A mutation affecting carbon catabolite repression suppresses growth defects in pyruvate carboxylase mutants from *Saccharomyces cerevisiae*

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Abstract **Yeasts with disruptions in the genes *PYC1* and *PYC2* encoding the isoenzymes of pyruvate carboxylase cannot grow in a glucose-ammonium medium (Stucka et al. (1991) Mol. Gen. Genet. 229, 307–315). We have isolated a dominant mutation, *BPC1-1*, that allows growth in this medium of yeasts with interrupted *PYC1* and *PYC2* genes. The *BPC1-1* mutation abolishes catabolite repression of a series of genes and allows expression of the enzymes of the glyoxylate cycle during growth in glucose. A functional glyoxylate cyle is necessary for suppression as a disruption of gene *ICL1* encoding isocitrate lyase abolished the phenotypic effect of *BPC1-1* on growth in glucose-ammonium. Concurrent expression from constitutive promoters of genes *ICL1* and *MLS1* (encoding malate synthase) also suppressed the growth phenotype of *pyc1 pyc2* mutants. The mutation *BPC1-1* is either allelic or closely linked to the mutation *DGT1-1*.**

Key words: Pyruvate carboxylase; Glyoxylate cycle; Catabolite repression; Yeast

1. Introduction

The citric acid cycle performs two important roles: not only is it the final oxidation pathway for different types of nutrients but also several of its intermediates are used for synthesis of different cell constituents [1]. This last function implicates that anaplerotic reactions shall function to replenish the cycle and compensate for the withdrawal of intermediates. When *Saccharomyces cerevisiae* grows in a medium with glucose as carbon source and ammonium as nitrogen source the citric acid cycle is replenished by the action of pyruvate carboxylase (for reviews see [2,3]). Hence disruption of the *PYC1* and *PYC2* genes, encoding the two isoenzymes of pyruvate carboxylase, abolishes growth in the mentioned medium and results in a requirement for aspartate for yeasts growing in synthetic media with glucose as carbon source [4]. Aspartate replenishes the cycle by providing oxaloacetate most likely via transamination with α-ketoglutarate. We observed that yeast strains with disrupted *PYC1* and *PYC2* genes were able to grow in glycerol or pyruvate with ammonium as nitrogen source without added aspartate. This observation indicated that glucose interferes with the operativity of anaplerotic reactions that are functional in glycerol or pyruvate growing cells but do not operate during growth in glucose. In fact, glucose represses isocitrate lyase and malate synthase, the two specific enzymes of the glyoxylate cycle which has an anaplerotic role during growth in 2-C compounds [5–8]. Therefore, mutations that would allow growth of *pyc1 pyc2* mutants in a glucose-ammonium medium could affect catabolite repression of genes encoding enzymes that participate in anaplerotic reactions used in the absence of glucose. Alternatively, other reactions bypassing pyruvate carboxylase could be functional in the new mutants. This article presents our results in the search for mutants with the mentioned phenotype.

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2. Material and methods

2.1. Strains

All strains used in this work are isogenic and are derived from strain W303–1A, *MATa ade2–1 his3–11,15 leu2–3,112 ura3–52 trp1–1*. The strain defective in both pyruvate carboxylase isoenzymes used as starting material was strain 22B, *MATa ade2–1 his3–11,15 leu2–3,112 ura3 trp1–1 pyc1::LEU2 pyc2::URA3* [4]. Strain 22BHL has the same genetic background but is *pyc1::LEU2 pyc2::HIS3* (see below for its construction). Crosses were carried out either with W303–1A or with W303–1B (*MATα ade2–1 his3–11,15 leu2–3,112 ura3–52 trp1–1*). Diploids were isolated as zygotes. Sporulation and asci dissection were performed by conventional methods.

2.2. Media and growth conditions

Rich media contained 1% yeast extract, 2% peptone and 2% glucose or 2% pyruvate. Minimal media were 0.17% YNB without ammonium sulfate (Difco), 40 mM nitrogen source as indicated, 2% glucose or 2% pyruvate as carbon and energy sources and the required auxotrophic requirements. Growth was at 30°C and liquid cultures were grown with shaking.

2.3. Mutagenesis

About 10^7 cells of strain 22B were irradiated on a YNB glucose-ammonium plate with an UV germicidal lamp. Colonies appearing after several days were picked and purified on plates with a medium of the same composition.

2.4. Plasmids

The following plasmids were used: pUC18 [9], YEp352 [10], YEplac112 [11], pUC-HIS3 [12] and pGEM-T (Clontech). Plasmid pAAH5 carries the promoter and terminator of yeast *ADH1* [13]. It was digested with *Hin*dIII, blunt ended, and *Nco*I linkers were added to generate plasmid pAAH5-NcoI.

2.5. Gene disruption

Gene *PYC2* was disrupted with *HIS3* as follows: The 4.1 kb *Hin*dII-*Sma*I fragment from pRS2 [4] with the gene *PYC2* was introduced into pUC18 digested with *Hin*dIII and *Sma*I. The resulting plasmid was digested with *Bam*HI and the 1.1 kb *Bam*HI-*Bam*HI fragment from plasmid pUC-HIS3 was inserted, yielding pIC5. For the construction of 22BHL, strain 22A (*pyc1::LEU2 PYC2*) [4] was transformed with the 5.2 kb *Eco*RI-*Eco*RI fragment of pIC5. Gene *ICL1* was disrupted with *HIS3* as follows: plasmid pUC18 was digested with *Eco*RI and *Bam*HI blunt ended and religated. The resulting plasmid was cut with *Xba*I and *Sph*I and a 2.3 kb *Xba*I-*Sph*I fragment from plasmid pICL1.1–5 [6], carrying the *ICL1* gene inserted in it. The resulting plasmid was then digested with *Bam*HI and *Xho*I and a 1.3 kb DNA

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fragment from plasmid pUC-HIS3 carrying the *S. cerevisiae HIS3* gene was inserted. The chromosomal *ICL1* copy was exchanged with the interrupted one as described by Rothstein [12]. Integration was checked by Southern blot analysis and lack of enzymatic activity.

2.6. Construction of plasmids pADH1-ICL1 and pADH1-MLS1

Plasmid pADH1-ICL1 carrying the gene *ICL1* under the control of the promoter of the *ADH1* gene was constructed as follows: The coding region from *ICL1* was amplified with DNA from plasmid pICL1.1-S [6] as template using the following oligonucleotides as primers: upstream, 5'-CCCATGGTGCCTATCCCCGTTGG-3'; downstream 5'-GGCCATGGCCTATTTCTTTACGCC-3'. Two *Nco*I sites were added to facilitate subcloning (underlined). The1.6 kb *Nco*I-*Nco*I fragment carrying the structural *ICL1* gene obtained from PCR was inserted into plasmid pAAH5-NcoI (see above). From this plasmid a 2.3 kb fusion carrying the *ADH1* promoter fused with the *ICL1* gene was excissed by partial digestion with *Bam*HI and inserted into YEplac112.

Plasmid pADH1-MLS1 carrying the gene *MLS1* under the control of the promoter of the *ADH1* gene was constructed as follows: The coding region of *MLS1* was amplified by PCR using DNA from plasmid YE352-MLS (sent by A. Hartig) using the following oligonucleotides as primers: upstream, 5'-GGTGTCGACACCTATATGACTG-3'; downstream 5'-GGGTCGACTGGGGCAAGGGGAGATC-3'. Two *Sal*I sites were added to facilitate subcloning (underlined). The PCR products were cloned into pGEM-T. Plasmid pAAH5-NcoI was digested with *Nco*I blunt ended, digested with *Bam*HI and the 1.5 kb fragment carrying the *ADH1* promoter was ligated into YEp352 previously cut with *Xba*I blunt ended and digested with *Bam*HI. The resulting plasmid was digested with *Sal*I and the 1.3 kb *Sal*I-*Sal*I fragment carrying the structural gene from *MLS1* obtained by PCR was inserted in it. This plasmid carries *URA3* as yeast marker.

2.7. Enzymatic assays

Enzymatic activities were determined in supernatants of crude extracts obtained as described by Blázquez et al. [14]. Glutamate dehydrogenase (NAD) was assayed as in [15], NADH:cytochrome *c* oxidase as in [16], fructose-1,6-bisphosphatase according to Gancedo and Gancedo [17], isocitrate lyase after Dixon and Kornberg [18] and malate dehydrogenase as described by Witt et al. [19]. Protein was assayed according to Lowry et al. [20].

3. Results

3.1. Isolation and genetic characterization of the mutants

A yeast strain with a disruption in each of the genes *PYC1* and *PYC2* encoding pyruvate carboxylase does not grow in a glucose-ammonium medium but grows in one with glucose and aspartate [4] (Fig. 1). After UV irradiation of a *pyc1::LEU2 pyc2::URA3* strain (see section 2) we isolated several colonies able to grow in the non-permissive medium. Each of these colonies was purified and crossed with an isogenic strain also disrupted in both pyruvate carboxylase genes. All diploids grew on glucose-ammonium medium indicating that the mutations were dominant. The segregation of the phenotype of growth on minimal medium glucose was $2^+:2^-$ in all complete tetrads analyzed for each mutant indicating that the mutations were nuclear and monogenic. We provisionally named them *BPC* for bypass of pyruvate carboxylase. Adequate crosses among independent mutants showed that they belonged to two complementation groups *BPC1-* and *BPC2* We have studied and further characterized the mutation *BPC1-1.*

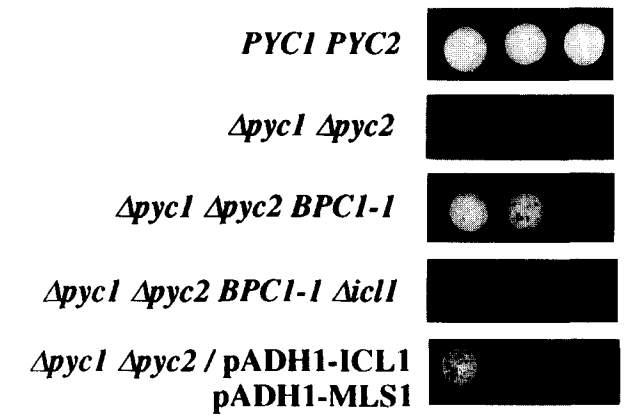

Fig. 1. Growth of strains carrying mutations in both PYC1 and PYC2 genes. The yeasts were grown in minimal glucose-ammonium medium with the adequate auxotrophic requirements in each case. The relevant genotype of the strains is shown at the left. The three spots in each row originated from decreasing 1/10 dilutions of the corresponding culture. The relevant genotypes and plasmids of each strain are shown on the left.

Table 1
Generation times (min) of strains carrying the *BPC1-1* mutation, in media with different carbon and nitrogen sources

Relevant genotype	Nitrogen source			
	Amonium		Aspartate	
	Glucose	Pyruvate	Glucose	Pyruvate
PYC1 PYC2 BPC1	150	345	145	230
pyc1 pyc2 BPC1	NG	360	145	230
pyc1 pyc2 BPC1-1	520	310	260	240
PYC1 PYC2 BPC1-1	240	335	260	240

Yeasts were grown in minimal media with 2% glucose or pyruvate as carbon sources and the indicated nitrogen sources at a final concentration of 40 mM. NG, no growth.

The mutation *BPC1-1* was taken out of the *pyc1::LEU2 pyc2::URA3* background after dissection of 53 complete tetrads derived from a cross between the mutant and a wild type. It can be calculated that with a probability of 1/36 a tetrad with two spores *pyc1::LEU2 BPC1*, *pyc2::URA3 BPC1* and two *PYC1 PYC2 BPC1-1* will be found. We identified a tetrad with these characteristics and a *PYC1 PYC2 BPC1-1* spore was selected and used in this work. Its phenotypic behaviour (see below) showed that it had the expected genotype.

3.2. Generation times

The generation times on different culture media of strains carrying the *pyc1::LEU2 pyc2::HIS3* interruptions, the double interruption and the *BPC1-1* mutation or the *BPC1-1* mutation alone are shown in Table 1. As expected, the *pyc1 pyc2* disruptant does not grow in minimal glucose-ammonium medium. A strain bearing the *BPC1-1* mutation grew in this medium although its generation time was over 8 h as compared with 2 h 30 min for the wild type. The *BPC1-1* mutation by itself decreased the growth rate in glucose media but had no effect in media with pyruvate as carbon source.

3.3. Biochemical characterization of the mutants

We assayed several enzymes repressed by glucose and related with the replenishment of the citric acid cycle like isocitrate lyase, malate dehydrogenase and glutamate dehydrogenase, and others not directly related with this function. These activities were assayed during growth in glucose or pyruvate containing media in strains carrying the double *PYC1 PYC2* interruption, the double interruption and the *BPC1-1* mutation or the

BPC1-1 mutation alone. The results of the assays are shown in Table 2. It can be seen that in the *BPC1-1* mutants all enzymes tested had lost the repression by glucose although to different extents. The strongest effect was observed with cytochrome *c* oxidase which was totally derepressed. Malate dehydrogenase and glutamate dehydrogensae were derepressed to intermediate levels while isocitrate lyase and fructose-1,6-bisphosphatase reached values of 17 and 25%, respectively, of the levels measured in pyruvate grown cells. It appears therefore that a defect in the *BPC1* gene affects the correct functioning of catabolite repression of a variety of genes.

3.4. Suppression of the pyruvate carboxylase-less phenotype requires function of the glyoxylate cycle

The *BPC1-1* mutation could suppress the pyruvate carboxylase-less phenotype either by relieving the repression of the glyoxylate cycle enzymes or by allowing the operation of some reaction that would bypass pyruvate carboxylase function. If the first idea is correct, disruption of one of the genes encoding a specific enzyme of the cycle would abolish the effect of *BPC1-1*. Therefore, we disrupted the gene *ICL1* encoding isocitrate lyase in a wild type and in a strain with the relevant genotype *pyc1::LEU2 pyc2::URA3 BPC1-1*. While disruption of *ICL1* in the wild type was without effect on growth in glucose (not shown) it abolished growth in glucose-ammonium in the strain carrying the *BPC1-1* suppressor (Fig. 1). Growth in glucose-aspartate of this strain was unaffected by the *ICL1* disruption (not shown). This result shows that functionality of the glyoxylate cycle is necessary for the suppression effected by *BPC1-1*. To test if functionality of the cycle would be sufficient to compensate for the defect caused by the double *pyc1 pyc2* mutation we constructed plasmids with the genes *ICL1* and *MLS1*, encoding isocitrate lyase and malate synthase, respectively, under the control of the *ADH1* promoter. These plasmids expressed isocitrate lyase and malate synthase when the yeasts grew in glucose to 75 and 50%, respectively, of the levels found in fully derepressed cultures. When these plasmids were introduced in a strain carrying disrupted *PYC1 PYC2* genes the transformants carrying these constructs were able to grow in glucose-ammonium (Fig. 1) although growth was slower than that of those carrying the *BPC1-1* mutation.

3.5. The mutation BPC1-1 is allelic or closely linked with DGT1-1

While this work was in progress another dominant mutation, *DGT1-1*, showing a similar effect on the derepression of glucose repressed enzymes, was isolated in our laboratory by a different approach [24]. When this mutation was introduced in a pyruvate carboxylase-less strain we found that it suppressed the growth phenotype. We therefore tested the possible allelism of *DGT1-1* and *BPC1-1*. The progeny of a cross between strains of genotypes *pyc1::LEU2 pyc2::URA3 BPC1-1* and *pyc1::LEU2 pyc2::URA3 DGT1-1* was analyzed. Eight complete tetrads gave a $4^+:0^-$ segregation for growth in glucose-ammonium. This result indicates that *BPC1-1* and *DGT1-1* are either allelic or closely linked.

4. Discussion

We have isolated a dominant mutation, *BPC1-1*, that allows yeasts with both *PYC1* and *PYC2* genes interrupted to grow in glucose-ammonium media. The molecular basis of the suppression appears to be the release from carbon catabolite repression of the genes encoding the enzymes of the glyoxylate cycle. That the glyoxylate cycle plays a predominant role in the suppression of the pyruvate carboxylase-deficient phenotype is highlighted by two results: (a) the inability of the *BPC1-1* mutation to suppress the pyruvate carboxylase-deficient phenotype when the gene *ICL1* is disrupted and (b) the ability of the pyruvate carboxylase-deficient mutants to grow in glucose-ammonium medium when both *ICL1* and *MLS1* are expressed from the non-repressible *ADH1* promoter. The fact that these last cells grew somewhat slower in glucose-ammonium than those carrying the *BPC1-1* mutation may be due to the fact that in the *BPC1-1* cells respiratory enzymes – and likely other tricarboxylic acid cycle enzymes – were derepressed and the glucose could be used more effectively for energy production.

Although it has been reported that interruption of *PYC1* results in an aspartate requiring phenotype during growth in ethanol [21], we have consistently found that in our strain each gene could substitute for the other for growth in the carbon sources we tested and that the requirement for aspartate was only observed during growth in repressing carbon sources. Different genetic backgrounds that influence the expression of each of the genes may account for this difference in results.

The dominant character of the mutation *BPC1-1* suggests that the product of the wild type gene could be a repressor acting on several systems controlled by glucose. Some of the properties of the mutant described were reminiscent of those reported for the mutations *CCR80* [22] or *CCR91* [23]. Unfortunately, these mutants were not available so that it was not possible to test their possible allelism with the mutation reported here. However, we found that the mutation described was allelic or very closely linked with the mutation *DGT1-1* reported by Gamo et al. [24]. This mutation, isolated as a suppressor of the toxic effect caused by glucose in a phosphoglycerate mutase mutant, presents a decreased glucose transport and releases from catabolite repression a series of

Table 2
Specific activities (mU/mg protein) of several enzymes repressed by glucose in strains carrying the *BPC1-1* mutation

Relevant genotype	ICL		MDH		GluDH		CytcOx		FbPase	
	G	P	G	P	G	P	G	P	G	P
PYC1 PYC2 BPC1	<1	49	159	2670	10	203	15	100	<1	65
pyc1 pyc2 BPC1	1	81	190	2350	17	265	20	78	1	60
pyc1 pyc2 BPC1-1	17	79	586	2840	126	185	110	108	13	58
PYC1 PYC2 BPC1-1	7	41	380	2100	100	190	100	107	15	60

Yeasts were grown in rich medium with 2% glucose (G) or pyruvate (P) as carbon source and harvested during the exponential phase of growth. Enzymes were assayed as described in section 2. Figures are mean values of at least three different cultures. ICL, isocitrate lyase; MDH, malate dehydrogenase; GluDH, glutamate dehydrogenase (NAD); CytcOx, cytochrome *c* oxidase; FbPase, fructose-1,6-bisphosphatase.

enzymes. The results presented show that carbon catabolite repression may in some cases affect the growth behaviour of yeasts in different nitrogen sources and also demonstrate the potentiality to find new types of carbon catabolite repression mutations of searching for suppressors of other mutations.

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