Expression of PEP carboxylase from *Escherichia coli* complements the phenotypic effects of pyruvate carboxylase mutations in *Saccharomyces cerevisiae*

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Abstract We investigated the effects of the expression of the *Escherichia coli ppc* gene encoding PEP carboxylase in *Saccharomyces cerevisiae* mutants devoid of pyruvate carboxylase. Functional expression of the *ppc* gene restored the ability of the yeast mutants to grow in glucose-ammonium medium. Growth yield in this medium was the same in the transformed yeast than in the wild type although the growth rate of the transformed yeast was slower. Growth in pyruvate was slowed down in the transformed strain, likely due to a futile cycle produced by the simultaneous action of PEP carboxykinase and PEP carboxylase.

Key words: PEP carboxylase; Pyruvate carboxylase; TCA cycle; Glucose metabolism (yeast)

1. Introduction

Intermediates of the tricarboxylic acid (TCA) cycle are continuously withdrawn for synthesis of cell constituents. Therefore replenishment of the cycle is critical to maintain its functionality. Different reactions are used by diverse organisms to this end (for reviews see [1–3]). In yeast growing in a medium with glucose and ammonium the replenishment of the TCA cycle is carried out by pyruvate carboxylase that synthesizes oxaloacetate from pyruvate. The important anaplerotic role of this enzyme in yeast is shown by the phenotype exhibited when the two genes *PYC1* and *PYC2* encoding two isoenzymes of pyruvate carboxylase are disrupted. These mutants are unable to grow on a glucose-ammonium medium while they grow on a medium with glucose-aspartate [4].

Escherichia coli synthesizes oxaloacetate not from pyruvate, but from phosphoenolpyruvate (PEP) in a reaction catalyzed by PEP carboxylase [2,5]. Since it is not obvious why different organisms have evolved different starting points to replenish the TCA cycle we decided to investigate if the phenotype of a yeast devoid of pyruvate carboxylase could be complemented by expression of a foreign PEP carboxylase. Functional expression of *E. coli* PEP carboxylase will create a branch point at the level of PEP that could result in a rerouting of metabolism during growth on glucose or in a futile cycle during growth on gluconeogenic substrates (Fig. 1). We document in this article the positive phenotypic complementation of the pyruvate carboxylase mutation and some differences observed between the wild-type yeast strain and the one expressing the *E. coli* protein.

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2. Materials and methods

2.1. Yeast strains and culture conditions

S. cerevisiae W303-1A *Mat* a *ade2-1 his3-11,15 leu 2,3-112 trp1-1 ura3-52* transformed with plasmid pAN10, a yeast-*E. coli* shuttle plasmid carrying the *URA 3* marker and a *Hin*dIII site between the yeast *ADH1* promoter and terminator [6] was used as wild-type control. *S. cerevisiae* CJM 238 *ade2-1 his3-11,15 leu 2,3-112 trp1-1 ura3-52 pyc1::LEU2 pyc2::HIS3* carries disruptions in *PYC1* and *PYC2* genes as described in [4]. The strains were grown at 30°C in 0.17% yeast nitrogen base with 40 mM ammonium sulfate or aspartate as nitrogen source and the adequate auxotrophic requirements. Glucose 2% or pyruvate 2% were used as carbon sources.

2.2. Plasmid construction

Plasmid pAN10-ppc carrying the *E. coli ppc* gene under the control of the yeast *ADH1* promoter was constructed as follows: The *ppc* gene was isolated by PCR using plasmid pS2 [7] as template. The primers used were: upstream 5′-GGGAAGCTTAATATGAACGAACAATATTCC-3′ and downstream 5′-GCAGAAAAGCTTGATTAGCCGGTA-3′. *Hin*dIII sites (underlined) were introduced to facilitate cloning into pAN10. The 2.7-kbp fragment obtained was cloned into pGEMT (Promega) and the resulting plasmid digested with *Hin*dIII. The fragment containing the *ppc* gene was cloned into the *Hin*dIII site of pAN10.

2.3. Enzyme assays

Cell extracts were obtained by shaking 100 mg of cells (wet weight) in 0.5 ml of 50 mM Tris hydrochloride, 1 mM dithiothreitol pH 7.4 with 1 g of glass beads (0.5 mm diameter) in a vortex for five periods of 1 min each, with 1 min intervals on ice. PEP carboxylase was assayed as in [8]. The reaction mixture contained: 0.1 M Tris-HCl, 0.1 mM NADH, 10 mM $KHCO_3$, 10 mM magnesium acetate, 5 mM PEP, 0.5 mM acetyl CoA, 1 unit malic dehydrogenase and the adequate amount of extract. When tested as an inhibitor, aspartate was routinely added at 0.8 mM. Pyruvate kinase was assayed as in [9]. Protein was determined with the Pierce reagent (Cultek), using bovine serum albumin as standard.

2.4. Other methods

Measurements of respiration and fermentation were carried out at 30^0 C in a conventional Warburg respirometer. The vessels contained in a final volume of 1 ml: 1% glucose, 25 mM potassium phosphate pH 6 and an adequate amount of yeast (usually 10–20 mg wet weight). Growth was followed measuring the optical density of the culture at 660 nm. For growth yield determinations, samples of cultures were filtered through glass fiber filters (Whatman GF/C) and dried until constant weight. Glucose was determined in parallel in the corresponding supernatants as in [10]. All determinations were performed in three independent cultures in different days.

3. Results and discussion

3.1. Expression of E. coli PEP carboxylase in S. cerevisiae mutants lacking pyruvate carboxylase

A multicopy plasmid carrying the coding region of *E. coli*

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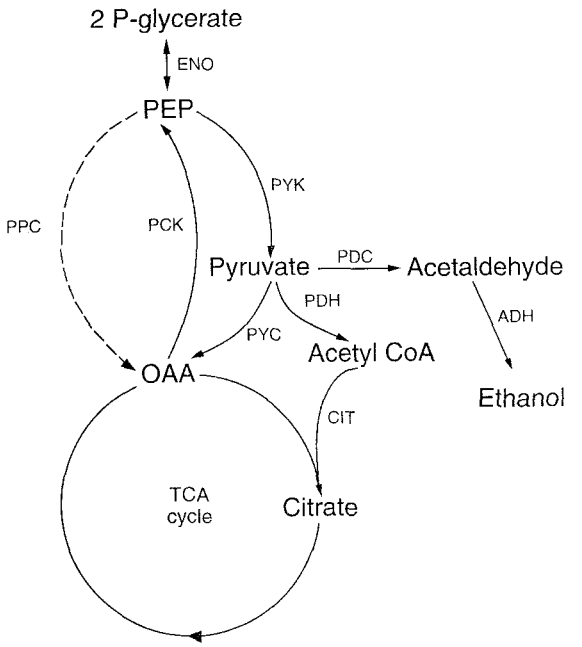

Fig. 1. Situation of pyruvate carboxylase, PEP carboxylase and the possible PEP-OAA futile cycle in the metabolism of a yeast expressing *E. coli* PEP carboxylase. Branching at the PEP level (dotted line) does not occur in wild-type yeast. (For a discussion on branching at the pyruvate level, see [18]. Abbreviations: PPC, PEP carboxylase; PYK, pyruvate kinase; PYC, pyruvate carboxylase; PCK, PEP carboxykinase; PDC, pyruvate decarboxylase; PDH, pyruvate dehydrogenase complex; ADH, alcohol dehydrogenase; ENO, enolase; CIT, citrate synthase; OAA, oxaloacetate; TCA, tricarboxylic acid cycle.

PEP carboxylase under the control of the yeast *ADH1* promoter was constructed as described in Section 2. We transformed with this plasmid a *S. cerevisiae* strain whose *PYC1* and *PYC2* genes were disrupted. Transformants were selected on a permissive medium for the double *PYC* disruptants (glucose-aspartate), and then transferred to a non-permissive one (glucose-ammonium). All transformants grew on this medium indicating that the activity of the *E. coli* enzyme could alleviate the phenotype produced by the absence of the two *PYC* genes. To discard the unlikely possibility that the growth were caused by some extragenic suppressor [11] we used two controls: measurement of PEP carboxylase activity and plasmid loss. The transformants showed an enzymatic activity that was dependent on PEP, strongly activated by acetyl CoA and inhibited by aspartate. This activity was not detected in a control strain transformed with the same plasmid without the *ppc* gene (Fig. 2). Loss of the plasmid was accompanied by loss of the ability to grow on glucose ammonium medium (Fig. 3). These results demonstrate that the activity of the *E. coli* PEP carboxylase complements the phenotype of *S. cerevisiae* mutants lacking pyruvate carboxylase.

3.2. *Effect of PEP carboxylase expression on different physiological parameters*

Replacement of pyruvate carboxylase by *E. coli* PEP carboxylase might produce qualitative and quantitative differences with respect to a wild-type yeast. Qualitatively, the organism will have a new branching point at the level of PEP and pyruvate kinase and PEP carboxylase will compete for PEP (Fig. 1) and quantitatively the proportion of glycolytic intermediates funneled into oxaloacetate could be different. It is difficult to predict the distribution of PEP between pyruvate kinase and PEP carboxylase because the actual activities of both enzymes depend on the concentration of their respective allosteric activators, fructose-1,6-bisphosphate and acetyl CoA [12,8]. While reliable data exist for the concentrations of fructose-1,6-bisphosphate in yeast [13], this is not the case for acetyl CoA. For pyruvate kinase we measured an activity in glucose of 7 units/mg protein in the presence of 1 mM fructose-1,6-biphosphate and for PEP carboxylase activities varied between 40 mU/mg protein without addition of acetyl CoA and 2 mU/mg protein at 0.5 mM of the activator.

In a wild-type yeast pyruvate carboxylase, pyruvate decarboxylase and pyruvate dehydrogenase compete for pyruvate. The capacity of pyruvate dehydrogenase in a yeast growing in glucose is low, so that at a semiquantitative level its contribution may be neglected. The K_m values for pyruvate of pyruvate carboxylase and pyruvate decarboxylase are respectively 0.4 mM [4] and 1 mM [14] and the activities 20–40 mU/mg protein pyruvate carboxylase (4, our own results) compared with 1.5 units/mg protein of pyruvate decarboxylase [15,16]. Therefore it can be calculated that in the case of a wild-type yeast the deviation to oxaloacetate by pyruvate carboxylase will be about 1/50 of the incoming flux while in the case of the yeast expressing PEP carboxylase the proportion of PEP yielding oxaloacetate may range from 1/15 to 1/7000 of the flux depending on the actual concentration of acetyl CoA in the cytoplasm. Differences in the rate at which oxaloacetate is supplied to the TCA cycle may influence the fermentative behavior normally observed in glucose batch cultures [17,18]. To see if this was the case we measured the fermentation and respiration rates of a wild-type yeast and of a pyruvate carboxylase mutant expressing PEP carboxylase (Table 1).

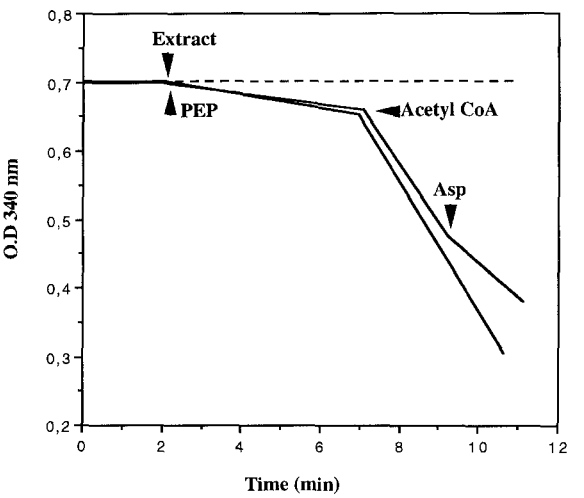

Fig. 2. Demonstration of PEP carboxylase activity in a pyruvate carboxylase mutant transformed with plasmid pAN10-ppc. *S. cerevisiae* CJM 238 (*pyc1::LEU2 pyc2::HIS3*) transformed with plasmid pAN10-ppc was used. Extracts were made and activity measured as described in Section 2. All the components of the reaction mixture were present initially, except those indicated that were added at the times shown by the arrows (aspartate was added only to one of the samples). The dashed line shows the variation of optical density of a complete mixture of an extract of the same strain transformed with plasmid pAN10. In the assay conditions a change of the optical density of 0.012 corresponds to the oxidation of 1 nmole of NADH.

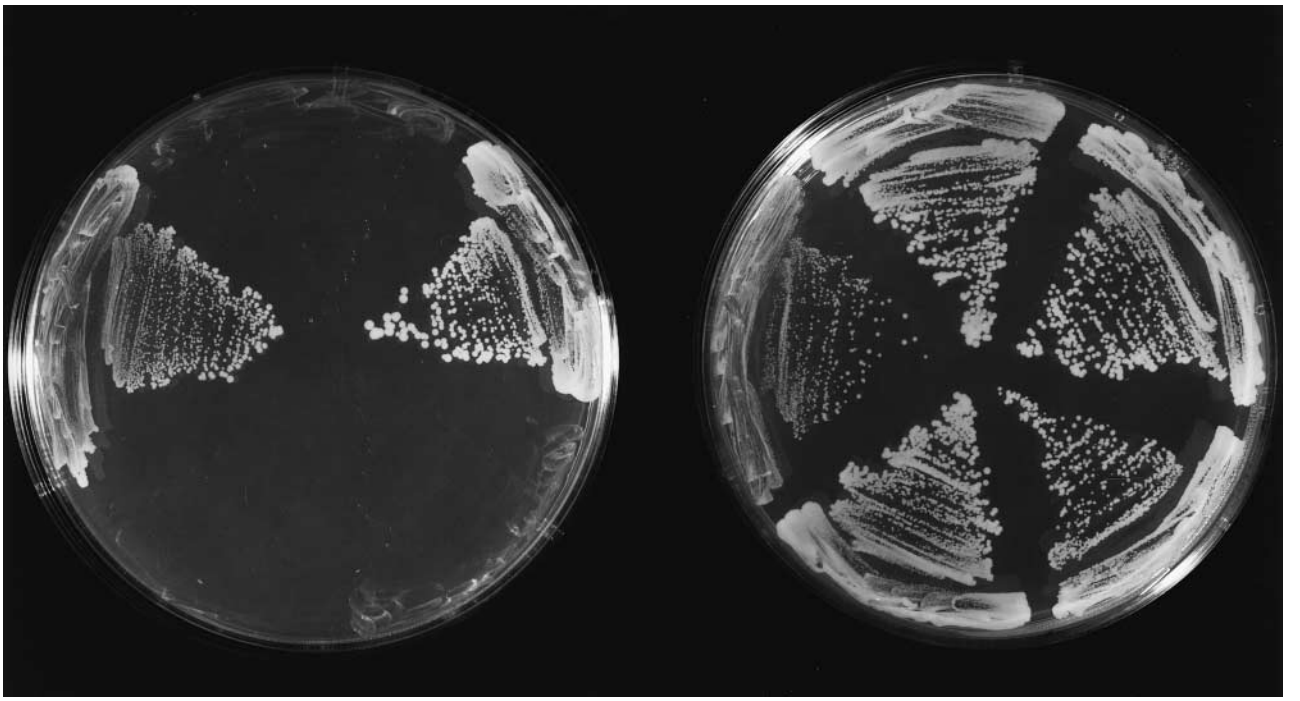

Fig. 3. Phenotypic complementation of *S. cerevisiae pyc* mutants by *E. coli* PEP carboxylase. The *S. cerevisiae* strains were plated on glucose-ammonium medium (left) or glucose-aspartate medium (right). Clockwise from the upper left quadrant: *S. cerevisiae* W303-1A (wild type) transformed with plasmid pAN10; *S. cerevisiae* CJM238 (*pyc1::LEU2 pyc2::HIS3*); *S. cerevisiae* CJM238 transformed with plasmid pAN10-ppc (carrying *E. coli ppc*); *S. cerevisiae* CJM238 transformed with plasmid pAN10; *S. cerevisiae* CJM238 transformed with plasmid pAN10-ppc after plasmid loss.

In resting cells the amount of glucose fermented was the same in both strains but the rate of respiration although low was twofold higher in the PEP carboxylase expressing strain than in the wild type. This suggests that a higher amount of the incoming glucose is being fuelled to the TCA cycle. Both strains showed the same glucose consumption in resting cells. During growth, both strains consumed glucose at higher rate than in the resting state, a phenomenon well known in yeast but not yet satisfactorily explained [19], but the transformed yeast used glucose at a slower rate than the wild type. This could indicate that modifications in the systems that metabolize PEP and pyruvate are able to influence the glycolytic flux. One possibility is that the increased respiration inhibits glucose uptake. Although no mechanism relating transport and respiration has been identified yet, glucose consumption appears to be related with the respiratory capacity of the yeast [20,21]. Whereas growth rate was decreased in the yeast expressing the heterologous protein, growth yield was similar in both cases (Table 1) and the value was in the same range as that found by others for yeast growing in minimal media [22].

When pyruvate was the carbon source the growth rate of the PEP carboxylase expressing strain was also impaired (Table 1). This is likely due to the existence of a futile cycle between PEP carboxylase and the antagonistic gluconeogenic PEP carboxykinase, active under these conditions [23,24]. In yeast, operation of futile cycles between glycolytic and gluconeogenic enzymes produces a decrease in the fitness of the organism although less deleterious than could have been anticipated [25].

From our results it appears that at least in a laboratory setting, there is no basic incompatibility between fermentative yeast metabolism and use of the PEP carboxylase instead of pyruvate carboxylase to replenish the TCA cycle. The differences observed in the glucose grown cultures could be related more with an excess in the amount of PEP carboxylase expressed than with the nature of the reaction catalyzed itself. It is therefore possible that the different solutions adopted by

Table 1
Effects of the expression of *E. coli* PEP carboxylase on different physiological parameters of *S. cerevisiae*

Strain	Fermentation (μmol CO_2/g yeast/min)	Respiration (μmol O_2/g yeast/min)	Glucose consumption (μmol/g yeast/min)		Growth yield (g yeast/g glucose)	Generation time (min)	
			Resting	Growing		Glucose	Pyruvate
W303-1A/pAN10	141 ± 35	18 ± 3	102	233	0.15	110	280
CJM238/pAN10-ppc	136 ± 26	36 ± 7	101	165	0.15	155	380

Yeasts were grown and parameters measured as described in Section 2. For fermentation and respiration measurements cells were grown on glucose and harvested in the exponential phase of growth. The rate of glucose consumption during growth was calculated using the formula (0.693/generation time) × (g glucose/g yeast). Yeast weights are dry weights.

diverse organisms to replenish the TCA cycle are the result of aleatory events and not a specific adaptation to different metabolic styles.

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