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Regulation of *pyc1* encoding pyruvate carboxylase isozyme I by nitrogen sources in *Saccharomyces cerevisiae*

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In *Saccharomyces cerevisiae,* the existence of *PYC1* and *PYC2* encoding cytosolic pyruvate carboxylase isoform I and II is rather puzzling, owing to the lack of potent differential gene regulation by the carbon sources. We report several findings indicating that these two genes are differentially regulated by the nature of the nitrogen source. In wild-type cells, the activity of pyruvate carboxylase, which is the sum of pyruvate carboxylase isoform I and II, was two- to fivefold lower in carbon medium containing aspartate, asparagine, glutamate or glutamine instead of ammonium as the nitrogen source, whereas it was 1.5- to threefold higher when the ammonium source was substituted by arginine, methionine, threonine or leucine. These enzymatic changes were independent of the nature of the carbon source and closely correlated to the changes in β-galactosidase from *PYC1-lacZ* gene fusion and in *PYC1* transcripts. Transfer of exponentially growing cells of the *pyc2* mutant from an aspartate or a glutamate medium to an ammonium medium caused a fivefold increase in *PYC1* mRNA in less than 30 min, whereas in the inverse experiment, *PYC1* transcripts returned within 30 min to the low levels found in aspartate/glutamate medium. By contrast, these conditions affected neither the pyruvate carboxylase activity encoded by *PYC2* nor *PYC2* mRNA. Considering that changes in *PYC1* expression inversely correlated with changes in α-ketoglutarate concentration or in α-ketoglutarate/glutamate ratio following the nitrogen shift experiments, and taking into account the pivotal role of this metabolite in ammonium assimilation, it is suggested that changes in α-ketoglutarate or in the α-ketoglutarate/glutamate ratio might be implicated in triggering the nitrogen effects on *PYC1* expression. The physiological significance of the differential sensitivity of *PYC1* and *PYC2* genes with respect to the nitrogen source in the growth medium is also discussed.

Keywords: metabolism; *PYC* genes; pyruvate carboxylase; transcriptional regulation; yeast.

Pyruvate carboxylase (EC 6.4.1.1) is a key enzyme in central metabolism. It catalyses the magnesium–ATP- and biotin-dependent carboxylation of pyruvate to oxaloacetate, a reaction necessary for the replenishment of the tricarboxylic cycle in all eukaryotic organisms [1,2]. This key function justifies the fine metabolic control of this enzyme by different effectors, including activators such as acetyl-CoA and palmitoyl-CoA and inhibitors such as aspartate [1–3]. *Saccharomyces cerevisiae* contains two different genes, *PYC1* and *PYC2*, encoding isoenzymes I and II of pyruvate carboxylase [4,5]. The physiological significance of the two genes is not yet clear, because even if both genes are expressed slightly differently depending on the growth phase of yeast cultures on glucose or on the carbon source of the medium, the differences appear minor as to physiological relevance [6,7]. It is also noteworthy that the two pyruvate carboxylase isoenzymes are localized in the cytosol [8–10], whereas in mammalian cells, this enzyme is associated with the mitochondrion, a localization which appears more befitting with its function of replenishing the mitochondrially localized tricarboxylic acid cycle [1].

As oxaloacetate, the product of pyruvate carboxylase, is the direct precursor of C4-amino acids and is implicated in purine and protein synthesis [1,2,11], it seems plausible to expect that the expression of the *PYC* genes could be influenced by the nature of the nitrogen source present in the growth medium. In fact, in 1975, Haarasilta and Oura [12] showed that, in *S. cerevisiae*, the substitution of ammonium ions for aspartate or asparagine in a glucose mineral medium caused a three- to fourfold decrease in the activity of pyruvate carboxylase. Although they indicated that this reduction was not due to the direct inhibition of the enzyme by aspartate, this initial observation has not been followed up. In this article we report that the expression of *PYC1*, but not that of *PYC2*, is regulated by the nature of the nitrogen source independently of the nature of the carbon source in the growth medium.

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MATERIALS AND METHODS

Yeast strains and culture conditions

The following *Saccharomyces cerevisiae* strains were used throughout this work: CEN.PK113–7 D (*Mat α MAL2–8C SUC2*) and the isogenic CEN.PK113–5D (*Mat **a** MAL2–8C SUC2 ura3–52*) were supplied by the EUROSCARF strain collection (K. D. Entian, Frankfurt, Germany). Strain CEN.JB4 (*Mat **a** MAL2–8C SUC2 Δpyc2::ILV2^{SMR} PYC1*) and CEN.JB8 (*Mat a MAL2–8C SUC2 Δpyc1::KanMX4 PYC2*) were a kind gift from P. Niederberger (Nestec Ltd, Lausanne, Switzerland). Strain JF1209 *(Mat a MAL2–8C SUC2 Δpyc1::KanMX4*

Δpyc2::ILV2^{SMR}) was obtained after a cross with CENPK113–1 A and CEN.JB4 followed by a second cross between a *pyc2* mutant segregant from the first cross with CEN.JB8. The *pyc1pyc2* double mutant was identified by its inability to growth in a glucose mineral medium in the absence of aspartate [4]. Transformation of strain CEN.PK113–5D with plasmid pJC1 or pJC2–2 carrying a *PYC1-lacZ* or *PYC2-lacZ* gene fusion, respectively [7] was made according to [13]. Plasmid pJC2–2 was constructed by inserting a 3.8 kbp *Sal*I–*Xba*I from pJC-2 [7] in the *Sal*I–*Xba*I sites of pRS316.

Unless otherwise stated, yeasts were grown at 30 °C in batch cultures on a mineral medium as described by Verduyn *et al.* [14] in the presence of 20 g·L^{-1} of glucose. As the NH_4^+ concentration in this medium is 50 mM, which correspond to 0.7 g-atoms of nitrogen per L, the concentration of amino acid added was enough to provide the same amount of g-atoms of N per L. Transfer of cells from a medium with a nitrogen source to another with a different one was carried out as follows: yeast cultures in the exponential phase of growth in a glucose medium with a certain nitrogen source ($D_{660} \approx 2$ or 0.6 mg dry mass per mL) were harvested by centrifugation (5 min at 2000 ***g*** at room temperature), washed once with sterile distilled water and resuspended either in a medium of the same composition or in a medium containing a different nitrogen source at an D_{660} of 0.5. At the times indicated, samples were taken either by rapid centrifugation for enzymatic assays and RNA extraction or by rapid filtration onto nylon filter (diameter 45 mm, pore size 0.45 μm) for determination of intracellular metabolites.

RNA extraction and Northern analysis

Total RNA was extracted from cells (about 5×10^9) harvested as described above by the procedure described by Siderius *et al.* [15]. The RNAs were separated in formaldehyde–agarose gels as described previously [16] and transferred to Hybond N$^+$ filters (Amersham, UK). Probes (the 1.1-kbp *Pst*I internal of *PYC1* from pCG50 [4] and a 1.5-kb internal fragment of the *ACT1* gene) were used for hybridization as recommended by the manufacturer. Blots were probed with ^{32}P-radiolabelled fragments of *PYC1* and *ACT1* using the megaprime™ DNA labelling system (Amersham, UK). RNA levels were quantified by PhosphoImager analysis (PhosphoFluorimager STORM 860 from Molecular Dynamic).

Preparation of extracts and enzymatic assays

Crude extracts were prepared by vortexing at 4 °C four times for 30 s, about 25 mg (dry mass) yeast cells with 1 g glass beads (0. 4–0.5 mm diameter) with 0.5 mL of Hepes 20 mM pH 7.1, KCl, 100 mM, EDTA 1 mM and dithiothreitol 1 mM. After centrifugation at 8000 ***g*** for 15 min, the supernatants were used for assay of pyruvate carboxylase and protein concentration. Pyruvate carboxylase was assayed in a coupled assay with citrate synthase, following the production of CoASH as described by Van Urk *et al.* [9]. The enzymatic activity is expressed as the amount of the enzyme that catalyses the production of 1 μmol CoASH per min under the assay conditions described in [9]. Preparation of crude extracts and determination of β-galactosidase were performed as described previously [18], and the activity is expressed in nmol of *o*-paranitrophenyl phosphate produced per min and per mg proteins. Protein was assayed by the Bradford method, using bovine serum albumin as the standard [17]

Extraction and assay of intracellular metabolites

The extraction of metabolites by the boiling buffered ethanol method of Gonzalez *et al.* [19] could not be used because it entirely destroyed oxaloacetate. However, the high stability of this metabolite in acidic solution at pH below three allowed its extraction in 5% perchloric acid or 0.1 M HCl. Due to the very low concentration of oxaloacetate in yeast (in the range of nanomole per g dry mass), samples (approx. 4×10^{10} cells·mL^{-1} or 75 mg dry mass) were quickly collected in several small portions (about 15 mg dry mass) by filtration through several nylon membranes (diameter 45 mm, pores size 0.45 μm). The cakes were scraped with a spatula, immediately frozen at −80 °C, and pooled in the same tube. Oxaloacetate was extracted from the frozen samples in 1 mL of 5% perchloric acid containing 0.5 g of glass beads by five cycles of freezing in liquid nitrogen/thawing in ice/10 s vortexing. After centrifugation at 3000 ***g*** for 5 min at 4 °C, the supernatant was neutralized with a solution of 0.3 M Mops/2 M KOH, centrifuged 1 min in a microcentrifuge, and a portion of the neutralized supernatant (0.1–0.2 mL) was immediately used for the oxaloacetate determination. Failure to determine oxaloactetate within 30 min after neutralization of the supernatant resulted in a loss of its concentration due to its instability in neutral pH. The remaining part of the neutralized solution was kept at −80 °C until use. Oxaloacetate as well as other metabolites (pyruvate and α-ketoglutarate) were measured enzymatically as described by Bergmeyer [20]. It is worth noting that some uncertainty about the true intracellular values of oxaloacetate could remain, as the whole procedure of sample collection by filtration took about 15–30 s. However, a good reproducibility was found for oxaloacetate measurement in independent experiments. Amino acids were determined with an amino-acid analyser (AminoQuant from Hewlett Packard). The concentration of metabolites is expressed in mol per g dry mass yeast. Conversion into mole·L^{-1} can be performed by considering that 1 g dry mass yeast corresponds to 2.7 mL of cell sap [21].

RESULTS

Effect of nitrogen sources on pyruvate carboxylase activity in wild type and *pyc* mutants

Pyruvate is situated at a major branch point in the central metabolism that makes a junction between carbon and nitrogen metabolism [11]. We therefore measured some enzymes (e.g. pyruvate decarboxylase, citrate synthase, acetyl-CoA hydrolase, pyruvate dehydrogenase complex) located at this node in yeast grown either in a glucose medium with ammonium sulphate (mineral medium) or peptones (rich medium) as the nitrogen source. Among the enzymes tested, only pyruvate carboxylase was fourfold lower in cells cultivated in the rich medium (70 mU·mg^{-1} protein vs 15 mU·mg^{-1} protein). A similar four- to fivefold lower pyruvate carboxylase activity was found in a glucose mineral medium in which peptones or aspartate was used as the nitrogen source in the place of ammonium sulphate (Fig. 1). Other amino acids including glutamate (Fig. 1), asparagine and glutamine (not shown) also reduced by two- to fourfold pyruvate carboxylase specific activity. However, arginine, threonine, alanine, methionine, leucine (Fig. 1), valine or serine (not shown) did not have much change the activity of total pyruvate carboxylase with respect to that measured in ammonium cultures. Table 1 shows that the

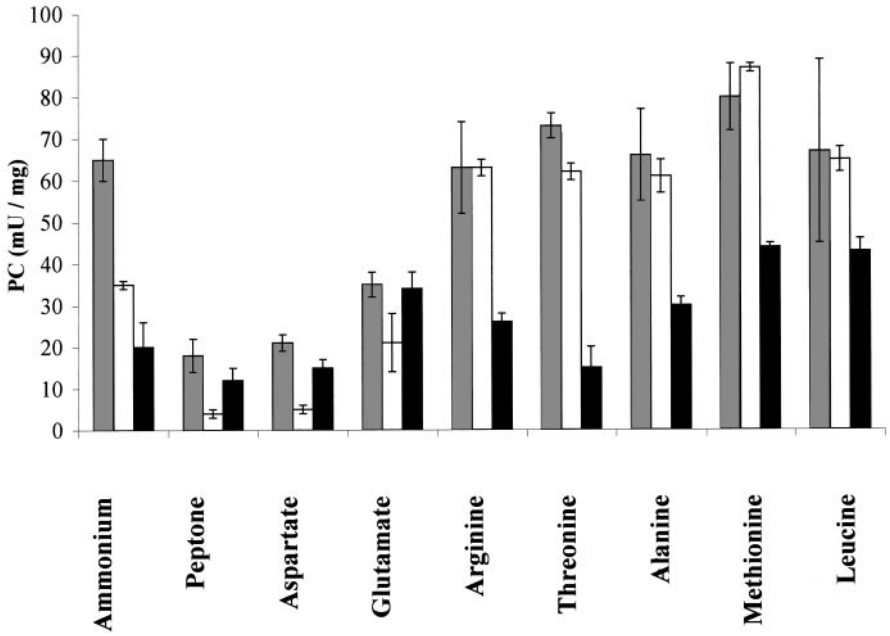

Fig. 1. Activity of pyruvate carboxylase determined in wild type and *pyc* mutants during the exponential phase of growth on glucose in the presence of different amino acids used nitrogen sources. Strains used were CEN PK 113–7D (*PYC1 PYC2*, lightly shaded histograms), CEN.JB4 (*PYC1* Δ*pyc2*, open histograms) and CEN.JB8 (Δ*pyc1 PYC2*, shaded histograms). The activities are the average ± SD of 4 independent culture experiments.

repressive effect exerted by aspartate on pyruvate carboxylase was independent of the nature of the carbon source present in the growth medium. Curiously, threonine led to a decrease in pyruvate carboxylase activity when a carbon source other than glucose was used and to the inability of cell growth in the presence of ethanol. Nevertheless, these results confirmed the previous observation of Haarasilta & Oura [12] that levels of pyruvate carboxylase are influenced by the nature of the nitrogen source, and raised the question of whether the nitrogen effects affected either one or the two pyruvate carboxylase isoforms.

In *S. cerevisiae*, the measured activity of pyruvate carboxylase is the sum of the activities resulting from *PYC1* and *PYC2* gene products [4–5]. Therefore, the effects of nitrogen sources on each of the two isoenzymes could be examined using strains bearing a deletion in *PYC1* or *PYC2* genes, owing to the fact that the growth behaviour is not impaired in the respective *pyc* mutants. As previously reported [4,22], mutants in only one of the *PYC* genes or the double *pyc1pyc2* mutant grew as the wild type in a glucose medium containing aspartate as the nitrogen source [4,22]. However, loss of *PYC1* function resulted in a twofold reduction in the growth rate in a glucose/ammonium medium (Table 2), suggesting that the function of this gene in anabolic pathways (i.e. biomass production) is more important than *PYC2*. Consistent with this hypothesis, is the result shown in Fig. 1 that the nitrogen sources affected principally the activity of pyruvate carboxylase encoded by *PYC1*, with a much lesser effect on the enzyme encoded by *PYC2*. It is worth noting that total pyruvate carboxylase activity in the wild-type cells growing on a glucose medium with ammonium, peptones, aspartate, and to a lesser extent glutamate, used as the nitrogen source roughly corresponded to the sum of the activity measured in each of the individual *pyc* mutants, whereas total pyruvate carboxylase activity in wild type cultivated in the presence of other amino acids such as arginine, alanine, methionine or leucine was always lower than the sum of activity from the

Table 1. Effect of the nature of the nitrogen source on the activity of pyruvate carboxylase during growth on different carbon sources. Wild-type strain CENPK113–7D was cultivated in a mineral medium containing 2% of each of the different carbon sources and a concentration of amino acids to provide an equivalent of 50 mM NH_4^+. The activity of pyruvate carboxylase was determined with exponential growing cells ($D_{660} \approx 2$). The experimental results are the average ± SD of 3 independent culture experiments.

	Pyruvate carboxylase activity (mU·mg protein^{-1})				
	carbon source				
Nitrogen source	Glucose	Maltose	Sucrose	Galactose	Ethanol
Ammonium	65 ± 5	56 ± 11	52 ± 5	70 ± 2	239 ± 31
Aspartate	18 ± 4	12 ± 2	15 ± 2	29 ± 10	86 ± 13
Glutamate	23 ± 2	22 ± 7	23 ± 2	24 ± 5	75 ± 15
Threonine	73 ± 3	40 ± 5	25 ± 5	26 ± 6	no growth

Table 2. Generation times (h) of yeast strains harbouring single or double null mutations for *PYC1* and *PYC2*. Cultures were made in glucose mineral medium containing ammonium or aspartate as nitrogen source as described in Materials and methods. Cells were grown from $D_{660} = 0.2$ to $D_{660} = 4$ and generation time (tg, in hours) was estimated during the exponential phase.

Strain/genotype	NH_4^+ medium (tg)	Aspartate medium (tg)
CEN. PK113–7D (*PYC1 PYC2*)	1.75	1.75
CEN.JB4 (*PYC1* Δ*pyc2*)	1.75	1.75
CEN.JB8 (Δ*pyc1 PYC2*)	3	1.75
JF1209 (Δ*pyc1* Δ*pyc2*)	no growth	1.85

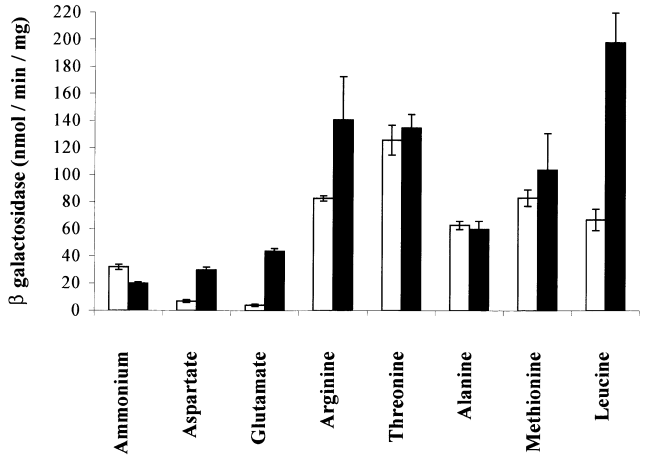

Fig. 2. Levels of β-galactosidase from *PYC-lacZ* gene fusions in exponential cells of yeast cultivated in the presence of different amino acid used as the nitrogen source. The strain CEN.PK113–5D carrying either *PYC1-lacZ* (open histogram) or *PYC2-lacZ* (filled histogram) on a centromeric plasmid was cultivated on glucose mineral medium in the presence of different amino acids at a concentration that provided an equivalent of 50 mM NH_4^+. The activity of β-galactosidase as determined with log phase cultures ($D_{660} \approx 2$) is the average ± SD of at least four independent cultures.

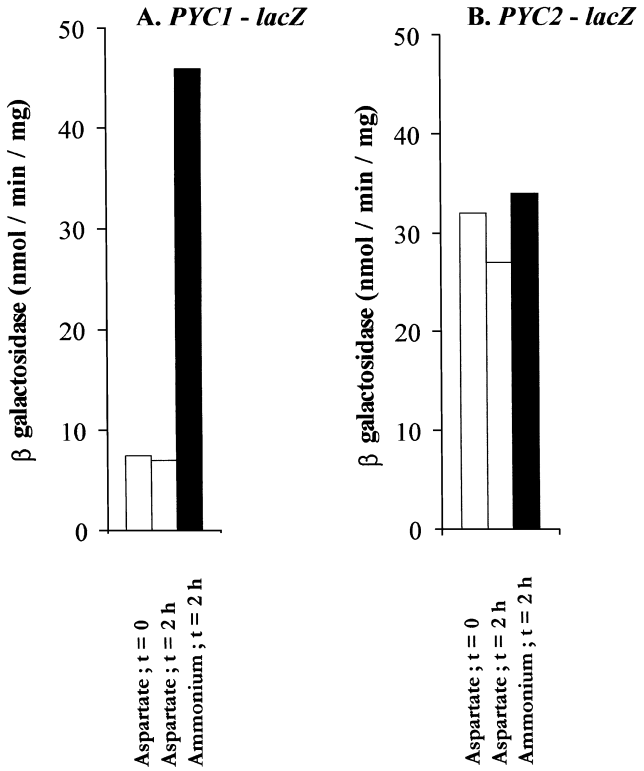

Fig. 3. Activity of β-galactosidase from *PYC-lacZ* fusion genes before and after the transfer of a yeast culture from one nitrogen source to another nitrogen source. Strain CEN.PK113–5D carrying *PYC1-lacZ* or *PYC2-lacZ* cultivated on a glucose/aspartate medium were collected at $D_{660} \approx 2$ by centrifugation, and resuspended at $D_{660} \approx 0.5$ either in the same medium (open histogram) or in a glucose/NH_4^+ medium (filled histogram). β-galactosidase was determined before and 2 h after the shift. Values reported are from one experiment, which was reproduced two times with consistent results.

PYC1 and *PYC2* mutants. A possible explanation for this difference could be that the positive effect exerted by these amino acids on *PYC1* and *PYC2* expressed individually is cancelled by some feedback control when both genes are expressed.

Effect of nitrogen sources on *PYC1* and *PYC2* expression

To investigate whether the nitrogen effects were exerted at the transcriptional level, we measured β-galactosidase levels in *S. cerevisiae* cultivated on a glucose mineral medium supplemented with different nitrogen sources and expressing the *PYC1* and *PYC2lacZ* fusions. As shown in Fig. 2, the expression of both genes appeared to be affected by the nature of the amino acid used as the nitrogen source. However, only *PYC1* was repressed by aspartate and this repression correlated with the reduced activity of pyruvate carboxylase isoenyme 1 measured in the same condition (see Fig. 1). It can also be seen that glutamate exerted a repressive effect on the *PYC1-lacZ* fusion that was as potent as that of aspartate, while the enzymatic activity was reduced by only twofold (compare Fig. 1 and Fig. 2). In contrast, arginine, threonine, alanine, methionine and leucine enhanced by 1.5- to threefold the expression of *PYC1* and *PYC2lacZ* fusion genes, with a major effect observed with leucine on the *PYC2-lacZ* construct. While changes in β-galactosidase activity from *PYC1-lacZ* expression were closely correlated to those in enzyme activity encoded by *PYC1* in response to these amino acids, it is not easy to explain how these same amino acids exerted a potent stimulation of *PYC2-lacZ* expression while they were without marked effect on the enzyme activity encoded by this gene (compare Figs 1 and 2). Further evidence supporting *PYC1* transcription control by the nature of the nitrogen source is shown in Fig. 3. When yeast cells carrying either the *PYC1-lacZ* or *PYC2-lacZ* fusion genes were transferred from a glucose/aspartate to a glucose/$NH4^+$ medium, the β-galactosidase activity from *PYC1*-lacZ fusion increased from 7 to 45 mU·mg^{-1} protein within two hours after the shift, whereas that of *PYC2-lacZ* fusion remained unchanged. As a control, the transfer of the yeast cells from a glucose/aspartate medium to the fresh medium of the same composition did not modify β-galactosidase levels from *PYC1-lacZ.*

To validate the hypothesis of a transcription control of *PYC1* by nitrogen source, *PYC1* and *PYC2* mRNAs were determined during nitrogen source shift experiments. As the two genes are more than 80% identical at the sequence level [4–6], we performed these experiments using a *pyc1* (strain CENJB8) and a *pyc2* (strain CEN.JB4) mutant in order to distinguish the effects on the transcripts of the *PYC1* and *PYC2* genes. As shown in Fig. 4A, *PYC1*mRNA was raised by more than 5 times within 30 min upon the transfer of *pyc2* cells from a glucose/aspartate to a glucose/$NH4^+$ mineral medium, while the corresponding pyruvate carboxylase activity increased more slowly and reached about 10 times its initial value only 2 h after the shift. In contrast, *PYC2* mRNAs and the corresponding gene product were not modified during this transfer. Reciprocally, the transfer of yeast cells from the nonrepressing threonine-containing medium to the repressing aspartate-containing medium (Fig. 5) caused in less than 30 min a decrease of *PYC1* mRNA to the levels measured in the nitrogen repressing medium, while the corresponding enzyme activity started to decline only after 2 h and, as expected, expression of *PYC2* was barely affected. Finally, we found that the transfer of

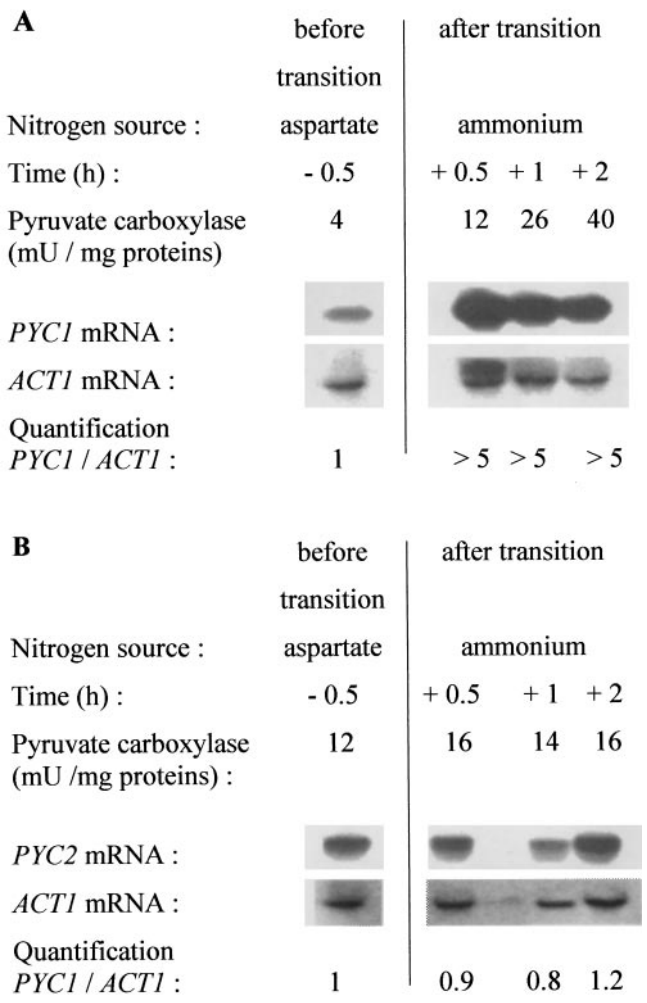

Fig. 4. Northern blots analysis of *PYC* mRNA upon transfer of yeast cells from a glucose/aspartate to a glucose/ammonium medium. The mutant strains CEN.JB8 (*PYC1Δpyc2*; A) and CEN.JB4 (*Δpyc1PYC2*; B) were used for this experiment, which was carried out as outlined in Fig. 3. Quantification of mRNA levels is expressed as the ratio of *PYC1/ACT1* messages at different times divided by the *PYC1/ACT1* ratio measured 30 min before the shift.

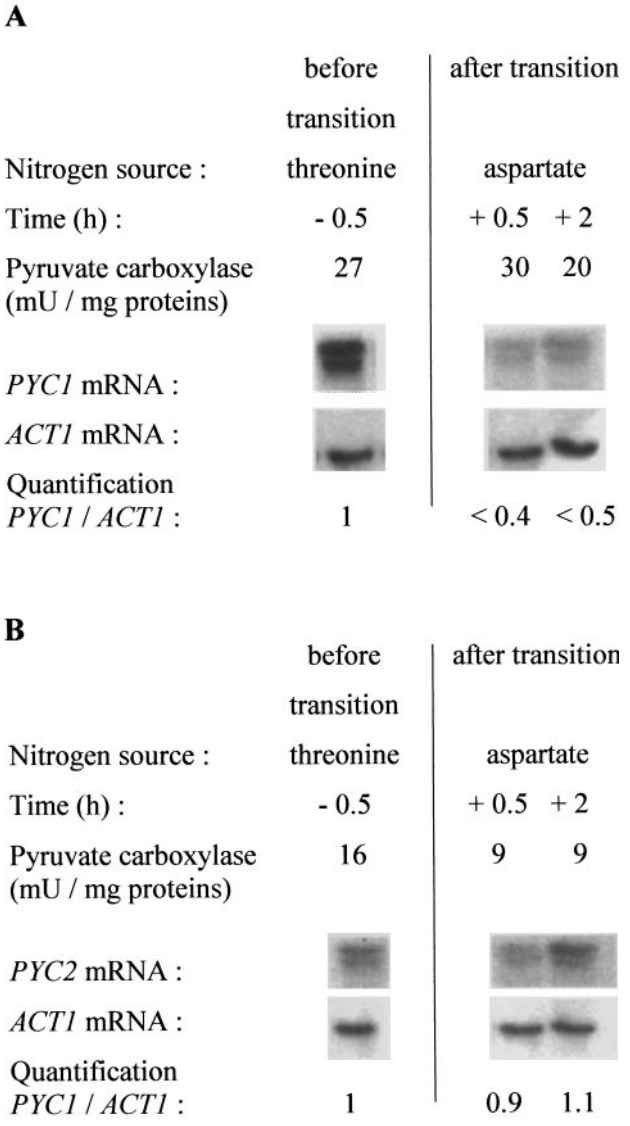

Fig. 5. Northern blots analysis of *PYC*mRNA upon transfer of yeast cells from a glucose/threonine to a glucose/aspartate medium. Same strains and procedures as in Fig. 4.

yeast cells from a glucose/$NH4^+$ to glucose/threonine caused a 1.5- to twofold increase in *PYC1* (not shown), which correlated with the twofold increase in β-galactosidase activity from *PYC1-LacZ* constructs (see Fig. 2) and with the twofold increase in enzyme activity encoded by *PYC1* (see Fig. 1). Taken together, these results are consistent with a transcriptional control of *PYC1* by the nitrogen source, although alteration of the *PYC1* mRNA stability cannot be excluded.

Changes in levels of intracellular metabolites following nitrogen shift experiments

As the concentration of the substrates of pyruvate carboxylase as well as that of some key amino acids can vary according to the nitrogen sources present in the growth medium [24], one may hypothesize that the effects of the nitrogen source on *PYC1* expression is triggered by change in one or several of these intracellular metabolites. We therefore investigated a possible correlation between changes in the levels of pyruvate, oxaloacetate, aspartate, glutamate, glutamine and α-ketoglutarate with that of *PYC1* expression in yeast cells subjected to nitrogen shift experiments. As illustrated in Table 3, pyruvate and oxaloacetate could not be considered as potential triggers because the concentration of these metabolites did not change very much under the different nitrogen shift experiments investigated. Aspartate could also be excluded from the list as its concentration did not vary correlatively with change in *PYC1-lacZ* expression. Good candidates that remained as potential triggers were glutamate, glutamine and α-ketoglutarate. Huge drop in α-ketoglutarate and an accumulation of glutamine following transfer to a repressing nitrogen source (aspartate or glutamate) could be expected as α-ketoglutarate is the initial intracellular acceptor of NH_4^+ whereas glutamine represents a nitrogen store in yeast [23–25]. It is also relevant to note that changes in levels of these metabolites were much more potent than that of *PYC1* expression. However, experiments of transfer from glutamate to glutamine indicated a good proportionality between changes in glutamate/α-ketoglutarate ratio and that in β-galactosidase activity from *PYC1-lacZ* construct.

Table 3. Levels of intracellular metabolites, amino acids and of β-galactosidase activity from *PYC1-lacZ* following transfer of yeast culture from a nitrogen source to another one. Exponential cells of the strain CEN.PK113–5D on a glucose mineral medium containing one nitrogen source were transferred to a glucose mineral medium containing another nitrogen source as described in Materials and methods. Collection for metabolites were performed 15 min before and after the shift, whereas β-galactosidase activity was determined 6 h after the shift. The experimental results are the mean of two independent experiments. The concentration of metabolites are expressed in μmol/g dry mass, except for oxaloacetate which was in nmol·g dry mass^{-1}. ND, not determined

Metabolite	Asp to NH_4^+		NH_4^+ to Asp		Glu to NH_4^+		NH_4 to Glu		Glu to Gln		Gln to Glu	
Pyruvate	4	4	4	4	4	4	4	4	4	4	4	4
Oxaloacetate	0.26	< 0.01	< 0.01	0.56	< 0.01	< 0.01	<0.01	< 0.01	< 0.01	<0.01	<0.01	< 0.01
α-ketoglutarate	0.8	<0.01	<0.01	4.3	12	<0.01	<0.01	13.1	14	1	1	15
Aspartate	248	12	19	107	13	14	19	6	11	17	9	14
Glutamate	97	27	70	125	493	28	70	245	351	55	108	173
Glutamine	18	160	50	<0.1	47	316	50	15	88	586	244	57
Threonine	48	20	11	8	13	1	11	<0.1	10	9	8	7
Arginine	42	45	19	18	43	45	19	19	56	60	17	28
Glu/α-keto	121	>2700	>7000	29	41	>2800	>7000	19	25	55	108	11
PYC1-PYC1-lacZ	11	55	ND	ND	9	57	ND	ND	8	14	ND	ND

DISCUSSION

The physiological significance of the existence of two genes encoding pyruvate carboxylase in *S. cerevisiae* without any striking differences in their expression patterns and without known differences in the properties of their encoded enzymes has been a puzzle [4,5,7]. We have shown in this work, both by enzymatic assays and by Northern analysis, that there is an important difference in the response of *PYC1* and *PYC2* to the nitrogen source of the medium with independence of the carbon source. With respect to the expression in ammonium, a group of amino acids such as aspartate, asparagine, glutamate and glutamine repressed *PYC1*, while a second group including arginine, threonine, leucine and methionine exerted a weak but reproducible 1.5 to threefold activation of *PYC1* expression. The repressive effect may be explained considering that the amino acids of the first group directly supply the central metabolism with oxaloacetate, which could reduce the carbon flow through pyruvate carboxylase. However, the positive effect exerted by the second group of amino acids could be a consequence of their entrance in the central metabolism at the level of pyruvate or acetyl-CoA, which are, respectively, the substrate and a potent activator of the pyruvate carboxylase [1,2]. It is not easy to explain the effect caused by threonine and methionine assimilation by oxidative deamination [23–26], which releases $NH4^+$ and a C-4 carbon skeleton and would have expected to behave as $NH4^+$.

Determination of β-galactosidase levels from a *PYC1-LacZ* gene fusion and analysis of *PYC1* transcripts during nitrogen transfer experiments supported the notion that the control of *PYC1* by the nitrogen source occurred mainly at the transcriptional level. A modification of the *PYC1* mRNA turnover rates by the nature of the nitrogen source could also be invoked, as indicated by the fast decay of *PYC1* mRNA observed upon the transfer of a yeast culture from a nonrepressing (i.e. glucose/threonine) to a repressing nitrogen condition (i.e. glucose/aspartate medium). However, our data do not allow a firm conclusion on this point. With respect to the transcriptional control of *PYC1*, an extensive analysis of its 5′ untranslated region (about 1000 bp from the ATG) did not reveal consensus sequences related to the nitrogen metabolism [27]. However, other preliminary data indicate that the repressing effect by aspartate or glutamate is related with two distinct regions located between −800 and −350 bp from the initiation codon (Huet *et al*. in preparation).

As the expression of *PYC1* showed variations in response to different nitrogen sources used for growth, it is likely that the effects on this gene are directly triggered by a common intracellular metabolite whose concentration changes with the nature of nitrogen source used for the growth. Such a mechanism has been reported for *GLN1*, which encodes glutamine synthetase. This gene is down-regulated by glutamine, which, at increased intracellular concentrations, activates the expression of *URE2* encoding the transcriptional repressor factor Ure2p [25]. Also, it is likely that an increase in the intracellular concentration of ammonium is responsible for the repression of *GDH1*, which encodes NAD^+ glutamate dehydrogenase [28]. In the case of *PYC1*, good candidates for a triggering role are α-ketoglutarate, glutamate and glutamine. Although the present data do not allow discrimination between them, α-ketoglutarate appears, from a metabolic point of view, to be the candidate. Growth in ammonium causes a high demand for α-ketoglutarate, which would increase that of oxaloactetate and put a demand on a higher activity of pyruvate carboxylase. In contrast, growth in the presence of aspartate, glutamate or glutamine would largely supply α-ketoglutarate, thus decreasing the need of an increased oxaloacetate supply.

Our findings on the specific control of *PYC1* and the lack of apparent control of *PYC2* by the nitrogen source may support an idea put forward by Scrutton some 20 years ago [1]. Taking into account the kinetic differences and the different localization of the yeast pyruvate carboxylase with respect to the mammalian enzyme, Scrutton [1] proposed that the yeast pyruvate carboxylase may have a biosynthetic function rather than an anaplerotic role. Our finding that *PYC1* is repressed by amino acids such as aspartate and glutamate, which are direct precursors of proteins, purines, pyrimidines and porphyrins synthesis, and that the growth rate of a *pyc1* mutant in glucose/ammonium mineral medium is two times lower than that of the wild type, would agree with an anabolic role for *PYC1*. However, the anaplerotic role of pyruvate carboxylase cannot be forgotten. Yeast mutants lacking both pyruvate carboxylase isoenzymes do not grow in minimal media with glucose as carbon source and anmmonium as nitrogen source, owing to an inability to replenish the tricarboxylic acid cycle. Apparently any of the isoenzymes may play this role [4,22]. The

localization of both isoenzymes in the cytosol [8–11] implicates a need to transport oxaloacetate from the cytosol to the mitochondrion when the enzyme plays an anaplerotic role. The lack of a significant regulation of *PYC2* by nutrients including carbon [6,7] and nitrogen (this work) suggests that pyruvate carboxylase isoform II may be implicated in a function additional to the replenishment of the tricarboxylic cycle. A possible one could be to serve as a coadjuvant in the reoxidation of cytosolic NADH, providing oxaloacetate, which could be reduced by malate dehydrogenase. Such a function would be consistent with the results of Bauer *et al*. [29], who found that in glucose-limiting continuous cultures, an overproduction of pyruvate carboxylase encoded by *PYC2* led to an increase in malate production.

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