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Overproduction of Threonine Aldolase Circumvents the Biosynthetic Role of Pyruvate Decarboxylase in Glucose-Limited Chemostat Cultures of *Saccharomyces cerevisiae*

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Pyruvate decarboxylase-negative (Pdc$^-$) mutants of *Saccharomyces cerevisiae* require small amounts of ethanol or acetate to sustain aerobic, glucose-limited growth. This nutritional requirement has been proposed to originate from (i) a need for cytosolic acetyl coenzyme A (acetyl-CoA) for lipid and lysine biosynthesis and (ii) an inability to export mitochondrial acetyl-CoA to the cytosol. To test this hypothesis and to eliminate the C_2 requirement of Pdc$^-$ *S. cerevisiae*, we attempted to introduce an alternative pathway for the synthesis of cytosolic acetyl-CoA. The addition of L-carnitine to growth media did not restore growth of a Pdc$^-$ strain on glucose, indicating that the C_2 requirement was not solely due to the inability of *S. cerevisiae* to synthesize this compound. The *S. cerevisiae GLY1* gene encodes threonine aldolase (EC 4.1.2.5), which catalyzes the cleavage of threonine to glycine and acetaldehyde. Overexpression of *GLY1* enabled a Pdc$^-$ strain to grow under conditions of carbon limitation in chemostat cultures on glucose as the sole carbon source, indicating that acetaldehyde formed by threonine aldolase served as a precursor for the synthesis of cytosolic acetyl-CoA. Fractionation studies revealed a cytosolic localization of threonine aldolase. The absence of glycine in these cultures indicates that all glycine produced by threonine aldolase was either dissimilated or assimilated. These results confirm the involvement of pyruvate decarboxylase in cytosolic acetyl-CoA synthesis. The Pdc$^-$ *GLY1* overexpressing strain was still glucose sensitive with respect to growth in batch cultivations. Like any other Pdc$^-$ strain, it failed to grow on excess glucose in batch cultures and excreted pyruvate when transferred from glucose limitation to glucose excess.

Pyruvate decarboxylase (PDC), encoded by *PDC1*, *PDC5*, and *PDC6*, catalyzes the first, irreversible step of alcoholic fermentation by yeasts and has therefore long been considered a strictly catabolic enzyme. Consistent with this notion, PDC-negative (Pdc$^-$) mutants of *Saccharomyces cerevisiae* exhibit a drastically reduced specific growth rate in batch cultures on complex medium with glucose, in which glucose metabolism of wild-type *S. cerevisiae* is predominantly fermentative (35).

At a low residual glucose concentration in aerobic glucose-limited chemostat cultures, the glucose repression of respiratory enzymes is alleviated (4, 37). When grown at low specific growth rates in such cultures, wild-type cells dissimilate glucose exclusively via respiration (4, 37). Nevertheless, under these conditions, Pdc$^-$ *S. cerevisiae* strains were unable to grow on glucose as the sole carbon source, but growth could be restored by addition of small amounts of ethanol or acetate to the medium (10, 12). The C_2 compound requirement of Pdc$^-$ *S. cerevisiae* has been proposed to reflect an essential role of PDC in the synthesis of cytosolic acetyl coenzyme A (acetyl-CoA), which is required for the synthesis of lipids and lysine (10). PDC catalyzes the first reaction of a pathway for the cytosolic conversion of pyruvate into acetyl-CoA, which also involves acetaldehyde dehydrogenase and acetyl-CoA synthetase (17, 31, 32). Consistent with this proposed biosynthetic role of PDC, the experimentally determined minimum requirement of Pdc$^-$ mutants for C_2 compounds matched the theoretical demand for cytosolic acetyl-CoA (10).

An essential role of PDC in the synthesis of cytosolic acetyl-CoA is to some extent surprising, since several yeast species are known to grow rapidly on glucose in the absence of PDC. For example, Pdc$^-$ strains of *Kluyveromyces lactis* grow rapidly on glucose as the sole carbon source (2, 14). Furthermore, the lipid-accumulating yeast *Yarrowia lipolytica*, which lacks PDC, uses ATP-citrate lyase for the export of acetyl-CoA units to the cytosol from the mitochondrial matrix, where acetyl-CoA is formed by the pyruvate-dehydrogenase complex (8). ATP-citrate lyase does not occur in *S. cerevisiae*, thus precluding the involvement of this enzyme in cytosolic acetyl-CoA synthesis (33).

It is generally assumed that in eukaryotic cells, including *S. cerevisiae* (19, 31), the carnitine shuttle plays a key role in the transport of acetyl-CoA across the mitochondrial inner membrane. Although *S. cerevisiae* contains the genetic information encoding carnitine transferases and acetyl-carnitine translocase (1, 7, 27, 36, 39), it has recently emerged that *S. cerevisiae* is unable to synthesize L-carnitine (39). Since it has not been investigated whether L-carnitine supplementation enables growth of Pdc$^-$ strains of *S. cerevisiae* on glucose, it remains unclear whether the carnitine shuttle can catalyze the export of acetyl-CoA from the mitochondrial matrix.

The C_2 requirement of Pdc$^-$ *S. cerevisiae* is not only of

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TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype
CEN.PK182	*MAT***a** *pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP*
CEN.PK111-61A	*MAT*α *ura3-52 leu2-112 his3-Δ1*
RWB882	*MAT***a** *pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP leu2-112 his3-Δ1*
RWB893	*MAT***a** *pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP leu2-112 his3::*pYX022-Aat

fundamental scientific interest. The absence of alcoholic fermentation in Pdc$^-$ strains may be beneficial in biomass-directed applications. Another demonstrated application of such strains is the introduction of lactate dehydrogenase to utilize the glycolytic NADH to produce lactic acid (29), a chemical with commercial value. In all of these applications, elimination of the C_2 compound requirement would facilitate process design.

The aims of the present study were to verify the hypothesis that PDC is essential for cytosolic acetyl-CoA synthesis in glucose-grown *S. cerevisiae*, to investigate a possible role of the carnitine shuttle in the export of acetyl-CoA from the mitochondrial matrix, and to eliminate the C_2 requirement of Pdc$^-$ *S. cerevisiae* via metabolic engineering. The latter goal was pursued by overexpressing the *GLY1* gene encoding threonine aldolase, which catalyzes the cleavage of threonine to glycine and acetaldehyde (21, 25), in a Pdc$^-$ strain.

MATERIALS AND METHODS

Strains and maintenance. The *S. cerevisiae* strains used and constructed in the present study (Table 1) are congenic members of the CEN.PK family (38). Stock cultures were grown at 30°C in shake flasks containing 100 ml of synthetic medium with 20 g of glucose liter^{-1}. When the stationary phase was reached, 20% (vol/vol) glycerol was added and 2-ml aliquots were stored at −80°C.

Plasmid construction. *GLY1* was cloned behind the constitutive *TPI1* promoter on the 2μ-based expression vector YEplac181 (15). To this end, the *GLY1* open reading frame was isolated by performing a PCR amplification on chromosomal DNA from *S. cerevisiae* M5 (34) with the following oligonucleotide primers: 5′-GGAATTCTAGAATGACTGAGTTCGAATTGCCTCCAAAATATAC-3′ and 5′-CCGCTCGAGACATGATGCAACTGGAACGC-3′.

The PCR mix was separated on an agarose gel, after which the desired fragment was isolated and digested with *Eco*RI and *Xho*I. The resulting fragment was ligated into pYX042-AatII, which was digested with *Eco*RI and *Xho*I. This pYX042-AatII plasmid is derived from pYX042 (R&D Systems, Minneapolis, Minn.) by digesting it with *Aat*II and inserting a linker which destroys the *Aat*II site and introduces four other restriction sites: *Xho*I, *Bam*HI, *Sma*I, and *Nhe*I. The resulting plasmid, pRWGLY1, was then digested with *Nhe*I and *Sac*I. The P_{TPI}-*GLY1* fragment thus obtained was ligated to YEplac181, which was cut with *Xba*I and *Sac*I. The result of this procedure was YEpGLY1.

Strain construction. RWB882 was derived from a cross between CEN.PK182 and CEN.PK111-61A (both provided by P. Kötter, Frankfurt, Germany). The resulting diploid was sporulated, and the spore mixture was heated for 15 min to 56°C. Subsequently, the mixture was plated on YP medium with 0.2% sodium acetate as the carbon source. The resulting colonies were checked for growth on glucose. Colonies that could not grow on glucose were tested by PCR for the presence of a disrupted *PDC6* gene. Subsequent selection on synthetic medium for the presence of the desired auxotrophic marker(s), in this case *leu2-112*, resulted in RWB882. To eliminate the histidine auxotrophy, RWB882 was first transformed with pYX022-Aat to give RWB893. Transformation of this strain with the plasmids YEpGLY1 and YEplac181 (15) resulted in the *GLY1*-overexpressing strain RWB893(YEpGLY1) and the corresponding empty-vector strain RWB893(YEplac181), respectively.

PCR. PCR was performed with *Vent* DNA polymerase (New England Biolabs) according to the manufacturer's specifications. The PCR was performed as follows: 30 cycles of denaturation for 1 min at 94°C, followed by annealing for 1 min at 65°C, followed by an extension period of 3.5 min at 75°C.

Media. The synthetic medium for chemostat cultivation contained per liter of demineralized water 5 g of $(NH_4)_2SO_4$, 3 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.05 ml of silicon antifoam (BDH), and trace element concentrations according to Verduyn et al. (41). After heat sterilization of the medium for 20 min at 120°C, a filter-sterilized vitamin solution, prepared as described by Verduyn et al. (41), was added. The concentration of substrate carbon in the reservoir medium was always 250 mM. The carbon substrate consisted either of a mixture of glucose (6.75 g liter^{-1}) and acetate (0.75 g liter^{-1}) or of glucose alone (7.5 g liter^{-1}) as the sole carbon source. Glucose was added separately after heat sterilization at 110°C. Pure acetic acid was added to the autoclaved medium without prior sterilization. Synthetic media for batch cultivation and precultures contained 1.5% ethanol as the sole carbon source and were otherwise identical to the chemostat media. In cultures supplemented with L-carnitine, its final concentration was 0.4 g liter^{-1}.

Chemostat cultivation. Aerobic chemostat cultivation was performed at 30°C in 2-liter fermentors (Applikon, Schiedam, The Netherlands) with a working volume of 1 liter. The pH was controlled at 5.0 via automated addition of 2 M KOH (Applikon ADI 1030 biocontroller). A stirrer speed of 800 rpm and an airflow of 0.5 liter min^{-1} were applied to keep the dissolved-oxygen concentration higher than 60% of air saturation, as measured with an oxygen electrode, in all chemostat cultivations performed. The addition of medium was regulated by a peristaltic pump. The working volume of the cultures was kept constant by means of an electric level sensor. Cultures were assumed to be in steady state when, after at least five volume changes, the culture dry weight, glucose concentration, carbon dioxide production rate and oxygen consumption rate, changed by <2% during one volume change. Sustained oscillations of the dissolved-oxygen concentration (20) were not observed. There was no significant difference (<1%) between the biomass concentrations in effluent and in samples taken directly from the cultures.

Glucose-pulse experiments. Glucose-pulse experiments were performed by adding glucose to steady-state glucose-limited chemostat cultures. Just before the start of the pulse experiment, the medium pump was switched off. To achieve a 50 mM glucose pulse, 18 ml of a 50% (wt/vol) glucose solution was injected aseptically through a rubber septum. During glucose consumption and the subsequent consumption of metabolites, the optical densities of the culture samples at 660 nm and the concentrations of glucose and metabolites in the supernatant samples were determined at appropriate intervals.

Determination of culture dry weight. To determine the biomass dry weight, a known culture volume containing 0.01 to 0.03 g (dry weight) was filtrated over predried nitrocellulose filters of known weight (pore size, 0.45 mm; Gelman Sciences). The filters were washed with 20 ml of demineralized water and dried for 20 min in a microwave oven at 360 W, and the increase in the filter weight was measured. Duplicate samples varied by <1%.

Metabolite analysis. Acetate, glucose, glycerol, and pyruvate concentrations in supernatants were determined by HPLC analysis with a Bio-Rad Aminex HPX-87H column at 60°C. The column was eluted with 5 mM sulfuric acid at a flow rate of 0.6 ml min^{-1}. Pyruvate and acetate were detected by a Waters 2487 dual-wavelength absorbance detector at 214 nm. Glucose and glycerol were detected by a Waters 2410 refractive index detector. Glucose concentrations were confirmed enzymically with a commercial Roche diagnostics kit (no. 716251).

Gas analysis. The exhaust gas of chemostat cultures was cooled and dried with a Permapure dryer (Inacom Instruments) before analysis of the O_2 and CO_2 concentrations with a Rosemount NGA 2000 analyzer. The gas flow rate was determined with an Ion Science Saga digital flowmeter. Calculations of specific O_2 consumption and CO_2 production for chemostat cultures were performed according to the method of van Urk et al. (40).

Enzyme activity assays. Cell extracts for enzyme activity assays were prepared as described previously (6). Subcellular fractionation was performed according to the method of Luttik et al. (23). The marker enzymes, cytochrome *c* oxidase (EC 1.9.3.1; Douma et al. [5]) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49; Postma et al. [30]), used for the localization study were assayed at 30°C in a Hitachi model 100-60 spectrophotometer according to previously published methods. PDC was measured as described by Flikweert et al. (12). The assay mixture for threonine aldolase contained 0.1 mM HEPES buffer (pH 7.0) 50 μM pyridoxal-5-phosphate, 88 U of alcohol dehydrogenase (EC 1.1.1.1.) ml^{-1}, and 150 μM NADH in demineralized water. The reaction was started by the addition of 10 mM threonine. Oxidation of NADH was followed by monitoring its absorbance at 340 nm with a Hitachi 100-60 spectrophotometer. The protein concentration of cell extracts was estimated by the Lowry method with bovine serum albumin as the standard (22).

TABLE 2. Recovery of threonine aldolase and marker enzymes in the particulate and soluble fractions of homogenates of the *GLY1*-overexpressing Pdc$^-$ strain RWB893(YEpGLY1) harvested from aerobic glucose-limited chemostat cultures[a]

Enzyme	% Recovery of enzyme activity in:	
	Particulate fraction	Soluble fraction
Threonine aldolase	1	94
Glucose-6-phosphate dehydrogenase	1	101
Cytochrome *c* oxidase	98	12

[a] Data are presented are from a single representative experiment. A duplicate experiment yielded similar results.

RESULTS

L-Carnitine addition to chemostat cultures of Pdc$^-$ *S. cerevisiae*. It has recently been shown that *S. cerevisiae* is unable to synthesize L-carnitine (39). If carnitine auxotrophy is the reason for the C_2 compound requirement of Pdc$^-$ *S. cerevisiae*, growth in aerobic, glucose-limited chemostat cultures should be possible after L-carnitine supplementation. To investigate this possibility, duplicate chemostat cultures of the Pdc$^-$ strain RWB893(YEplac181) on glucose and acetate as the carbon source were supplemented with 0.4 g of carnitine liter^{-1}. The addition of L-carnitine to the medium did not influence the biomass yield on carbon (14.1 $g_{biomass}$ Cmol^{-1}), nor the fluxes of glucose (1.07 mmol $g_{biomass}^{-1}$ h^{-1}), acetate (0.4 mmol $g_{biomass}^{-1}$ h^{-1}), O_2 (2.95 mmol g of biomass^{-1} h^{-1}), or CO_2 (3.00 mmol $g_{biomass}^{-1}$ h^{-1}) compared to cultures on glucose and acetate without L-carnitine (Table 3). After a steady state was reached, the culture was switched to a medium with glucose as the sole carbon source but still containing L-carnitine. On this medium, the Pdc$^-$ strain washed out of the chemostat cultures. Apparently, L-carnitine supplementation could not rescue Pdc$^-$ *S. cerevisiae* in chemostat cultures on glucose as the sole carbon source.

Overproduction of threonine aldolase in Pdc$^-$ *S. cerevisiae*. In addition to PDC, the metabolic network of *S. cerevisiae* contains at least one alternative reaction that may provide cytosolic C_2 compounds. Threonine aldolase (EC 4.2.1.5), a key enzyme in glycine biosynthesis encoded by the *GLY1* gene, catalyzes the cleavage of threonine to glycine and acetaldehyde (21, 25). To investigate whether the threonine aldolase may replace PDC in its biosynthetic role, it was attempted to overexpress *GLY1* in a *pdc1*Δ *pdc5*Δ *pdc6*Δ strain.

To determine whether introduction of the *GLY1* expression vector resulted in a higher activity of threonine aldolase, the activity of the enzyme was measured in cell extracts. The threonine aldolase activity of a Pdc$^-$ strain carrying the *GLY1* expression vector was 0.75 ± 0.01 U mg of protein^{-1}, whereas the activity in the corresponding empty-vector strain was below the detection limit of 0.005 U mg of protein^{-1}.

To investigate the subcellular localization of the overproduced Gly1p, the threonine aldolase activity was determined in both the soluble and particulate fractions of the cell homogenate obtained from a glucose-limited chemostat culture. The cytosolic enzyme glucose-6-phosphate dehydrogenase was fully recovered in the soluble fraction of the homogenate. The activity of cytochrome *c* oxidase, a mitochondrial marker enzyme, was almost exclusively located in the particulate fraction. Threonine aldolase activity in the overproducing strain was almost exclusively found in the soluble fraction of cell homogenate (Table 2), indicating a cytosolic localization of Gly1p.

***GLY1* overexpression eliminates the C_2 compound requirement of Pdc$^-$ *S. cerevisiae*.** Growth of Pdc$^-$ *S. cerevisiae* in glucose-limited chemostat cultures requires the addition of small amounts of ethanol or acetate to the glucose media (10). Like other Pdc$^-$ mutants the Gly1p-overproducing Pdc$^-$ strain did not grow in batch culture on glucose, not even in the presence of small amounts of ethanol or acetate (data not shown). Therefore, to test the ability of the Gly1p-overproducing Pdc$^-$ strain and the empty-vector reference strain to grow on glucose as the sole carbon source, aerobic mixed-substrate cultures grown at a dilution rate of 0.10 h^{-1} were switched to a medium containing glucose as the sole carbon source.

As expected, both strains were able to grow in chemostat cultures on a mixture of glucose and acetate (Table 3). Under these conditions, key physiological parameters of the cultures, such as biomass yields and respiratory quotient (RQ), were not significantly different for the two strains (Table 3). Consistent with a complete (>97%) recovery of substrate carbon in biomass and carbon dioxide, no significant accumulation of metabolites, such as ethanol, acetate, or glycerol, was observed in culture supernatants.

TABLE 3. Physiology of the threonine aldolase-overproducing Pdc$^-$ *S. cerevisiae* strain RWB893(YEpGLY1) and the empty-vector reference Pdc$^-$ strain RWB893(YEplac181) in aerobic chemostat cultures[a]

Parameter	Value for:			
	Medium with acetate		Medium without acetate	
	Empty vector (D = 0.10 h^{-1})	*GLY1* ↑ (D = 0.10 h^{-1})	*GLY1* ↑ (D = 0.10 h^{-1})	*GLY1* ↑ (D = 0.15 h^{-1})
Y_{sx} ($g_{biomass}$ Cmol^{-1})	14.0 ± 0.1	14.0 ± 0.1	14.0 ± 0.2	14.7 ± 0.0
$q_{glucose}$ (mmol $g_{biomass}^{-1}$ h^{-1})	1.11 ± 0.02	1.08 ± 0.03	1.17 ± 0.03	1.66 ± 0.02
$q_{acetate}$ (mmol $g_{biomass}^{-1}$ h^{-1})	0.4 ± 0.0	0.4 ± 0.0		
q_{CO_2} (mmol $g_{biomass}^{-1}$ h^{-1})	3.09 ± 0.07	3.12 ± 0.09	2.85 ± 0.02	4.06 ± 0.05
q_{O_2} (mmol $g_{biomass}^{-1}$ h^{-1})	3.01 ± 0.08	3.04 ± 0.09	2.82 ± 0.06	3.93 ± 0.05
RQ ($mmol_{CO_2}$ $mmol_{O_2}$)	1.03 ± 0.00	1.03 ± 0.00	1.02 ± 0.02	1.04 ± 0.00
Carbon recovery (%)	100 ± 0	97 ± 2	100 ± 0	98 ± 1

[a] Averages and mean deviations were obtained from duplicate experiments with independent steady-state cultures on synthetic medium containing a mixture of glucose and acetate (0.25 M substrate carbon and 10% acetate on a carbon basis) or glucose alone (0.25 M substrate carbon). Calculations of the carbon recovery were based on a biomass carbon content of 48% (wt/wt). *GLY1* indicates strain RWB893(YEpGLY1).

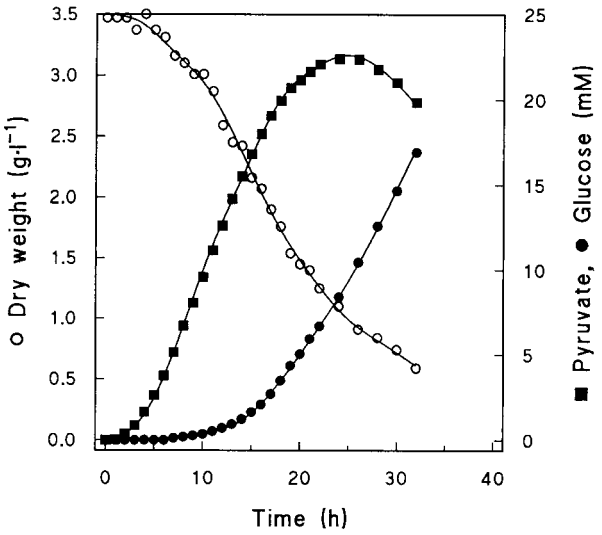

FIG. 1. Concentrations of glucose, metabolites, and biomass after a switch to an aerobic chemostat culture (dilution rate = 0.10 h^{-1}) of the *pdc1*Δ *pdc5*Δ *pdc6*Δ reference strain RWB893(YEplac181) from growth on synthetic medium containing a mixture of glucose and acetate (0.25 M substrate carbon and 10% acetate on a carbon basis) to growth on a synthetic medium containing glucose (0.25 M substrate carbon) as the sole carbon source. The graph shows the washout profile of a single representative culture. An independent replicate experiment yielded the same results.

After a switch to a medium with glucose as the sole carbon source, the empty-vector Pdc⁻ reference strain washed out of the chemostat cultures (Fig. 1). The exponential decrease of the biomass concentration, accompanied by the accumulation of glucose and pyruvate, was consistent with a low residual growth rate of 0.03 h^{-1}. It has previously been shown that a nonisogenic Pdc⁻ *S. cerevisiae* strain washed out of the chemostat cultures in a similar manner (12). Evidently, the reference strain was unable to sustain glucose-limited growth without acetate in the medium at a dilution rate of 0.10 h^{-1}.

Under identical conditions, the threonine aldolase-overproducing Pdc⁻ strain was capable of growth on glucose as the sole carbon source (Table 3), indicating that threonine aldolase could provide the cells with sufficient precursors for the synthesis of cytosolic acetyl-CoA. The obtained biomass yield on glucose of the Gly1p-overproducing strain (0.47 ± 0.01 $g_{biomass}$ g of $glucose^{-1}$) was comparable to the yield obtained for the wild-type strain (0.48 to 0.49 $g_{biomass}$ g of $glucose^{-1}$) under similar conditions. Byproduct formation in steady-state cultures of the Gly1p-overproducing strain was negligible. This indicated that the additional glycine produced by threonine aldolase was either dissimilated or assimilated by the glucose-limited chemostat cultures. The engineered strain was also capable of glucose-limited growth at a dilution rate of 0.15 h^{-1} (Table 3). However, attempts to further increase the dilution rate to 0.20 h^{-1} resulted in washout.

Byproduct formation upon exposure of glucose-limited cultures to excess glucose. To investigate the short-term response of the threonine aldolase-overexpressing Pdc⁻strain to excess glucose, a glucose pulse was administered to a steady-state, aerobic, glucose-limited chemostat culture. After a 50 mM glucose pulse was administered, the prototrophic reference strain CEN.PK113-7D required 2 h to completely consume the added glucose. Glucose consumption was accompanied by the production of 50 mM ethanol and 10 mM acetate (Fig. 2). Under identical conditions, the *GLY1*-overproducing Pdc⁻ strain required 4 h for complete consumption of the glucose pulse. No ethanol or acetate was produced but, in contrast to the wild-type strain and similar to a nonisogenic Pdc⁻ strain (13), the engineered strain produced substantial amounts of pyruvate (up to 30 mM; Fig. 2).

DISCUSSION

The C_2 compound requirement of Pdc⁻ *S. cerevisiae* has been proposed to reflect an essential role of PDC in the synthesis of cytosolic acetyl-CoA (10, 31). The available evidence

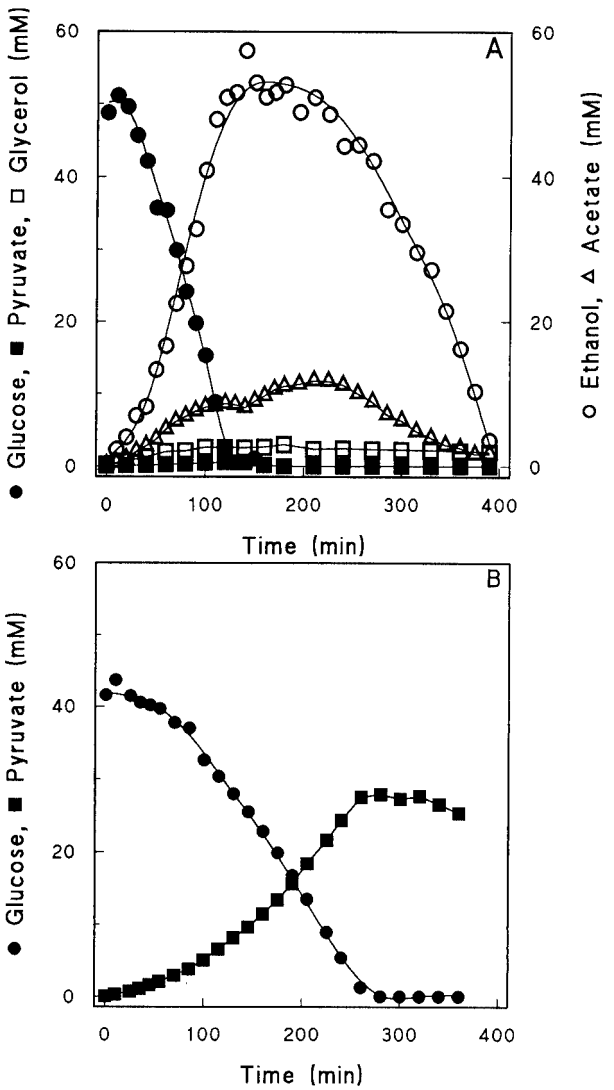

FIG. 2. Metabolic responses of aerobic, glucose-limited chemostat cultures (dilution rate = 0.10 h^{-1}) to a 50 mM glucose pulse. (A) *S. cerevisiae* CEN.PK 113-7D (prototrophic wild-type strain). (B) *GLY1*-overproducing Pdc⁻ strain RWB893(YEpGLY1). The graphs show single representative glucose pulse experiments for each strain. Independent replicate experiments yielded essentially the same results.

indicates that *S. cerevisiae* is not capable of de novo synthesis of L-carnitine (39). During growth in synthetic media that lack this cofactor, this might preclude the involvement of the L-carnitine shuttle (39) in export of mitochondrial acetyl-CoA to the cytosol. Our results demonstrate that the C_2 requirement of Pdc$^-$ *S. cerevisiae* is not caused by a simple L-carnitine auxotrophy. This does not necessarily imply that a mitochondrial carnitine shuttle in *S. cerevisiae* is unidirectional, as has earlier been proposed based on the phenotype of Pdc$^-$ strains and on the assumption that this yeast was capable of L-carnitine biosynthesis (31). Instead, the absence of an effect of L-carnitine addition might reflect a limitation in L-carnitine uptake over the yeast plasma membrane, as has recently been demonstrated in a different *S. cerevisiae* genetic background (37). Once the biochemistry and regulation of L-carnitine uptake and metabolism in *S. cerevisiae* are better understood, Pdc$^-$ strains may be useful for studies into the role of the L-carnitine shuttle in mitochondrial acetyl-CoA transport.

The observation that, at low through moderate specific growth rates, threonine aldolase-overproducing Pdc$^-$ strains were capable of growth on glucose as the sole carbon source (Table 3) is consistent with the proposed essential role of PDC in cytosolic acetyl-CoA biosynthesis (10, 31). Synthesis of acetaldehyde via threonine aldolase overproduction is accompanied by the formation of equimolar amounts of glycine. The minimum cytosolic acetyl-CoA requirement for the lipid and lysine (3, 9) biosynthesis during glucose-limited growth has previously been estimated at 1.05 mmol $g_{biomass}^{-1}$ (10). Therefore, at least 1.05 mmol of glycine $g_{biomass}^{-1}$ will be produced if all cytosolic acetyl-CoA is produced via threonine aldolase. Multiple pathways may be involved in the metabolism of this glycine in the engineered Pdc$^-$, *GLY1*-overexpressing strain. In addition to direct incorporation in cellular protein (the glycine content of yeast biomass is ca. 0.29 mmol $g_{biomass}^{-1}$ [26]), glycine may be used for the synthesis of serine via serine hydroxymethyl transferase and the glycine cleavage system (18, 28). If all serine is produced in this way, consuming two molecules of glycine per serine produced, an additional 0.37 mmol of glycine $g_{biomass}^{-1}$ can be incorporated in the biomass (26). Furthermore, additional glycine may be converted via the glycine cleavage system in conjunction with either methionine biosynthesis or one-carbon metabolism (18, 28).

The inability of Pdc$^-$ strains to grow on glucose as the sole carbon source indicates that regulatory properties of the *GLY1* gene and/or the regulatory and kinetic properties of Gly1p prevent the native *GLY1* gene from meeting the cellular demand for cytosolic acetyl-CoA. In terms of regulatory properties, it seems likely that regulation of the native *GLY1* gene will be primarily based on its role in nitrogen metabolism. In terms of kinetic properties, the low affinity of threonine aldolase for threonine (K_m = 55 mM [21]) may limit the flux through the enzyme at physiological intracellular threonine concentrations (5 to 10 mM [16, 24]). We cannot exclude the possibility that a low expression level of *GLY1* may have contributed to the low residual specific growth rates observed upon switching chemostat cultures of a Pdc$^-$ reference strain to a medium containing glucose as the sole carbon source (Fig. 1). It will be of interest to investigate whether threonine aldolase is involved in cytosolic acetyl-CoA biosynthesis in eukaryotes that lack PDC.

The aerobic production of ethanol and acetate by wild-type *S. cerevisiae* is considered a substantial problem in biomass- and protein-directed industrial applications. The engineered Pdc$^-$, *GLY1*-overexpressing strain combines the absence of this alcoholic fermentation with the ability to grow on glucose as the sole carbon source in aerobic carbon-limited chemostat cultures. However, several growth characteristics of this strain limit the industrial application as a host for the expression of heterologous proteins or as a strain platform for the production of L-lactate (29). First, similar to a strain with reduced expression of PDC (11), the engineered strain exhibited a reduced maximum specific growth rate of 0.20 h^{-1} in glucose-limited chemostat cultures compared to 0.38 h^{-1} of the wild type. Second, like other strains of *S. cerevisiae* with reduced or zero PDC activity (11, 13, 35), it produced substantial amounts of pyruvate during exposure to glucose excess (Fig. 2). Third, growth of this strain on glucose in batch culture was not possible.

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