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Pyruvate Decarboxylase: An Indispensable Enzyme for Growth of *Saccharomyces cerevisiae* on Glucose

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In *Saccharomyces cerevisiae*, the structural genes *PDC1*, *PDC5* and *PDC6* each encode an active pyruvate decarboxylase. Replacement mutations in these genes were introduced in a homothallic wild-type strain, using the dominant marker genes *APT1* and *Tn5ble*. A pyruvate-decarboxylase-negative (Pdc$^-$) mutant lacking all three *PDC* genes exhibited a three-fold lower growth rate in complex medium with glucose than the isogenic wild-type strain. Growth in batch cultures on complex and defined media with ethanol was not impaired in Pdc$^-$ strains. Furthermore, in ethanol-limited chemostat cultures, the biomass yield of Pdc$^-$ and wild-type *S. cerevisiae* were identical. However, Pdc$^-$ *S. cerevisiae* was unable to grow in batch cultures on a defined mineral medium with glucose as the sole carbon source. When aerobic, ethanol-limited chemostat cultures ($D = 0{\cdot}10\ h^{-1}$) were switched to a feed containing glucose as the sole carbon source, growth ceased after approximately 4 h and, consequently, the cultures washed out. The mutant was, however, able to grow in chemostat cultures on mixtures of glucose and small amounts of ethanol or acetate (5% on a carbon basis). No growth was observed when such cultures were used to inoculate batch cultures on glucose. Furthermore, when the mixed-substrate cultures were switched to a feed containing glucose as the sole carbon source, wash-out occurred. It is concluded that the mitochondrial pyruvate dehydrogenase complex cannot function as the sole source of acetyl-CoA during growth of *S. cerevisiae* on glucose, neither in batch cultures nor in glucose-limited chemostat cultures.

KEY WORDS — pyruvate decarboxylase; sugar metabolism; *Saccharomyces cerevisiae*; metabolic compartmentation; acetyl-CoA

INTRODUCTION

In yeasts, respiratory dissimilation of pyruvate is initiated by its conversion into acetyl-CoA. This can occur in two ways: via a direct reaction catalysed by the mitochondrial pyruvate dehydrogenase complex or via an indirect route, involving pyruvate decarboxylase, acetaldehyde dehydrogenase and acetyl-coenzyme A synthetase (Figure 1; Holzer and Goedde, 1957; Pronk *et al.*, 1994).

Experiments with isogenic *Saccharomyces cerevisiae* mutants defective in the synthesis of an active pyruvate dehydrogenase complex have demonstrated that, during glucose-limited aerobic growth of wild-type cells, this enzyme is predominantly or even exclusively responsible for respiratory pyruvate dissimilation. Under these conditions, the indirect route apparently does not play an important role in respiratory pyruvate metabolism (Pronk *et al.*, 1994).

To study the metabolic significance of the pyruvate dehydrogenase bypass route, it is of interest to investigate the physiology of mutants affected in pyruvate decarboxylase (EC 4.1.1.1). *S. cerevisiae* contains three structural genes that each encode an active pyruvate decarboxylase; *PDC1*, *PDC5* and *PDC6* (Hohmann, 1991a). Strains in which *PDC1* and *PDC5* or all three *PDC* genes have been disrupted lack pyruvate decarboxylase activity. Such pyruvate decarboxylase (Pdc$^-$) mutants showed a reduced growth rate in complex (yeast extract–peptone) media supplemented with glucose (Hohmann, 1991a). Although, under all growth

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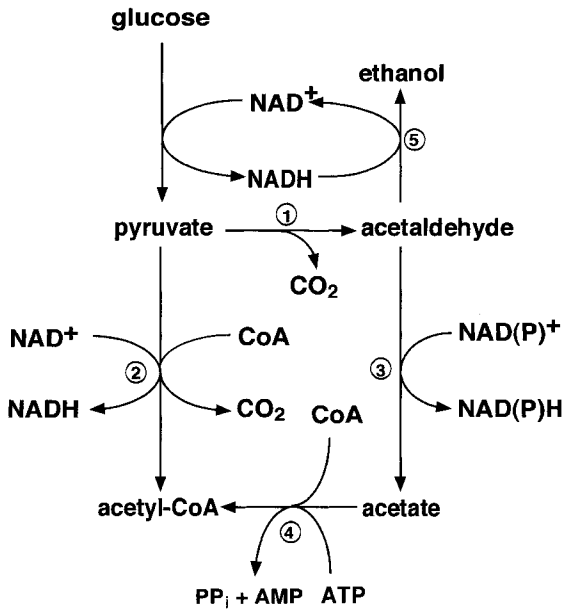

Figure 1. Enzymes of pyruvate metabolism in *Saccharomyces cerevisiae*. Numbered reactions are catalysed by the following enzymes: 1, pyruvate decarboxylase; 2, pyruvate dehydrogenase complex; 3, acetaldehyde dehydrogenase; 4, acetyl-coenzyme A synthetase; 5, alcohol dehydrogenase.

conditions tested, expression of *PDC6* was either very low or absent, revertants of *pdc1-pdc5* double mutants have been isolated, in which a recombination event had caused a fusion of the *PDC1* promotor and the *PDC6* open reading frame (Hohmann, 1991b). Therefore, physiological studies on Pdc$^-$ mutants should preferably be performed with stable strains in which all three *PDC* genes are disrupted.

Physiological characterization of the *S. cerevisiae* Pdc$^-$ mutants described in the literature has been restricted to growth studies in complex media (Hohmann, 1991a). The auxotrophic markers present in these strains make them unsuited for quantitative studies in defined media. In particular, the addition of amino acids to growth media may lead to substantial changes in the metabolism of the carbon source. For example, approximately 5% of yeast biomass consists of leucine (Oura, 1972). Furthermore, use of a genetic background containing auxotrophic markers may obscure effects of gene disruptions on amino acid metabolism: a partial leucine deficiency of a pyruvate dehydrogenase (Pdh$^-$) mutant was initially overlooked because the *pda1* strain was constructed in a *leu2* background, which required the inclusion of leucine in the growth media (Wenzel *et al.*, 1992a).

The aim of the present work was to construct isogenic pyruvate decarboxylase mutants of a homothallic, prototrophic *S. cerevisiae* strain and to characterize their growth in mineral media.

MATERIALS AND METHODS

Strains

The *S. cerevisiae* strains used in this study are listed in Table 1. *PDC* genes were disrupted in the homothallic, prototrophic, homozygous diploid strain T2–3D (Wenzel *et al.*, 1992a; Pronk *et al.*, 1994; de Jong-Gubbels *et al.*, 1995). *Escherichia coli*, strain XL1 blue (Bullock *et al.*, 1987) was used for plasmid amplification.

Maintenance of strains

Wild-type *S. cerevisiae* and *pdc* mutants were grown to stationary phase in shake-flask cultures

Table 1. *S. cerevisiae* strains used in the present study. Strain T2-3D (Wenzel *et al.*, 1993, Pronk *et al.*, 1994, de Jong-Gubbels *et al.*, 1995) is a homozygous diploid strain, derived from the heterozygous strain CBS8066 (Centraal Bureau voor Schimmelcultures, Delft, The Netherlands). Strains GG 562-GG 570 are isogenic mutants of T2-3D.

Strain	Genotype
T2-3D	*HO/HO PDC1/PDC1 PDC5/PDC5 PDC6/PDC6*
GG 562	*HO/HO pdc1::Tn5ble/pdc1::Tn5ble PDC5/PDC5 PDC6/PDC6*
GG 564	*HO/HO PDC1/PDC1 pdc5::Tn5ble/pdc5::Tn5ble PDC6/PDC6*
GG 566	*HO/HO PDC1/PDC1 PDC5/PDC5 pdc6::APT1/pdc6::APT1*
GG 568	*HO/HO pdc1::Tn5ble/pdc1::Tn5ble pdc5::Tn5ble/pdc5::Tn5ble PDC6/PDC6*
GG 569	*HO/HO pdc1::Tn5ble/pdc1::Tn5ble PDC5/PDC5 pdc6::APT1/pdc6::APT1*
GG 567	*HO/HO PDC1/PDC1 pdc5::Tn5ble/pdc5::Tn5ble pdc6::APT1/pdc6::APT1*
GG 570	*HO/HO pdc1::Tn5ble/pdc1::Tn5ble pdc5::Tn5ble/pdc5::Tn5ble pdc6::APT1/pdc6::APT1*

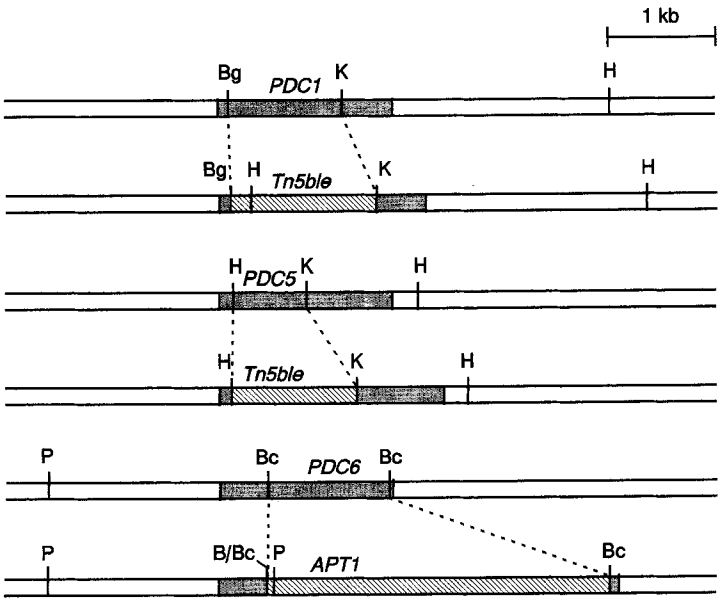

Figure 2. Schematic representation of the gene disruptions in *PDC1*, *PDC5* and *PDC6*. Restriction sites are indicated by the following abbreviations: B=*Bam*HI, Bc=*Bcl*I, Bg=*Bgl*II, H=*Hin*dIII, K=*Kpn*I, P=*Pst*I.

on complex medium containing 2% (v/v) ethanol. After adding glycerol (15% v/v), 2-ml aliquots were stored in sterile vials at −70°C. Prior to growth experiments, samples from a frozen stock culture were streaked on complex medium–ethanol agar plates. Precultures were inoculated directly from these plates.

Recombinant DNA techniques

Standard protocols were followed for plasmid isolation, restriction, ligation, Southern blotting, hybridization and gel electrophoresis (Maniatis *et al.*, 1982). Yeast chromosomal DNA was isolated by the method of Holm *et al.* (1986). *S. cerevisiae* and *E. coli* strains were transformed with a Bio-Rad gene pulser (Dower *et al.*, 1988). Sporulation, dissection and mating of *S. cerevisiae* strains was performed according to published procedures.

Construction of pdc mutants

Subclones of *PDC1*, *PDC5* and *PDC6* in pUC vectors were kindly provided by Dr S. Hohmann. The one-step gene-disruption method (Rothstein, 1983) was used to inactivate the *PDC1*, *PDC5* and *PDC6* genes in *S. cerevisiae* T2–3D (Figure 2).

PDC1 was disrupted by replacing an internal 1058 bp *Kpn*I-*Bgl*II fragment with a 1·35 kb *Kpn*I-*Bgl*II fragment from the plasmid pUT332 (Gatignol *et al.*, 1990) containing the marker gene *Tn5ble* under the control of the *S. cerevisiae TEF1* promoter and *CYC1* terminator. A 1·15 kb *Hin*dIII-*Kpn*I fragment from pUT332, carrying the same marker gene, was used to replace an internal 691 bp *Hin*dIII-*Kpn*I fragment of *PDC5*. *PDC6* was disrupted by replacing an internal 1190 bp *Bcl*I fragment with a 3.2 kb *Bam*HI-*Bcl*I fragment from the plasmid pBEJ24 (Hadfield *et al.*, 1990) containing the marker gene *APT1* under the control of the *S. cerevisiae PGK1* promoter and *CYC1* terminator. After transformation of *S. cerevisiae* T2–3D with linear restriction fragments containing the disrupted genes, transformants were selected on YPD plates containing either phleomycin (strains expressing *Tn5ble*) or G418 (transformants expressing *APT1*), as described by Wenzel *et al.* (1992b) and Hadfield *et al.* (1990), respectively. Since the strains are homothallic, spore-to-spore matings were used to obtain strains in which two or three *PDC* genes were disrupted. The following combinations were used: GG 562 × GG 564; GG 562 × GG 566, GG 564 × GG 566 and GG 562

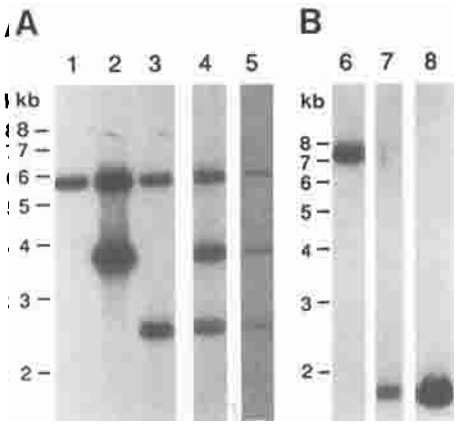

Figure 3. Southern analyses of genomic DNA restriction digests. Panel A: *Hin*dIII digests. The probe contained the *TEF1* promoter and the *Tn5ble* gene from the phleomycin-resistance cassette. The largest hybridizing fragment in lanes 1–5 contains the native *TEF1* promoter. Panel B: *Pst*I digests. The probe contained the 5′ region of the *PDC6* gene. Relevant restriction sites are indicated in Figure 1. Lane 1: *S. cerevisiae* T2–3D (wild type); lane 2: GG 562 (*pdc1*::*Tn5ble*); lane 3: GG 564 (*pdc5*::*Tn5ble*); lane 4: GG 568 (*pdc1*::*Tn5ble pdc5*::*Tn5ble*); lane 5: GG 570 (*pdc1*::*Tn5ble pdc5*::*Tn5ble pdc6*::*APT1*); lane 6: T2–3D (wild type); lane 7: GG 566 (pdc6::APT1); lane 8: GG 570 (*pdc1*::*Tn5ble pdc5*::*Tn5ble pdc6*::*APT1*).

× GG 567. The resulting heterozygous diploid strains were again sporulated and dissected to obtain the homozygous strains. Spore-to-spore matings were performed on CY plates with 2% (v/v) ethanol instead of glucose. Spore viability was low, probably due to the *pdc* mutations (Hohmann, 1991a). Dissection on glucose or galactose media did not significantly improve spore viability. The genotype of all strains containing single or multiple disrupted *PDC* genes was confirmed by Southern analysis (Figure 3).

Media

The mineral medium contained per litre of demineralized water: $(NH_4)_2SO_4$, 5 g; KH_2PO_4, 3 g; $MgSO_4 \cdot 7H_2O$, 0·5 g; EDTA, 15 mg; $ZnSO_4 \cdot 7H_2O$, 4·5 mg; $CoCl_2 \cdot 6H_2O$, 0·3 mg; $MnCl_2 \cdot 4H_2O$, 1 mg; $CuSO_4 \cdot 5H_2O$, 0·3 mg; $CaCl_2 \cdot 2H_2O$, 4·5 mg; $FeSO_4 \cdot 7H_2O$, 3·0 mg; $Na_2MoO_4 \cdot 2H_2O$, 0·4 mg; H_3BO_3, 1·0 mg; KI, 0·1 mg; silicone antifoam (BDH), 0·05 ml. After heat sterilization (120°C) of the medium, filter-sterilized vitamins were added, to final concentrations per litre of: biotin, 0·05 mg; calcium pantothenate, 1·0 mg; nicotinic acid, 1·0 mg; inositol, 25·0 mg; thiamin-HCl, 1·0 mg; pyridoxin-HCl, 1·0 mg and para-aminobenzoic acid, 0·2 mg. The concentration of ethanol or glucose in the reservoir medium was 5·75 g l^{-1} or 7·5 g l^{-1} respectively (0·25 Cmol l^{-1}). Complex medium contained per litre: yeast extract (Difco), 10 g; peptone from casein (Merck), 20 g; and 2% (v/v) ethanol (YPE) or 20 g D-glucose (YPD). CY plates contained per litre: yeast extract (Difco), 5 g; bactopeptone (Difco), 5 g; agar (Difco), 20 g; and glucose, 20 g.

Shake-flask cultivation

Precultures were prepared by inoculating 100 ml YPE (2% ethanol) with a few colonies from a plate. Cultures were incubated on an orbital shaker (200 rpm) at 30°C for 2 days. For growth curves, 1 ml of the preculture was inoculated in a 500 ml Erlenmeyer flask with 100 ml YPE (2% ethanol) or 100 ml YPD (2% glucose) and then shaken (200 rpm) at 30°C. Optical-density measurements were performed at appropriate intervals as described by Weusthuis *et al.* (1994). For induction of pyruvate decarboxylase, 10 ml of a preculture was inoculated in a 100 ml shake flask with either 50 ml YPE (2% ethanol) or 50 ml YPD (8% glucose) and shaken for 6 h at 30°C (Hohmann, 1991a).

Batch cultivation in fermenters

Batch cultivation was performed at 30°C in laboratory fermenters (Applikon, Schiedam, The Netherlands) with a working volume of 1·5 l. The pH was controlled at 5·0 ± 0·1 by automatic addition of 2 mol l^{-1} KOH and 1 mol l^{-1} H_2SO_4. The fermenter was flushed with air at a flow rate of 1·5 l min^{-1} and stirred at 800 rpm. The dissolved-oxygen concentration was continuously monitored with an oxygen electrode (Ingold, 34 100 3002) and remained above 60% of air saturation. Cultures were grown on the mineral medium described above, with glucose (25 g l^{-1} initial concentration) or ethanol (7·9 g l^{-1} initial concentration) as the sole carbon source. 25 ml samples were withdrawn at appropriate intervals for determination of dry weight and metabolite concentrations.

Chemostat cultivation in fermenters

Aerobic chemostat cultivation was performed at 30°C in laboratory fermenters (Applikon, Schiedam, The Netherlands), at a stirrer speed of 750 rpm and a dilution rate of 0·10 h^{-1}. The working volume of the cultures was kept at 1·0 l by a peristaltic effluent pump coupled to an electrical

level sensor. This set-up ensured that under all growth conditions, biomass concentrations in samples taken directly from the cultures differed by less than 1% from biomass concentrations in samples taken from the effluent line (Noorman *et al.*, 1991). The pH was kept constant at 5·0 by an ADI 1020 biocontroller, via the automatic addition of 2 mol l^{-1} KOH. The fermenter was flushed with air at a flow rate of 0·7 l min^{-1} using a Brooks 5876 mass-flow controller. The dissolved-oxygen concentration was continuously monitored with an oxygen electrode (Ingold, 34 100 3002) and remained above 50% of air saturation. Steady-state data refer to cultures without detectable oscillations. Chemostat cultures were checked for purity using phase-contrast microscopy.

Determination of culture dry weight

The dry weight of washed culture samples was determined using 0·45 μm membrane filters and a microwave oven as described by Postma *et al.* (1989). Parallel samples varied by less than 1%.

Metabolite analysis

Organic acids, ethanol and glycerol in culture supernatants were determined by HPLC analysis using a Phenomenex column (Rezex ROA Organic acid 00H-0138-KO) at 60°C. The column was eluted with 0·5 g l^{-1} sulphuric acid at a flow rate of 0·5 ml min^{-1}. Organic acids were detected by a Waters 441 UV-meter at 214 nm coupled to a Waters 741 Data module. Ethanol and glycerol were detected by an Erma ERC 7510 refractive-index detector coupled to a Hewlett Packard 3390A RI integrator. 20 μl samples were injected using a Hamilton syringe. Glucose in reservoir media and supernatants was determined enzymically using the GOD-PAP method (Merck Systems kit 14144; detection limit ca. 5 μM). Ethanol was assayed colorimetrically with an alcohol oxidase/peroxidase kit (Leeds Biochemicals; detection limit ca. 100 μM).

Preparation of cell-free extracts

For preparation of cell-free extracts, culture samples were harvested by centrifugation, washed twice with 10 mM-potassium-phosphate buffer, pH 7·5, containing 2 mM-EDTA, concentrated four-fold and stored at −20°C. Before assaying, the samples were thawed at room temperature, washed and resuspended in 100 mM-potassium phosphate buffer, pH 7·5, containing 2 mM-$MgCl_2$ and 1 mM-dithiothreitol. Extracts were prepared by sonication with 0·7 mm diameter glass beads at 0°C for 2 min at 0·5-min intervals with an MSE sonicator (150 W output, 7 μm peak-to-peak amplitude). Unbroken cells and debris were removed by centrifugation at 4°C (20 min at 36,000 *g*). The supernatant was used as the cell-free extract.

Pyruvate decarboxylase assays

Pyruvate decarboxylase activity was assayed at 30°C immediately after preparation of the extracts, using a Hitachi model 100–60 spectrophotometer set at 340 nm. Reaction rates were linearly proportional to the amount of cell-free extract added. The assay mixture consisted of: 40 mM-imidazole–HCl buffer (pH 6·5), 0·2 mM-thiamine pyrophosphate, 0·15 mM-NADH, alcohol dehydrogenase 88 U ml^{-1} (Boehringer), 5 mM-$MgCl_2$ and cell-free extract. The reaction was started with 50 mM-pyruvate.

Protein determination

Protein concentrations in cell-free extracts were determined by the Lowry method. Bovine serum albumin (fatty-acid-free; Sigma Chemical Co.) was used as a standard. The protein content of whole cells was determined by a modified biuret method (Verduyn *et al.*, 1990).

RESULTS

Specific activities of pyruvate decarboxylase in pdc mutants

Effects of the gene disruptions on pyruvate decarboxylase expression were investigated by measuring enzyme activities in cell-free extracts of wild-type *S. cerevisiae* T2–3D and in homozygous mutant strains containing one, two or three disrupted *PDC* genes. To discriminate between constitutive and glucose-inducible pyruvate decarboxylase activity, cells were pregrown in complex medium with ethanol as the carbon source and then either incubated in the ethanol medium used for growth or induced by incubation in glucose medium (Hohmann, 1991a). The wild-type strain T2–3D exhibited a high pyruvate decarboxylase activity after induction in complex medium with glucose (ca. 3 U mg protein $^{-1}$, Table 2). An approximately three-fold lower activity was measured in extracts from non-induced wild-type cells grown on ethanol.

When strain GG562 carrying the *pdc1::Tn5ble* mutation was induced with glucose, its pyruvate

Table 2. Specific pyruvate-decarboxylase activity and growth rates of wild-type (T2-3D) and *pdc* mutant strains. For enzyme activity assays, cells pregrown on complex medium with ethanol were induced on either 8% (w/v) glucose of 2% (v/v) ethanol in complex medium. Growth rates were determined in complex medium containing either 2% (v/v) ethanol of 2% (w/v) glucose.

Strain	Genotype			Ethanol		Glucose	
				PDC activity U.(mg prot)$^{-1}$	μ_{max} (h^{-1})	PDC activity U.(mg prot)$^{-1}$	μ_{max} (h^{-1})
T2-3D	*PDC1*	PDC5	PDC6	1·0 ± 0·10	0·30 ± 0·02	3·1 ± 0·55	0·54 ± 0·02
GG 562	*Δpdc1*	PDC5	PDC6	0·1 ± 0·05	0·30 ± 0·01	2·1 ± 0·15	0·53 ± 0·02
GG 564	*PDC1*	*Δpdc5*	PDC6	1·0 ± 0·20	0·29 ± 0·01	2·9 ± 0·20	0·55 ± 0·02
GG 566	*PDC1*	PDC5	*Δpdc6*	1·0 ± 0·25	0·30 ± 0·01	2·9 ± 0·35	0·56 ± 0·02
GG 568	*Δpdc1*	*Δpdc5*	PDC6	<0·01	0·29 ± 0·01	<0·01	0·15 ± 0·01
GG 569	*Δpdc1*	PDC5	*Δpdc6*	0·1 ± 0·05	0·29 ± 0·01	2·0 ± 0·15	0·54 ± 0·02
GG 567	*PDC1*	*Δpdc5*	*Δpdc6*	1·2 ± 0·10	0·29 ± 0·02	2·7 ± 0·00	0·55 ± 0·02
GG 570	*Δpdc1*	*Δpdc5*	*Δpdc6*	<0·01	0·27 ± 0·00	<0·01	0·15 ± 0·01

decarboxylase activity, determined in cell-free extracts, was only ca. 30% lower than that of glucose-induced wild-type cells (Table 2). In non-induced cells, disruption of *PDC1* resulted in a ten-fold reduction of the pyruvate decarboxylase activity in comparison with the wild-type strain. Single gene disruptions in either *PDC5* or *PDC6* did not significantly affect enzyme activities, neither in induced nor in non-induced cells (Table 2).

Pyruvate decarboxylase activities in strains which, in addition to a disrupted *PDC1* or *PDC5* gene, contained a disruption in *PDC6*, were not significantly different from the activities in strains carrying the corresponding single gene disruptions (Table 2). When both *PDC1* and *PDC5* were disrupted, leaving *PDC6* as the only intact *PDC* gene, no enzyme activity was detected in cell-free extracts prepared from induced or non-induced cells. A complete absence of pyruvate decarboxylase activity was also observed in extracts of a triple mutant (strain GG 570), in which all three *PDC* genes had been disrupted (Table 2).

Growth rates in complex medium

For an initial physiological characterization, and to enable comparison with *pdc* mutations introduced in a different *S. cerevisiae* genetic background (Hohmann, 1991a), growth rates of the *PDC* mutant strains were determined in shake-flask cultures on complex media with glucose or ethanol.

In complex medium with ethanol, growth rates of strains carrying one, two or three disrupted *PDC* genes did not differ significantly from those of the isogenic wild type (Table 2). This result is consistent with the fact that pyruvate decarboxylase is not involved in ethanol metabolism. Nevertheless, it differs from the observation of Hohmann (1991a) that strains in which both *PDC1* and *PDC5* had been disrupted showed a 20–25% reduction of the specific growth rate on ethanol.

Disruption of any single *PDC* gene did not affect the growth rate in complex medium with glucose. In double mutants, growth rates on glucose were not significantly reduced when combinations of *PDC6* and either *PDC1* or *PDC5* were disrupted (Table 2). However, disruption of both *PDC1* and *PDC5* resulted in a 70% decrease of the specific growth rate on glucose. This negative effect on growth rate was not enhanced by the additional disruption of *PDC6* (Table 2).

Our results confirm the conclusion of Hohmann (1991a) that, during growth in ethanol- or glucose-containing media, *PDC6* expression is either very low or absent. However, it has been demonstrated that recombination events may lead to the activation of *PDC6* (Hohmann, 1991b). Since such instability is not desirable in physiological studies, it was decided to use the triple mutant strain GG 570 for further physiological investigations on the effects of pyruvate decarboxylase deficiency during growth of *S. cerevisiae* in mineral media.

Batch cultivation in defined mineral medium

Quantitative analysis of yeast physiology requires the use of defined mineral media. Therefore, aerobic growth of wild-type *S. cerevisiae* T2–3D in

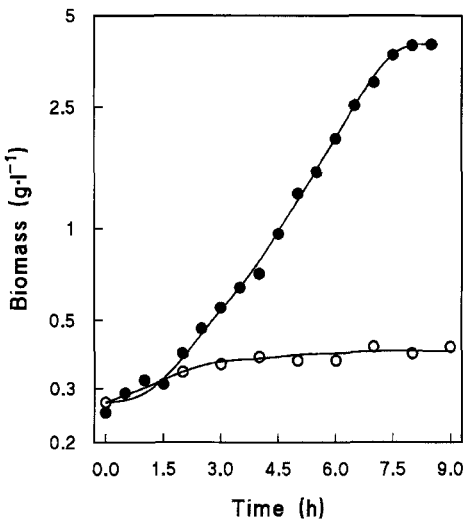

Figure 4. Growth of wild-type *S. cerevisiae* T2–3D (●) and Pdc⁻ triple mutant GG 570 (*pdc1::Tn5ble pdc5::Tn5ble pdc6::APT1*; (○) triple mutant on a defined mineral medium containing 25 g l^{-1} glucose as the sole carbon source. Batch cultivation was performed in pH-controlled, aerobic fermenters.

a defined mineral salts medium supplemented with vitamins was compared with growth of the isogenic pyruvate decarboxylase triple mutant GG 570, using pH-controlled fermenter cultures.

When grown on ethanol, there was no difference in growth rate between the wild-type strain and the pyruvate decarboxylase-deficient mutant: both strains grew exponentially with a specific growth rate of 0·13 ± 0·01 h^{-1}. The wild type grew exponentially on glucose, with a specific growth rate of 0·45 ± 0·01 h^{-1} (Figure 4). Growth on glucose was accompanied by the formation of ethanol and small amounts of pyruvate (3 mmol l^{-1}) and glycerol (0·3 mmol l^{-1}). In contrast, the Pdc⁻ strain GG 570 did not exhibit exponential growth on glucose. Instead, growth ceased after less than one biomass doubling (Figure 4). No ethanol or acetate was detected, but concentrations of pyruvate (8 mM) and glycerol (2 mM) attained higher values than in wild-type cultures, even though the biomass concentrations in mutant cultures were much lower.

Since a Pdc⁻ mutant cannot grow fermentatively, respiration is essential for its growth on glucose. In *S. cerevisiae*, many enzyme activities involved in respiratory sugar metabolism are subject to glucose catabolite repression (Gancedo, 1992). To investigate whether glucose repression of respiration might be responsible for the mutant's impaired growth on glucose in batch cultures, attempts were made to establish glucose-limited chemostat cultures.

Growth of pyruvate decarboxylase-deficient S. cerevisiae *in chemostat cultures*

The pyruvate dehydrogenase complex, rather than the bypass via pyruvate decarboxylase, is the predominant route of respiratory pyruvate metabolism during glucose-limited growth at D=0·10 h^{-1} (Pronk *et al.*, 1994). Furthermore, many key enzymes of glucose metabolism, including the pyruvate dehydrogenase complex, are expressed constitutively during growth of *S. cerevisiae* T2–3D on ethanol (Wenzel *et al.*, 1993; Pronk *et al.*, 1994; de Jong-Gubbels *et al.*, 1995). It was therefore anticipated that steady-state chemostat cultures growing on ethanol would readily adapt to growth on glucose under glucose limitation.

In ethanol-limited chemostat cultures (D= 0·10 h^{-1}) grown on a defined medium, the biomass yield of the pyruvate decarboxylase triple mutant GG 570 was not significantly different from that of the isogenic wild-type strain T2–3D (Table 3). To avoid glucose repression, ethanol-limited chemostat cultures (D=0·10 h^{-1}) of the triple mutant were switched to a medium containing glucose as the sole carbon source. During the first 4 h after the switch, the biomass concentration remained approximately constant and the glucose concentration in the culture remained below 0·2 g l^{-1} (Figure 5). This suggested that indeed, the culture rapidly adapted from ethanol-limited to glucose-limited growth. However, after this initial period, the biomass concentration decreased and glucose accumulated (Figure 5). The observed decrease of the biomass concentration was consistent with wash-out kinetics, indicating that growth had ceased completely. The wash-out of biomass and the accumulation of glucose was accompanied by the transient accumulation of pyruvate in the culture to a maximum concentration of 7 mM (Figure 5).

The observation that, both in batch and chemostat cultures, growth of the Pdc⁻ strain on glucose continued for a number of hours before growth ceased, can in theory be caused by a bottleneck in a biosynthetic pathway that requires pyruvate decarboxylase. This would be consistent with the ability of Pdc⁻ strains to grow, albeit poorly, in complex media with glucose (Table 2), in which precursors for biosynthesis can be obtained from yeast extract and/or peptone. Since growth of the

Table 3. Steady-state biomass yields (Y_{sx}, g biomass·[mol substrate carbon]$^{-1}$), protein contents and pyruvate-decarboxylase activities in ethanol- and glucose-limited, aerobic chemostat cultures of wild-type (T2-3D) and Pdc$^-$ (GG 570) *S. cerevisiae*. Relative concentrations of glucose and acetate in mixed-substrate cultures are presented as a percentage of the total carbon concentration (0·25 mol C.l^{-1}) in the feed. Growth conditions: D=0·10 h^{-1}, pH 5, T=30°C, dissolved-oxygen concentration >50% air saturation (n.d.: not determined).

Strain	Carbon source	Y_{sx} g.Cmol^{-1}	Protein content (%)	PDC-activity U.mg protein^{-1}
T2-3D (wildtype)	ethanol	14·4 ± 0·4	41 ± 2	0·7 ± 0·3
GG 570 (Pdc$^-$)	ethanol	14·3 ± 0·3	42 ± 2	<0·01
T2-3D (wildtype)	glucose	16·0 ± 0·3	40 ± 2	0·7 ± 0·1
T2-3D (wildtype)	95% glucose-5% acetate	16·5 ± 0·1	40 ± 2	n.d.
GG 570 (Pdc$^-$)	95% glucose-5% acetate	16·2 ± 0·4	40 ± 1	<0·01

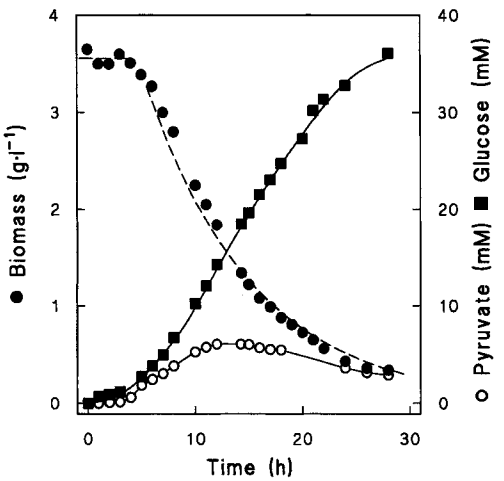

Figure 5. Concentrations of biomass, glucose and pyruvate after switching a chemostat culture (D=0·10 h^{-1}) of the Pdc$^-$ triple mutant *S. cerevisiae* GG 570 (*pdc1::Tn5ble pdc5::Tn5ble pdc6::APT1*) from growth on a mineral medium with ethanol (0·25 Cmol l^{-1}) to a medium containing glucose (0.25 Cmol l^{-1}) as the sole carbon source. The dashed line drawn through biomass data points represents wash-out kinetics, assuming a zero growth rate.

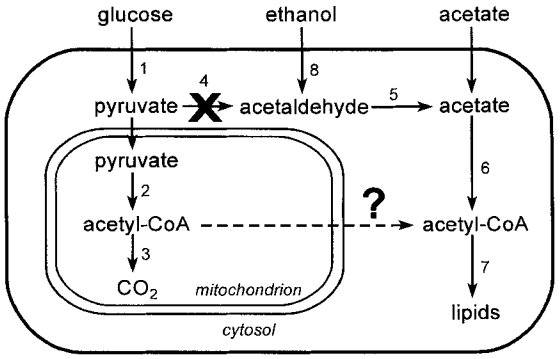

Figure 6. Hypothetical scheme of subcellular compartmentation of pyruvate and acetyl-CoA metabolism in *S. cerevisiae*, explaining the requirement of a Pdc$^-$ mutant for C2- compounds. If acetyl-CoA export from the mitochondria is restricted, glucose-grown cells depend on a source of cytosolic acetyl-CoA. In the absence of pyruvate decarboxylase, cytosolic acetyl-CoA cannot be synthesized from glucose, resulting in a requirement for exogenous C2-compounds. Numbered arrows indicate the following pathways or enzymes: 1, glycolysis; 2, pyruvate dehydrogenase complex; 3, TCA cycle; 4, pyruvate decarboxylase; 5, acetaldehyde dehydrogenase; 6, acetyl-coenzyme A synthetase; 7, lipid synthesis; 8, alcohol dehydrogenase.

mutant strain on mineral medium with ethanol appeared normal, formation of biosynthetic intermediates from ethanol was apparently not affected.

To study whether growth was possible on mixtures of glucose and C2-compounds, ethanol-limited chemostat cultures were switched to mineral medium containing a mixture of glucose (237·5 mmol C l^{-1}) and acetate (12·5 mmol C l^{-1}). This approach resulted in steady-state cultures, in which no residual glucose or acetate could be detected. Enzyme assays in cell-free extracts confirmed the absence of pyruvate decarboxylase activity (Table 3). The biomass concentration in the cultures did not differ significantly from that in similar cultures of the wild-type strain (Table 3). The same results were obtained when, instead of acetate, low concentrations of ethanol were added to the reservoir media (data not shown).

In the mixed-substrate cultures, glucose made up 95% of the substrate carbon fed to the cultures and was completely consumed. Nevertheless, when samples from such glucose-limited cultures were used to inoculate batch cultures on mineral medium with glucose, no growth was observed. The inability to grow on glucose in batch cultures could not be relieved by the addition of low concentrations of acetate or ethanol to the mineral media. Attempts to change the medium feed of chemostat cultures from a 95% glucose/5% acetate mixture to glucose as the sole carbon source reproducibly resulted in wash-out of the cultures (data not shown). This indicated that even cells utilizing high glucose-to-acetate ratios could not be readily adapted to growth on glucose as the sole carbon source.

DISCUSSION

Growth of PDC mutants in batch cultures

In batch cultures grown on complex media with ethanol or glucose, *PDC1* was expressed constitutively, whereas expression of *PDC5* appeared to be induced by glucose. *PDC6* did not contribute significantly to the overall level of pyruvate decarboxylase. The pattern of pyruvate decarboxylase (Table 2) was generally consistent with that observed by Hohmann (1991a), who studied the effect of *PDC* gene disruptions in a different *S. cerevisiae* genetic background. However, it should be borne in mind that the differential regulation of the three *PDC* genes has so far only been studied during growth on complex media in shake-flask cultures. The possibility that, under appropriate growth conditions, *PDC6* is transcribed at significant levels therefore cannot be excluded.

Growth experiments in defined and complex media with ethanol as the carbon source gave no indications of pleiotropic effects of the *PDC* mutations. This contrasts with the results of Hohmann (1991a), who observed significantly reduced growth rates for Pdc$^-$ strains on ethanol even though pyruvate decarboxylase deficiency should have no effect on ethanol metabolism. This discrepancy deserves further attention.

Previously, the Pdc$^-$ phenotype, established either by random mutagenesis (Schmitt and Zimmermann, 1982) or gene disruption (Hohmann, 1991a), has only been studied in cultures grown in complex media. These studies invariably demonstrated a reduction of the specific growth rates of the mutants in glucose-containing media. The residual growth rates in the mutant strains were consistently at least 25% of the wild-type rate (Schmitt and Zimmermann, 1982; Hohmann, 1991a). A similar effect was observed in the present study (Table 2).

In the absence of pyruvate decarboxylase activity, regeneration of NAD^+ and growth of *S. cerevisiae* become critically dependent on respiration. Indeed, Hohmann (1991a) demonstrated that growth of Pdc$^-$ mutants on glucose was completely arrested by the respiratory inhibitor antimycin A. It is well known that in *S. cerevisiae*, many respiratory enzymes are repressed in the presence of excess glucose (Entian, 1986; Gancedo and Serrano, 1989). However, glucose repression of respiratory enzymes is generally not complete (Gancedo, 1992). This is consistent with the reduced growth rate of Pdc$^-$ strains in complex medium with glucose (Table 2).

Surprisingly, Pdc$^-$ *S. cerevisiae* completely failed to grow in batch cultures on a mineral medium with glucose as the sole carbon source. Clearly, if glucose repression of respiratory enzymes were the sole factor affecting the growth rate of Pdc$^-$ mutants, a residual growth rate similar to that observed in complex medium would be expected. Pdc$^-$ strains retained the ability to grow in mineral medium with ethanol and were apparently able to convert glucose into pyruvate (Figure 5). It is therefore conceivable that the absence of growth on glucose in a defined medium is due to a shortage of acetyl-CoA.

An anabolic role of pyruvate decarboxylase

Inclusion of low concentrations of ethanol or acetate in the medium feed was required to enable growth of Pdc$^-$ *S. cerevisiae* in glucose-limited chemostat cultures. The most likely explanation for this dependency on C2-compounds is a limitation in the synthesis of acetyl-CoA, an important building block for the synthesis of TCA-cycle intermediates, lipids and some amino acids (Oura, 1972). An inability to synthesize one or more of these biosynthetic precursors would be consistent with the phenotype of the Pdc$^-$ mutants: growth in complex medium with glucose may still occur at the expense of biosynthetic precursors available from yeast extract and peptone. Moreover, the observation that growth in chemostat cultures continued for a short period after transfer from an ethanol feed to a glucose feed (Figure 5) may be

explained from depletion of an intracellular metabolite pool.

In the Pdc$^-$ strain, the only reaction that can lead to the formation of acetyl-CoA from pyruvate is the direct oxidative decarboxylation of pyruvate by the mitochondrial pyruvate dehydrogenase complex. As a result, formation of acetyl-CoA in the Pdc$^-$ mutant is confined to the mitochondrial matrix (Figure 6). This necessitates export of acetyl-CoA to the cytosol, where lipid synthesis occurs (Ratledge and Evans, 1989). Pyruvate decarboxylase, acetaldehyde dehydrogenase and acetyl-CoA synthetase all have been reported to be present in the cytosol of wild-type *S. cerevisiae* (van Urk *et al.*, 1989; Jacobson and Bernofsky, 1974; Kispal *et al.*, 1991). Consequently, the pyruvate dehydrogenase bypass may act as the major source of cytosolic acetyl-CoA in wild-type *S. cerevisiae*, which would preclude the necessity of acetyl-CoA export from the mitochondrial matrix.

In yeasts, two enzyme systems may catalyse transport of acetyl-CoA across the mitochondrial inner membrane. ATP-citrate lyase, a key enzyme of one system, is absent in *S. cerevisiae* (Ratledge and Evans, 1989). A second system, the acetylcarnitine/carnitine translocase is generally assumed to catalyse import of acetyl-CoA into the mitochondria (Kohlhaw and Tan-Wilson, 1977; Schmalix and Bandlow, 1993). However, to what extent this system can also catalyse the reverse reaction under physiological conditions is at present unclear.

The observation that small amounts of ethanol or acetate allow normal growth of the Pdc$^-$ mutant in glucose-limited chemostat cultures strongly suggests that pyruvate decarboxylase plays a crucial role in the supply of cytosolic acetyl-CoA in wild-type cells (Figure 5) and that this function cannot be fulfilled by the mitochondrial pyruvate dehydrogenase complex. In batch cultures, mixtures of glucose and ethanol or acetate did not support growth of the pyruvate decarboxylase-deficient mutant. The most simple explanation for the difference between batch and chemostat cultures is a differential effect of glucose repression and/or inactivation of respiratory enzymes, regulatory processes which are at least partially relieved in carbon-limited chemostat cultures.

Further studies on the reversibility of the acetylcarnitine shuttle under physiological conditions is required to further substantiate this hypothesis. Nevertheless, it can be concluded that in addition to its key role in alcoholic fermentation, pyruvate decarboxylase fulfils at least one other essential role in glucose metabolism in *S. cerevisiae*. Furthermore, this work exemplifies the necessity of defined growth conditions for studies on *S. cerevisiae* mutants affected in central metabolic pathways. Only by using mineral media and chemostat cultivation, could the unexpected behaviour of Pdc$^-$ mutants be unveiled.

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