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Energetic aspects of glucose metabolism in a pyruvate-dehydrogenase-negative mutant of *Saccharomyces cerevisiae*

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***Saccharomyces cerevisiae* T23C (*pda1*::Tn*5ble*) is an isogenic gene replacement mutant of the wild-type strain *S. cerevisiae* T23D. The mutation causes a complete loss of pyruvate dehydrogenase activity. Pyruvate metabolism in this pyruvate-dehydrogenase-negative (Pdh^-) strain was investigated in aerobic glucose-limited chemostat cultures, grown at a dilution rate of 0·10 h^{-1}, and compared with the metabolism in the isogenic wild-type strain. Under these conditions, growth of the Pdh^- strain was fully respiratory. Enzyme activities in cell-free extracts indicated that the enzymes pyruvate decarboxylase, acetaldehyde dehydrogenase and acetyl-coenzyme A (acetyl-CoA) synthetase could provide a functional bypass of the pyruvate dehydrogenase complex. Since this metabolic sequence involves ATP hydrolysis in the acetyl-CoA synthetase reaction, a negative effect of the *pda1*::Tn*5ble* mutation on the growth efficiency was anticipated. Indeed, the biomass yield of the Pdh^- strain [0·44 g biomass (g glucose)$^{-1}$] was significantly lower than that of wild-type *S. cerevisiae* [0·52 g biomass (g glucose)$^{-1}$]. The effect of the mutation on biomass yield could be quantitatively explained in terms of a lower ATP yield from glucose catabolism and an increased ATP requirement for the synthesis of acetyl-CoA used in anabolism. Control experiments showed that the *pda1*::Tn*5ble* mutation did not affect biomass yield in ethanol-limited chemostat cultures. The results support the view that, during aerobic glucose-limited growth of *S. cerevisiae* at low growth rates, the pyruvate dehydrogenase complex accounts for the major part of the pyruvate flux. Moreover, it is concluded that hydrolysis of pyrophosphate formed in the acetyl-CoA synthetase reaction does not contribute significantly to energy transduction in this yeast. Respiratory-deficient cells did not contribute to glucose metabolism in the chemostat cultures and were probably formed upon plating.**

Keywords: glucose metabolism, *Saccharomyces cerevisiae*, pyruvate dehydrogenase, *PDA1* gene, pyruvate decarboxylase

INTRODUCTION

Pyruvate is a key intermediate in sugar metabolism. In *Saccharomyces cerevisiae* (baker's yeast), pyruvate is located at a branching point between fermentative and respiratory sugar metabolism (Fig. 1). Under anaerobic and oxygen-limited growth conditions, pyruvate is converted to acetaldehyde by pyruvate decarboxylase (EC 4.1.1.1). Acetaldehyde subsequently serves as an electron acceptor to reoxidize NADH formed in glycolysis. Peculiarly, fermentative metabolism also occurs in *S. cerevisiae* under fully aerobic conditions at high rates of sugar metabolism (i.e. at high growth rates or in the presence of excess sugar). This phenomenon, called the Crabtree effect, has been attributed to an apparent limited respiratory capacity (Petrik *et al.*, 1983). In *S. cerevisiae*, completely respiratory

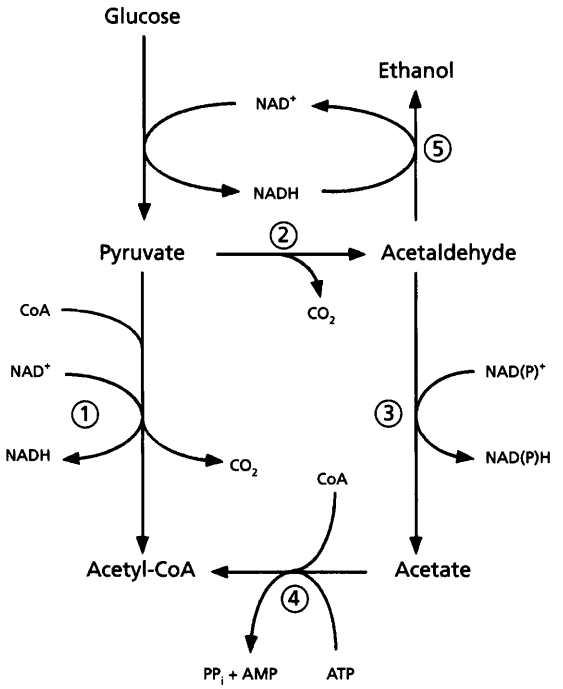

Fig. 1. Enzymes of pyruvate catabolism in *S. cerevisiae*. Numbered reactions are catalysed by the following enzymes: 1, pyruvate dehydrogenase complex; 2, pyruvate decarboxylase; 3, acetaldehyde dehydrogenase; 4, acetyl-CoA synthetase; 5, alcohol dehydrogenase.

catabolism of sugars only occurs at relatively low growth rates under sugar-limited growth conditions (Petrik *et al.*, 1983; van Urk *et al.*, 1989).

During respiratory growth of *S. cerevisiae* on sugars, pyruvate is converted to acetyl-coenzyme A (acetyl-CoA), which can subsequently be oxidized in the tricarboxylic acid cycle. Formation of acetyl-CoA can occur via two mechanisms (Holzer & Goedde, 1957; Fig. 1). Oxidative decarboxylation of pyruvate by the mitochondrial pyruvate dehydrogenase (EC 1.2.4.1) complex directly yields acetyl-CoA. Alternatively, the pyruvate dehydrogenase complex can be bypassed by the concerted action of the enzymes pyruvate decarboxylase, acetaldehyde dehydrogenase (EC 1.2.1.4 or EC 1.2.1.5) and acetyl-CoA synthetase (EC 6.2.1.1).

Isolated mitochondria as well as purified pyruvate dehydrogenase complex from *S. cerevisiae* exhibit a higher affinity for pyruvate than the cytosolic enzyme pyruvate decarboxylase (Holzer & Goedde, 1957; Kresze & Ronft, 1981; van Urk *et al.*, 1989; Postma *et al.*, 1989). In view of its higher affinity for pyruvate, it is assumed that during respiratory, glucose-limited growth of *S. cerevisiae*, pyruvate metabolism occurs predominantly via the pyruvate dehydrogenase complex (Petrik *et al.*, 1983; van Urk *et al.*, 1989). In contrast, at high sugar concentrations (e.g. in batch cultures), intracellular pyruvate concentrations will be high and pyruvate metabolism will also occur via pyruvate decarboxylase. Indeed, pyruvate-decarboxylase-negative disruption mutants of *S. cerevisiae* exhibit very low growth rates in glucose-grown batch cultures (Hohmann & Cederberg, 1990).

Quantitative determination of pyruvate dehydrogenase activities in cell-free extracts is complicated by the instability of this large multi-enzyme complex and by interference from pyruvate decarboxylase. For this reason, data on the activity of the pyruvate dehydrogenase complex in *S. cerevisiae* as a function of growth conditions are not available. An alternative approach to investigate the role of the pyruvate dehydrogenase complex in sugar metabolism is to study growth of pyruvate-dehydrogenase-negative mutants. Recently the *PDA1* gene, encoding the E1α subunit of the *S. cerevisiae* pyruvate dehydrogenase complex, has been cloned (Steensma *et al.*, 1990) and isogenic gene replacement mutants have been constructed using the dominant marker Tn*5ble*. These *pda*::Tn*5ble* mutants completely lack pyruvate dehydrogenase activity (Wenzel *et al.*, 1992).

In the present study, growth characteristics of wild-type *S. cerevisiae* during glucose-limited, respiratory growth are compared to those of an isogenic *pda1*::Tn*5ble* gene replacement mutant. The aim of the experimental work was to determine whether the enzymes pyruvate decarboxylase, acetaldehyde dehydrogenase and acetyl-CoA synthetase can provide a functional bypass for the pyruvate dehydrogenase complex during aerobic, glucose-limited growth. A model predicting the consequences of the absence of pyruvate dehydrogenase activity on growth efficiency is proposed and experimentally tested.

METHODS

Yeast strains and maintenance. *Saccharomyces cerevisiae* T23D (Wenzel *et al.*, 1992) is a homozygous diploid, homothallic wild-type strain, derived from the heterozygous strain *S. cerevisiae* CBS 8066. *S. cerevisiae* T23C (*pda1*::Tn*5ble*; Wenzel *et al.*, 1992) is isogenic to strain T23D, with the exception of the replacement of the complete *PDA1* open reading frame by a yeast expression cassette containing the prokaryotic Tn*5ble* gene (Wenzel *et al.*, 1992). This mutation confers resistance to the antibiotic phleomycin and completely abolishes pyruvate dehydrogenase activity (Wenzel *et al.*, 1992). Both strains were stored as frozen stock cultures with 20% (v/v) glycerol at −80 °C. Subcultures of these frozen stocks on YPD agar [Difco yeast extract, 2% (w/v); Difco peptone, 1% (w/v); glucose, 2% (w/v); and Difco agar, 2% (w/v)] slants were stored at 4 °C for no longer than 2 months.

Growth conditions and medium composition. Aerobic chemostat cultivation was performed at 30 °C in laboratory fermenters (Applikon), at a stirring speed of 800 r.p.m. The working volume of the cultures was kept at 1·0 litre by a peristaltic effluent pump coupled to an Applikon level controller. 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. An airflow of 1·5 l min^{-1} through the cultures was maintained using a Brooks 5876 mass flow controller. The dissolved-oxygen concentration was above 50% air saturation. The culture pH was maintained at 5·0 by addition of 2·0 M KOH, controlled by an Applikon ADI 1020 bio-processor.

The mineral medium, supplemented with vitamins, was prepared according to Verduyn *et al.* (1992). Vitamins were filter-sterilized and added after heat sterilization of the mineral medium. Glucose was sterilized separately at 110 °C. The concentration of ethanol or glucose in the reservoir medium was 5 g l^{-1}. Cultures were checked for the occurrence of oscillations (Parelukar *et al.*, 1986; Sonnleitner, 1991) by continuous registration of the dissolved-oxygen concentration and CO_2 production. All data presented refer to steady states without detectable oscillations.

Control of culture purity and homogeneity. The purity of chemostat cultures was routinely checked by phase contrast microscopy at 1000 × magnification, and by plating of culture samples on YPD agar plates. Stability of the strain carrying the *pda1*::Tn*5ble* mutation was checked by replica-plating on YPD agar plates containing 7·5 μg phleomycin ml^{-1}. To investigate if respiratory-deficient cells were present in the chemostat cultures, appropriate dilutions of culture samples were plated on YPD agar. After 48 h incubation at 30 °C, colonies were streaked on YPE [Difco yeast extract, 2% (w/v); Difco peptone, 1% (w/v); ethanol, 2% (v/v); and Difco agar, 2% (w/v)] and YPD plates. Colonies growing on YPD, but not on YPE plates were scored as respiratory deficient.

Determination of culture dry weight. Dry weights of washed culture samples were 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%.

Gas analysis and carbon recovery. Carbon recoveries were calculated from the production of biomass and CO_2. Production of CO_2 by the cultures was determined and calculated according to van Urk *et al.* (1988). The carbon content of biomass was assumed to be 45% of the dry weight (Verduyn *et al.*, 1990).

Analysis of metabolites. Organic acids in culture supernatants were determined by HPLC on an Aminex HPX-87H column (300 × 7·8 mm, Bio-Rad) at 30 °C. The column was eluted with 0·5 g H_2SO_4 l^{-1} at a flow rate of 0·6 ml min^{-1}. Detection was by means of a Waters 441 UV detector set at 214 nm, coupled to a Waters 741 data module. Peak areas were linearly proportional to concentrations. The detection limits (20 μl samples) for pyruvate and acetate were below 50 and 200 μM, respectively. Glucose in reservoir media was assayed with the Boehringer UV kit (no. 716 251); glycerol in media and cultures was assayed with Boehringer kit no. 148 270 (detection limit ca. 10 μM). Ethanol was assayed colorimetrically with an alcohol oxidase/peroxidase kit (Leeds Biochemicals; detection limit ca. 100 μM).

Protein determination. Protein concentrations in cell-free extracts were estimated by the Lowry method. The protein content of culture samples was assayed by a modified biuret method (Verduyn *et al.*, 1990). In both assays, bovine serum albumin (BSA; fatty-acid-free, Sigma) was used as a standard. BSA concentrations in standard solutions were determined with a Hitachi spectrophotometer at 280 nm, assuming an extinction coefficient of 0·661 l g^{-1} cm^{-1} (Wetlaufer, 1962).

Preparation of cell-free extracts. Samples of steady-state chemostat cultures were harvested by centrifugation, washed twice with 10 mM potassium phosphate buffer (pH 7·5) containing 2 mM EDTA, concentrated to a final concentration of 10–15 mg dry wt ml^{-1}, and stored at −20 °C in 5 ml aliquots. Before preparation of extracts, the samples were thawed at room temperature, washed, and resuspended in ice-cold 100 mM potassium phosphate buffer (pH 7·5) containing 1 mM dithiothreitol and 2 mM $MgCl_2$. Extracts were prepared by sonication with 0·1 mm diameter glass beads at 0 °C for 2 min at 0·5 min intervals, using an MSE 150 W sonicator. Unbroken cells and debris were removed by centrifugation at 75000 ***g*** (20 min at 4 °C). The clear supernatant was used as the cell-free extract.

Enzyme assays. Activity of the pyruvate dehydrogenase complex was measured as pyruvate-dependent consumption of coenzyme A by partially purified mitochondria, as described by Wenzel *et al.* (1992). Other enzymes were assayed in cell-free extracts, immediately after the extracts had been prepared. Reaction rates were proportional to the amount of extract added to the assays, which were performed at 30 °C. Pyruvate decarboxylase (EC 4.1.1.1), alcohol dehydrogenase (EC 1.1.1.1, assayed with either 100 mM ethanol or 25 mM pentanol as the substrate), NAD- and NADP-dependent acetaldehyde dehydrogenase (EC 1.2.1.5 and EC 1.2.1.4, respectively), acetyl-CoA synthetase (EC 6.2.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) were assayed according to Postma *et al.* (1989). Citrate synthase (EC 4.1.3.7) was assayed according to Srere (1969) and isocitrate lyase (EC 4.1.3.1) according to Dixon & Kornberg (1959). In all enzyme assays, 1 unit is defined as the amount of enzyme catalysing the conversion of 1 μmol substrate min^{-1}. Specific activities are given as U (mg protein)$^{-1}$.

Electron microscopy. For electron microscopy, cells were fixed and stained as described by Visser *et al.* (1990).

RESULTS

Prediction of the effect of the *pda1*::Tn*5ble* mutation on growth efficiency

Theoretically, conversion of pyruvate to acetyl-CoA in a Pdh⁻ mutant of *S. cerevisiae* can occur by the concerted action of the enzymes pyruvate decarboxylase, acetaldehyde dehydrogenase and acetyl-CoA synthetase (Holzer & Goedde, 1957; Fig. 1). If hydrolysis of pyrophosphate cannot be coupled to energy conservation, the equivalent of two molecules of ATP (when hydrolysed to ADP and inorganic phosphate) is used in the acetyl-CoA synthetase reaction. In contrast, direct oxidative decarboxylation of pyruvate to acetyl-CoA by the pyruvate dehydrogenase complex does not require ATP hydrolysis (Fig. 1). It is generally assumed that in wild-type *S. cerevisiae* grown under glucose limitation, the pyruvate dehydrogenase complex is primarily responsible for pyruvate catabolism (Petrik *et al.*, 1983; van Urk *et al.*, 1989). For a quantitative estimation of the effect of the *pda1*::Tn*5ble* mutation on growth efficiency during aerobic, glucose-limited growth, the assumption was made that in wild-type *S. cerevisiae* conversion of pyruvate to acetyl-CoA occurs solely via the pyruvate dehydrogenase complex.

The use of the pyruvate decarboxylase bypass in Pdh⁻ *S. cerevisiae* and the resulting increased ATP requirement for acetyl-CoA synthesis will cause a decrease of the net ATP yield from aerobic glucose catabolism. Furthermore, because acetyl-CoA is an important 'building block' for biosynthesis, the ATP requirement for anabolism will increase. Both factors will negatively influence the growth efficiency [g biomass (g glucose consumed)$^{-1}$].

Catabolic and anabolic fluxes of glucose and ATP in wild-type *S. cerevisiae*, grown in aerobic, glucose-limited chemostat cultures at a dilution rate of 0·10 h^{-1}, are shown in Fig. 2(a). For the formation of 100 g *S. cerevisiae*

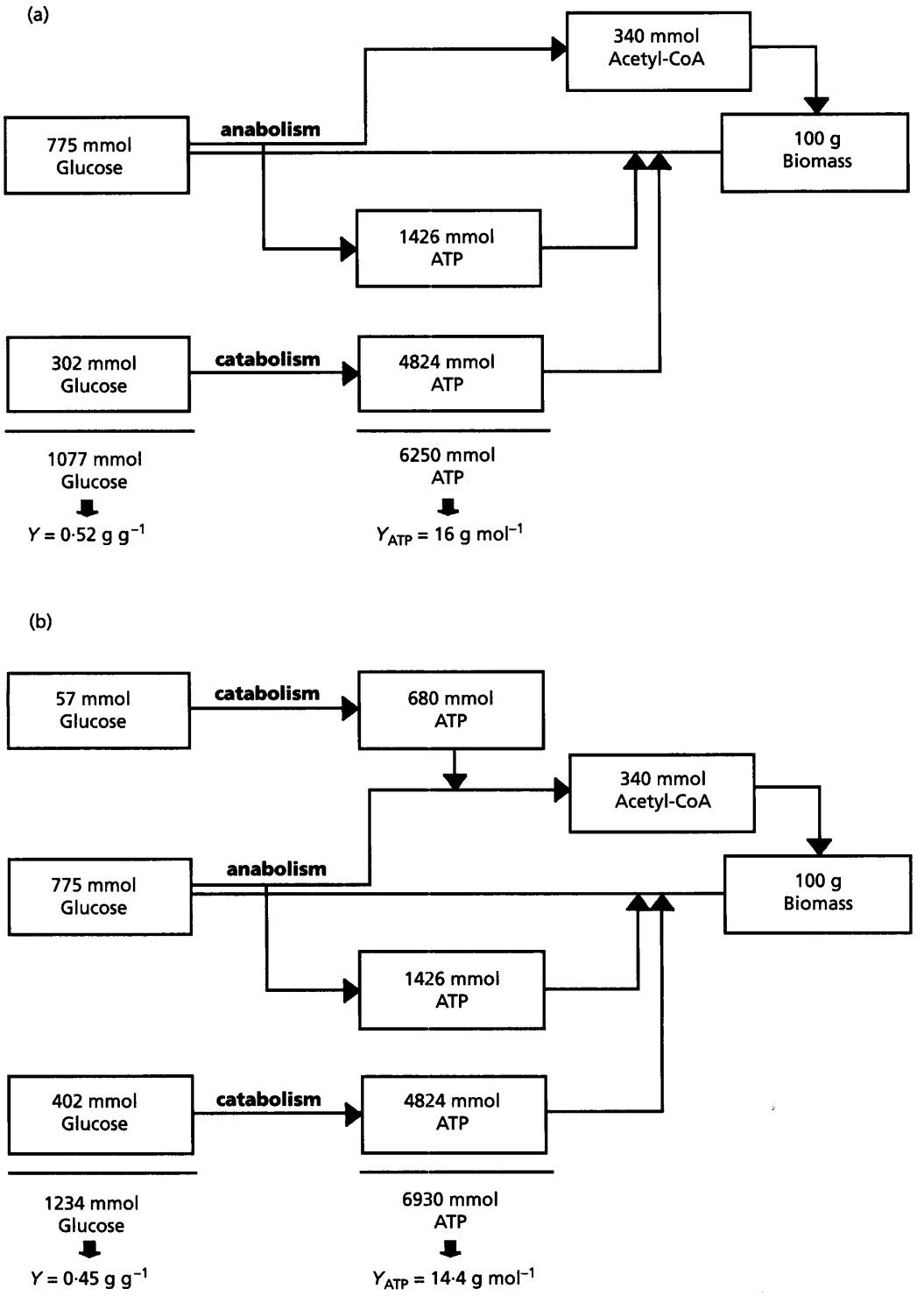

Fig. 2. Schematic representation of (a) metabolic fluxes of glucose and ATP in wild-type *S. cerevisiae*, grown aerobically in glucose-limited chemostat cultures and (b) predicted fluxes in a Pdh^- strain using the pyruvate decarboxylase bypass. It has been assumed that conversion of pyruvate to acetyl-CoA in wild-type *S. cerevisiae* occurs solely via the pyruvate dehydrogenase complex, and that the Pdh^- mutant uses the pyruvate decarboxylase bypass for this purpose. The 1426 mmol ATP which appear to be generated during anabolism are derived from the oxidation of reduced cofactors formed during biosynthetic reactions. Data on fluxes of glucose and ATP in wild-type *S. cerevisiae* are from Verduyn *et al.* (1991).

Table 1. **Estimation of acetyl-CoA requirement for synthesis of *S. cerevisiae* biomass**

The acetyl-CoA requirement for synthesis of the major biopolymers was calculated from Oura (1972). The protein content of *S. cerevisiae* strains T23D and T23C was determined experimentally (Table 2). Data on carbohydrate, nucleic acid and lipid contents of yeast biomass are from Oura (1972) and Verduyn *et al.* (1990, 1991).

Polymer	g polymer (100 g biomass)$^{-1}$	mmol acetyl-CoA (g polymer)$^{-1}$	mmol acetyl-CoA (100 g biomass)$^{-1}$
Carbohydrate	39	0	0
Protein	40	4·1	164
Nucleic acid	7	0	0
Lipid	7	25·1	176
Total...			340

biomass, 0·775 mol glucose is used in anabolism (Verduyn *et al.*, 1990). With a Y_{ATP} of 16 g biomass (mol ATP)$^{-1}$ (Verduyn *et al.*, 1990), the total ATP requirement for the formation of 100 g biomass is 6·25 mol ATP. Part of this requirement is met by oxidation of reduced cofactors formed during anabolism. The remainder is provided by complete catabolism of glucose to CO_2 (Fig. 2a). Complete respiratory catabolism of 1 mol glucose to CO_2 yields 4 mol ATP by substrate-level phosphorylation and 12 mol of reduced cofactors (NADH and FADH). Assuming an effective P/O ratio of 1 (Verduyn *et al.*, 1991), the total ATP yield from glucose catabolism will be 16 mol mol^{-1}.

In a Pdh⁻ mutant using the pyruvate decarboxylase bypass, glucose catabolism yields only 12 mol ATP (mol glucose)$^{-1}$, because the equivalent of 4 mol ATP (mol glucose)$^{-1}$ is used in the acetyl-CoA synthetase reaction. Consequently, the amount of glucose that has to be catabolized to provide ATP for biosynthesis will be 33% higher than in wild-type *S. cerevisiae* (Fig. 2b).

To calculate the expected effect of the absence of pyruvate dehydrogenase activity on the synthesis of acetyl-CoA used in anabolism, it was necessary to estimate the amount of acetyl-CoA required for biosynthesis. Based on the work of Oura (1972) it could be calculated that in the formation of 100 g *S. cerevisiae* biomass, 340 mmol acetyl-CoA are used for synthesis of fatty acids and amino acids (Table 1). In the Pdh⁻ mutant, synthesis of acetyl-CoA used in biosynthesis requires 2 mol ATP (mol acetyl-CoA)$^{-1}$. This results in an additional ATP requirement of 0·68 mol for the synthesis of 100 g biomass (Fig. 2b).

From the model presented in Fig. 2 (a, b), it can be predicted that in the Pdh⁻ mutant, redirection of pyruvate metabolism via the pyruvate decarboxylase bypass will lead to a significant decrease of the growth efficiency. This should be reflected both in a reduction of the biomass yield on glucose and in a decrease of Y_{ATP}. It is not possible to directly measure Y_{ATP} in aerobic cultures. However, the magnitude of the predicted decrease of the biomass yield [0·52 g biomass (g glucose)$^{-1}$ in wild-type *S. cerevisiae* versus 0·45 g g^{-1} in a Pdh⁻ strain] made it possible to verify the model by measuring growth yields in glucose-limited chemostat cultures.

Growth in ethanol- and glucose-limited chemostat cultures

The pyruvate dehydrogenase complex is not known to be involved in ethanol metabolism. Therefore, ethanol-limited chemostat cultures of wild-type and Pdh⁻ *S. cerevisiae* were used as a control for possible pleiotropic effects of the *pda1*::Tn*5ble* mutation. With both strains, substrate carbon was quantitatively recovered as biomass and CO_2 (data not shown). Neither the growth yield on ethanol nor the protein content of the biomass differed significantly between the two strains (Table 2). Furthermore, no significant changes were observed in the activities of a number of key enzyme activities in cell-free extracts (Table 3). Even after continuous cultivation for over 50 generations, all cells of the Pdh⁻ strain retained the phleomycin resistance marker (data not shown).

In glucose-limited chemostat cultures of wild-type and Pdh⁻ *S. cerevisiae*, substrate carbon could be quantitatively recovered as CO_2 and biomass also (carbon recoveries in all chemostat cultures were between 97% and 103%). No detectable amounts of ethanol, glycerol or acetate were found in the culture supernatants by HPLC analysis. Low concentrations (< 200 μM) of a number of organic acids (mainly TCA cycle intermediates) were detected in cultures of both wild-type and Pdh⁻ cultures. Apparently, the absence of pyruvate dehydrogenase activity did not result in aerobic ethanol formation or excretion of other metabolites. Also with the glucose-limited chemostat cultures, no loss of phleomycin resistance was observed in the strain carrying the *pda1*::Tn*5ble* mutation.

The biomass yield of the homozygous diploid wild-type strain *S. cerevisiae* T23D on glucose was identical to the growth yield of the heterozygous parent strain *S. cerevisiae* CBS 8066 (Verduyn *et al.*, 1991). The protein contents of the wild-type and Pdh⁻ strain were not significantly different (Table 2). However, the biomass yield of the Pdh⁻ strain was significantly lower (15·4%) than that of the wild-type strain (Table 2). It has been reported that in batch cultures grown on glucose, the *pda1*::Tn*5ble* mutation results in a partial leucine requirement (Wenzel *et al.*, 1992). Addition of leucine (0·2 g l^{-1}) to the reservoir medium of glucose-limited chemostat cultures of the Pdh⁻ strain did not significantly affect the biomass yield (data not shown). Quantitatively, the observed difference between the biomass yields of the wild-type and Pdh⁻ strains was in good agreement with the model presented in Fig. 2.

Enzymology of glucose and pyruvate metabolism

To establish if the *pda1*::Tn*5ble* mutation, which has previously been shown to cause a complete loss of pyruvate dehydrogenase activity in batch cultures (Wenzel *et al.*, 1992), indeed led to the absence of pyruvate

Table 2. **Growth yields and protein contents of *S. cerevisiae* T23D (wild-type) and *S. cerevisiae* T23C (*pda1*::Tn*5ble*) in ethanol- and glucose-limited chemostat cultures**

Growth conditions: $D = 0{\cdot}10\ h^{-1}$, pH 5·0, $T = 30\ °C$, $S_R = 5\ g\ l^{-1}$. Data are represented as means ± SD of samples from at least three independent steady-state chemostat cultures.

Growth substrate	*S. cerevisiae* T23D (wild-type)		*S. cerevisiae* T23C (*pda1*::Tn*5ble*)	
	Biomass yield (g dry wt g^{-1})	Protein content (%)	Biomass yield (g dry wt g^{-1})	Protein content (%)
Ethanol	0·59 ± 0·02	40·8 ± 1·2	0·59 ± 0·01	41·4 ± 1·8
Glucose	0·52 ± 0·01	40·4 ± 2·3	0·44 ± 0·01	39·6 ± 2·2

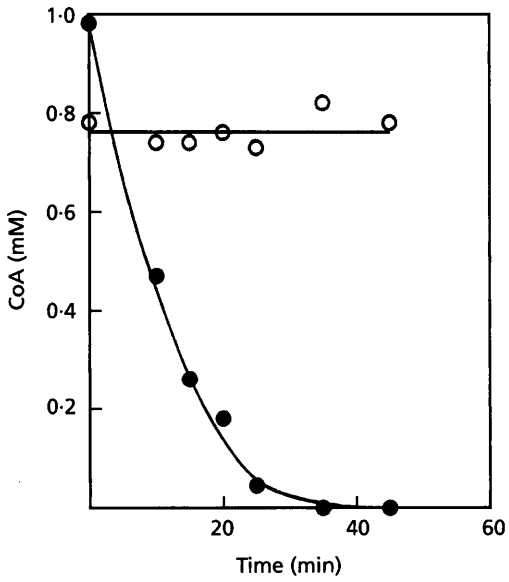

Fig. 3. Pyruvate-dependent consumption of coenzyme A by crude mitochondrial preparations of wild-type and Pdh⁻ *S. cerevisiae*, grown in aerobic, glucose-limited chemostat cultures ($D = 0{\cdot}1\ h^{-1}$, pH 5, $T = 30\ °C$). Symbols: ●, *S. cerevisiae* T23D (wild-type); ○, *S. cerevisiae* T23C (*pda1*::Tn*5ble*). The protein concentration in the assays was 1·3 mg ml^{-1}. The figure shows data from a representative experiment. Absence of activity in extracts of strain T23C was confirmed in five independent experiments.

dehydrogenase activity in glucose-limited chemostat cultures, consumption of CoA by crude mitochondrial preparations was studied. Indeed, pyruvate-dependent CoA consumption was demonstrated with extracts from wild-type cells grown in glucose-limited chemostat cultures, but not with extracts prepared from *S. cerevisiae* T23C (Fig. 3). This confirmed that, also during growth under substrate-limited conditions, the *pda1*::Tn*5ble* mutation led to a complete loss of pyruvate dehydrogenase activity.

Activities of the 'bypass' enzymes pyruvate decarboxylase, acetaldehyde dehydrogenase and acetyl-CoA synthetase in cell-free extracts prepared from glucose-limited chemostat cultures of the Pdh⁻ strain were similar to those observed in cultures of the wild-type strain (Table 3). The specific glucose consumption rate in chemostat cultures of the Pdh⁻ strain (equal to the dilution rate divided by the molar growth yield on glucose; see Table 2) was 21 nmol glucose min^{-1} (mg dry wt)$^{-1}$ or 63 nmol min^{-1} (mg protein)$^{-1}$ (assuming a soluble protein content of 33%; Postma *et al.*, 1989). The activities of the bypass enzymes measured in cell-free extracts were all higher than 100 nmol min^{-1} (mg protein)$^{-1}$ (Table 3) and hence sufficient to account for the *in vivo* substrate consumption rates.

Table 3. **Enzyme activities in cell-free extracts of *S. cerevisiae* T23D (wild-type) and *S. cerevisiae* T23C (*pda1*::Tn*5ble*), grown in aerobic ethanol- and glucose-limited chemostat cultures**

Growth conditions: $D = 0{\cdot}10\ h^{-1}$, pH 5·0, $T = 30\ °C$, $S_R = 5\ g\ l^{-1}$. Enzyme activities are presented as means ± SD of extracts prepared from at least three independent steady-state chemostat cultures.

Enzyme activity [μmol min^{-1} (mg protein)$^{-1}$]	T23D (wild-type) Ethanol	T23C (*pda1*::Tn*5ble*) Ethanol	T23D (wild-type) Glucose	T23C (*pda1*::Tn*5ble*) Glucose
Glucose-6-phosphate dehydrogenase	0·24 ± 0·01	0·23 ± 0·06	0·41 ± 0·14	0·46 ± 0·11
Hexokinase	0·63 ± 0·09	0·73 ± 0·12	1·50 ± 0·30	1·74 ± 0·17
Citrate synthase	0·88 ± 0·07	0·79 ± 0·09	0·55 ± 0·17	0·85 ± 0·09
Pyruvate decarboxylase	0·75 ± 0·33	0·47 ± 0·09	0·67 ± 0·05	0·78 ± 0·05
Acetyl-CoA synthetase	0·90 ± 0·21	0·50 ± 0·20	0·13 ± 0·03	0·11 ± 0·04
Isocitrate lyase	0·28 ± 0·07	0·30 ± 0·04	0·02 ± 0·01	< 0·003
Alcohol dehydrogenase (ethanol)	10·7 ± 2·2	13·0 ± 2·0	10·1 ± 0·5	9·84 ± 0·75
Alcohol dehydrogenase (pentanol)	7·32 ± 0·91	7·63 ± 1·84	5·65 ± 0·48	3·95 ± 0·23
Acetaldehyde dehydrogenase NAD-dependent	0·76 ± 0·11	0·67 ± 0·08	0·89 ± 0·07	1·31 ± 0·11
Acetaldehyde dehydrogenase NADP-dependent	0·27 ± 0·02	0·25 ± 0·04	0·31 ± 0·03	0·42 ± 0·06

Table 4. **Consumption and production of glycerol in aerobic, glucose-limited chemostat cultures of *S. cerevisiae* T23D (wild-type), *S. cerevisiae* T23C (*pda1*::Tn*5ble*) and *S. cerevisiae* T23C-RD (*pda1*::Tn*5ble*; spontaneous respiratory-deficient mutant)**

Growth conditions: $D = 0{\cdot}10\ h^{-1}$, $T = 30\ °C$, pH 5, $S_R = 5{\cdot}0$ g glucose l^{-1}.

Strain	Glycerol in medium (mM)	Glycerol in culture (mM)	Glycerol consumed (mM)
T23D	< 0·01	< 0·01	< 0·01
T23C	< 0·01	< 0·01	< 0·01
T23D	5·18	5·01	0·17
T23C	4·60	4·51	0·09
T23C-RD	< 0·01	5·20	−5·20

The lower growth yield of the Pdh⁻ strain results in an 18% increase of the specific glucose consumption rate, as compared to wild-type *S. cerevisiae*. The average activities of hexokinase and citrate synthase appeared to be higher in the Pdh⁻ strain than in wild-type *S. cerevisiae* (Table 3). However, the experimental variation in the enzyme activities was too large to decide if the increase of the catabolic fluxes in the Pdh⁻ strain is achieved by increased enzyme levels, increased metabolite concentrations, or a combination of these two parameters.

Absence of respiratory-deficient mutants in chemostat cultures

During growth of Pdh⁻ strains on glucose in batch cultures, a high incidence of respiratory-deficient ('petite') cells was observed (Wenzel *et al.*, 1992). When samples of steady-state, glucose-limited chemostat cultures of the Pdh⁻ mutant were plated on YPD agar, 20–30% of the colonies exhibited a typical 'petite' phenotype: colonies were small and, upon replica-plating, were unable to grow on YPE plates. This phenomenon was not observed with wild-type *S. cerevisiae* T23D or with ethanol-limited chemostat cultures of the Pdh⁻ strain. This observation suggested that respiratory-deficient cells were present in the glucose-limited chemostat cultures of the Pdh⁻ strain. Attempts to directly compare numbers of colonies on glucose plates with those on ethanol or acetate plates were not successful, because with wild-type *S. cerevisiae* also, the numbers of colonies on the latter plates were poorly reproducible and consistently lower than on glucose plates (data not shown).

If respiratory-deficient cells contributed to glucose metabolism in chemostat cultures of the Pdh⁻ strain, this would invalidate the model presented in Fig. 2, since the occurrence of alcoholic fermentation in the cultures would also result in a decrease of the growth efficiency. Therefore, the reliability of the replica-plating method in determining the presence of respiratory-deficient cells in the chemostat cultures was investigated by using different methods.

It has been reported that certain fluorescent dyes can be used to detect a mitochondrial membrane potential and, consequently, to discriminate between respiratory-competent and respiratory-deficient cells (Pringle *et al.*, 1989; Skowronek *et al.*, 1990). Attempts to detect respiratory-deficient cells by fluorescence microscopy, using the fluorescent dyes Rhodamine 123, DASPMI [2-(4-dimethyl-aminostyryl)-*N*-methylpyridinium iodide] and $DIOC_6$ (dihexyloxocarbocyanine iodide) were not successful (data not shown). Even with cultures of wild-type *S. cerevisiae*, large variations were observed in fluorescence among individual cells. With all three dyes, significant fluorescence of organelles was also observed with pure cultures of a Pdh⁻ respiratory-deficient strain. This observation suggests that in respiratory-deficient cells the mitochondria retain a membrane potential. Since direct quantification of the frequency of respiratory-deficient cells in the cultures by fluorescence microscopy was not successful, the presence of fermentation products in the cultures was studied.

A representative respiratory-deficient Pdh⁻ colony (strain T23C-RD) was isolated from a YPD plate. In aerobic, glucose-limited chemostat cultures, the growth yield of this strain [0·11 g (g glucose)$^{-1}$] was comparable to that of wild-type *S. cerevisiae* grown in anaerobic, glucose-limited chemostat cultures (Verduyn *et al.*, 1990). Consistent with this, ethanol and glycerol yields [0·36 g (g glucose)$^{-1}$ and 0·09 g (g glucose)$^{-1}$, respectively] also were equal to those in anaerobic cultures of wild-type *S. cerevisiae* (Verduyn *et al.*, 1990). No ethanol was detectable in culture supernatants of glucose-limited chemostat cultures of *S. cerevisiae* T23C (*pda1*::Tn*5ble*) but this might be explained by (co-)metabolism of ethanol by the respiratory-competent part of the population. However, the biomass yield of the respiratory-deficient cells in pure cultures [0·11 g (g glucose)$^{-1}$] corresponded to 25% of the growth yield of the Pdh⁻ strain in glucose-limited chemostat cultures (Table 2). If it is assumed that the frequency of respiratory-deficient colonies on plates (20–30%) accurately reflected the presence of respiratory-deficient cells in the chemostat cultures, this would imply that virtually all glucose supplied to the cultures was initially consumed by respiratory-deficient cells. The respiratory-competent population would then be restricted to the use of ethanol and/or glycerol as carbon sources. Consequently, this should lead to induction of enzymes involved in ethanol metabolism, such as, for instance, isocitrate lyase. This key enzyme of the glyoxylate cycle is induced during growth of wild-type and Pdh⁻ *S. cerevisiae* on ethanol (Table 3). However, no isocitrate lyase activity could be detected in cell-free extracts of glucose-limited cultures of the Pdh⁻ strain (Table 3), indicating that ethanol is not a major substrate for growth in these cultures. Clearly, this observation is not compatible with the presence of 20–30% actively growing respiratory-deficient cells in the cultures, as suggested by the plate counts.

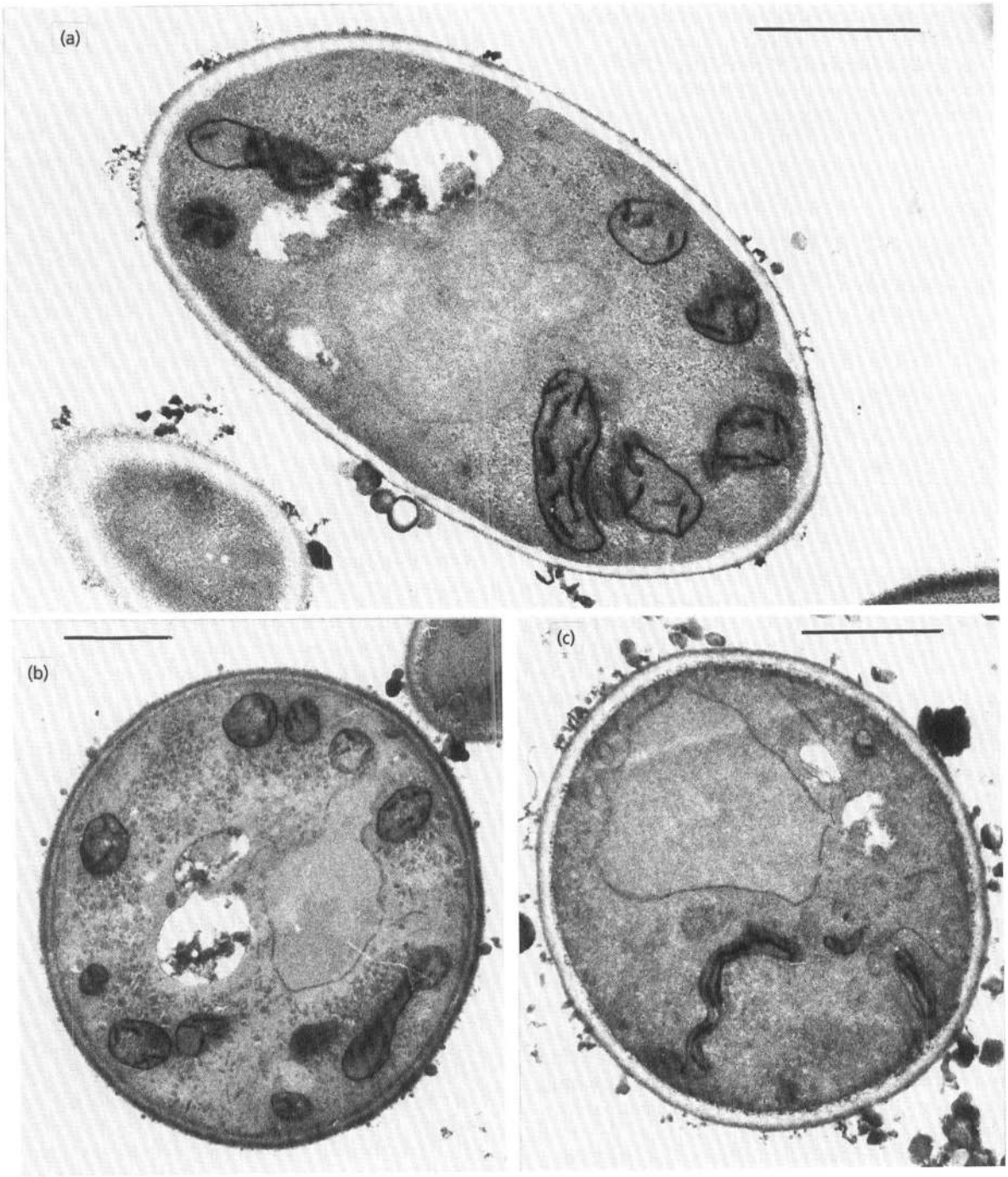

Fig. 4. Electron micrographs of thin sections of cells from glucose-limited chemostat cultures of (a) *S. cerevisiae* T23D (wild-type); (b) *S. cerevisiae* T23C (*pda1*::Tn*5ble*); (c) *S. cerevisiae* T23C-RD (*pda1*::Tn*5ble*; spontaneous respiratory-deficient mutant). The marker bars correspond to 1 μm.

No glycerol was detected in glucose-limited chemostat cultures of wild-type and Pdh$^-$ *S. cerevisiae*. Neither of these strains can use glycerol as a sole source of carbon and energy for growth (data not shown). However, it could not be excluded that small amounts of glycerol produced by respiratory-deficient cells might have been co-metabolized. Therefore, co-metabolism of glycerol was studied by adding low concentrations of glycerol to the reservoir medium of glucose-limited chemostat cultures of both wild-type and Pdh$^-$ *S. cerevisiae*. In all cases, glycerol added to the growth medium could be almost quantitatively recovered from the cultures (Table 4). Apparently, co-metabolism of glycerol does not occur to any significant extent under these growth conditions. The absence of glycerol in glucose-limited chemostat cultures of the Pdh$^-$ strain can therefore be interpreted as

additional proof that respiratory-deficient cells did not contribute significantly to glucose metabolism in these cultures.

Electron microscopy of thin sections of respiratory-deficient cells grown in aerobic, glucose-limited chemostat cultures revealed atypical mitochondrial structures (Fig. 4). In contrast, cells of wild-type and Pdh⁻ *S. cerevisiae* exhibited a normal mitochondrial morphology with well-defined cristae (Fig. 4). No atypical mitochondrial structures were observed in over 250 sections of cells from glucose-limited chemostat cultures of Pdh⁻ *S. cerevisiae*. This observation also indicated that respiratory-deficient cells were not present in the chemostat cultures, but were formed upon plating of Pdh⁻ cells.

DISCUSSION

Phenotypic characteristics of *S. cerevisiae* T23C

Inactivation of the *PDA1* gene results in a dramatic increase of the number of respiratory-deficient colonies after plating of samples from glucose-limited cultures onto YPD agar plates. This phenomenon also occurs with batch cultures of *S. cerevisiae* T23C and other strains in which the *PDA1* gene is disrupted (Wenzel *et al.*, 1992). For the interpretation of our experimental data, it was essential to assess whether or not respiratory-deficient cells contributed to glucose metabolism in the glucose-limited chemostat cultures. Both the absence of glycerol in culture supernatants (Table 4) and the absence of isocitrate lyase activity in cell-free extracts of glucose-limited chemostat cultures of Pdh⁻ *S. cerevisiae* (Table 3) indicate that, if the cultures contain any respiratory-deficient cells, these do not significantly contribute to glucose metabolism. This means that, if present at all in the cultures, the impact of respiratory-deficient cells on metabolic fluxes is comparable with that of dead cells, i.e. their main effect will be an increased growth rate of the remainder of the population. Since the biomass composition of the Pdh⁻ strain was not significantly different from that of wild-type *S. cerevisiae* (Table 2), such an increase in the specific growth rate would not affect the stoichiometric calculations leading to the model presented in Fig. 2.

If the plate counts reflect the presence of respiratory-deficient cells, non-growing in the chemostat cultures, this would imply a very high mutation rate (approximately 0·25 per generation). The absence of cells with an altered mitochondrial morphology in glucose-limited cultures of Pdh⁻ *S. cerevisiae* (Fig. 4) suggests that respiratory-deficient cells were not present in these cultures. Our current hypothesis is that cells from glucose-limited chemostat cultures of the Pdh⁻ strain contain high activities of a mitochondrial pyruvate transport system, but are unable to metabolize intramitochondrial pyruvate. Transfer to a situation of glucose excess (e.g. on glucose-containing agar plates) could lead to a rapid influx and accumulation of pyruvate into the mitochondria. If accumulation of pyruvate in the matrix impairs mitochondrial functions, including replication of mitochondrial DNA, this may explain the observed irreversible loss of respiratory activity.

Energetic implications of the *pda1*::Tn*5ble* mutation

The experimental data presented in this paper indicate that the enzymes pyruvate decarboxylase, acetaldehyde dehydrogenase and acetyl-CoA synthetase can provide a functional bypass of the pyruvate dehydrogenase complex. The observation that this bypass allows a fully respiratory metabolism demonstrates that redirection of glucose metabolism via pyruvate decarboxylase is not sufficient to trigger alcoholic fermentation. Apparently, the NADH/NAD ratio during aerobic glucose-limited growth is sufficiently low to allow acetaldehyde dehydrogenase to compete effectively with alcohol dehydrogenase. Also, the much higher affinity of acetaldehyde dehydrogenase for acetaldehyde as compared with that of alcohol dehydrogenase (Postma *et al.*, 1989) will favour oxidative metabolism of acetaldehyde.

Quantitatively, the decrease of the biomass yield on glucose caused by the *pda1*::Tn*5ble* mutation is in good agreement with the model outlined in Fig. 2. This model was based on a number of assumptions.

(1) In wild-type *S. cerevisiae* grown in aerobic, glucose-limited chemostat cultures, pyruvate catabolism occurs exclusively via the pyruvate dehydrogenase complex. However, the possibility cannot be excluded that in wild-type *S. cerevisiae* also, a fraction of the pyruvate flux is channelled through the pyruvate decarboxylase bypass. This should result in a smaller effect of the *pda1*::Tn*5ble* mutation on biomass yields.

(2) Conversion of ATP to AMP and pyrophosphate in the acetyl-CoA synthetase reaction is equivalent to the hydrolysis of two molecules of ATP to ADP and phosphate. This assumption would not hold if the chemical energy present in pyrophosphate can be conserved. In addition to soluble cytoplasmic pyrophosphatase, the presence of a vacuolar proton-translocating pyrophosphatase has been reported in *S. cerevisiae* (Lichko & Okorokov, 1991). Furthermore, it has been suggested that a yeast mitochondrial membrane-bound pyrophosphatase is proton-translocating and may therefore be energy-transducing (Lundin *et al.*, 1992). The relative contribution of these enzymes to the overall rate of pyrophosphate hydrolysis in yeast is unknown.

(3) The effective P/O ratio during glucose-limited growth of *S. cerevisiae* is near unity, as calculated by Verduyn *et al.* (1991). When, in fact, the effective P/O ratio is higher than one, the relative effect of additional ATP expenditure in the pyruvate decarboxylase bypass would be smaller than indicated by Fig. 2.

If either of the assumptions 1 or 2 are incorrect, the effect of the *pda1*::Tn*5ble* mutation on biomass yield would be smaller than observed. Hence, the observed effect of the *pda1*::Tn*5ble* mutation on biomass yield confirms that during respiratory, glucose-limited growth of *S. cerevisiae*, the pyruvate dehydrogenase complex accounts for the

major part of the pyruvate flux, as has previously been proposed on the basis of kinetic data (Petrik *et al.*, 1983; van Urk *et al.*, 1989). Furthermore, the observed biomass yield of Pdh$^-$ *S. cerevisiae* suggests that hydrolysis of pyrophosphate formed in the acetyl-CoA synthetase reaction does not contribute significantly to energy transduction. The decrease of the biomass yield in the Pdh$^-$ strain can be interpreted as further evidence for a rather low efficiency of oxidative phosphorylation (Verduyn *et al.*, 1991) in *S. cerevisiae* under the experimental conditions.

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