linear (Fig. 3) does not contradict this conclusion. The high fermentation rate of leaky mutants compared with the wild type may be brought about by the high pyruvate accumulation within the cells. Pyruvate and phosphate concentrations in wild-type cells are about 2 and 9 mM, respectively (this article; 1). Under these conditions, pyruvate decarboxylase is not saturated with substrate (3). None of the mutants formed pyruvate decarboxylase with reduced substrate affinity. But due to the accumulated pyruvate, they can work at considerably higher pyruvate concentrations. This explains the high in vivo activity in leaky mutants, but it also confirms the findings of Holzer (10), who showed that the pyruvate concentration in yeast cells determines the pyruvate decarboxylase activity and fermentation rate.

Some observations suggested that *pdcl* mutants are affected in a structural gene. The first evidence came from the enzyme activity of the heterozygous diploid *pdcl-8/PDC1*, which reached only one fourth of the wild-type enzyme level. The pyruvate decarboxylase has a dimeric structure (25). Dissociation into subunits occurs at pH 8.0 (8). Denaturing with sodium dodecyl sulfate is necessary to reveal a possible tetrameric structure of this enzyme (9). These four subunits, however, may resemble a dimeric structure if the monomers are not identical, and the enzyme would have an ( $\alpha\beta$ )<sub>2</sub> subunit composition (12). Assuming a dimeric structure, the simplest interpretation for the low specific activity in heterozygous diploids would be the assumption that the *pdcl-8* allele determines the

amino acid sequence of a defective enzyme which is still capable of forming hybrid dimers with the wild-type protein. If these hybrid enzymes were catalytically inactive, we should expect that the residual activity reflects the probability of the exclusive association of two wild-type gene products, which is 25%. Such a low residual activity was actually found in the heterozygous diploid carrying the tight *pdcl-8* mutation. Specific activities of only 10% of the wild-type level in mutant-wild type heterozygotes have already been reported by Zimmermann et al. (30) for mutants of the structural gene locus for threonine dehydratase, *ILVI*, in yeast.

The examination of the kinetics of the enzyme in *pdcl-8/PDC1* towards pyruvate and thiamine pyrophosphate did not reveal any differences in the wild-type enzyme. Alleles *pdcl-14* and *pdcl-30* alone, however, showed different kinetic properties, when tested with various thiamine pyrophosphate concentrations. This is all consistent with *PDC1* being a structural gene, but it does not necessarily mean that *PDC2* must have a regulatory function because yeast pyruvate decarboxylase may consist of two different kinds of subunits (9, 12). This ( $\alpha\beta$ )<sub>2</sub> structure, however, may explain that the effects of the *pdcl* and *pdcl-30* mutations are not purely additive when both are present in haploid cells, as shown by double mutants which show no residual activity even when leaky mutant alleles are combined. *pdcl-30* mutants, however, did not reveal any changes in the kinetics of their pyruvate decarboxylase activity towards pyruvate or thiamine pyrophosphate. Moreover, the *pdcl-30* mutations are fully recessive and show no gene dosage effect.

#### ACKNOWLEDGMENTS

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