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Futile cycles in *Saccharomyces cerevisiae* strains expressing the gluconeogenic enzymes during growth on glucose

(yeast/fructose-1,6-bisphosphatase/phospho*enol*pyruvate carboxykinase/^{13}C NMR)

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ABSTRACT **The systems which control the levels of the gluconeogenic enzymes in *Saccharomyces cerevisiae* have been bypassed to ascertain their physiological significance. The coding regions of the genes *FBP1* and *PCK1*, which encode fructose-1,6-bisphosphatase and phospho*enol*pyruvate carboxykinase, have been put under the control of the promoter of *ADC1* (alcohol dehydrogenase I), a gene not repressed by glucose, and introduced into yeast in multicopy plasmids. The transformed yeast cells show high levels of the gluconeogenic enzymes during growth on glucose. Generation time and growth yield of yeast expressing either fructose-1,6-bisphosphatase or phospho*enol*pyruvate carboxykinase are not significantly different from those of the wild-type strain. For a strain expressing both enzymes the increase in generation time is about 20% and the decrease in growth yield around 30%. The concentration of ATP is about 1.5 mM in the growing cells of the different strains. The extent of *in vivo* cycling was measured by ^{13}C NMR in cell-free extracts from yeast growing on [6-^{13}C]glucose. Cycling between fructose-6-phosphate and fructose-1,6-bisphosphate is <2%, most likely due to the very strong inhibition of fructose-1,6-bisphosphatase by fructose 2,6-bisphosphate. Cycling between phospho*enol*pyruvate and pyruvate is low, but a precise figure could not be obtained due to poor equilibration of label between carbons 2 and 3 of oxaloacetate.**

Saccharomyces cerevisiae can grow on sugars, metabolized through the glycolytic pathway, or on 2-C or 3-C compounds, which require gluconeogenesis. Gluconeogenesis proceeds by a reversal of glycolysis except for the reactions catalyzed by phosphofructokinase and pyruvate kinase. These steps are bypassed by fructose-1,6-bisphosphatase (FbPase) and phospho*enol*pyruvate carboxykinase (PEPCK) together with pyruvate carboxylase. Simultaneous operation of the antagonistic pairs phosphofructokinase/FbPase and pyruvate kinase/PEPCK will result in futile cycles with net hydrolysis of ATP. Therefore the shift between glycolysis and gluconeogenesis and vice versa raises the problem of how to control the antagonistic enzymes to prevent the operation of futile cycles. Although pyruvate carboxylase participates in the bypass of pyruvate kinase, it is not exclusively a gluconeogenic enzyme and its level remains fairly constant in yeast grown on different carbon sources (1).

Phosphofructokinase and pyruvate kinase are largely regulated at the level of activity: the differences in the amount of these enzymes in glycolytic and gluconeogenic conditions do not exceed 2-fold (2, 3). During gluconeogenesis, phosphofructokinase activity is low due to the low concentrations of its substrate fructose 6-phosphate and its activator fructose 2,6-bisphosphate (Fru-2,6-P_2) (4). Pyruvate kinase remains nearly inactive due to the low concentration of its allosteric activator fructose 1,6-bisphosphate (5). In contrast, the amount of the gluconeogenic enzymes FbPase and PEPCK is tightly regulated. Their synthesis is strongly repressed by glucose (6, 7) and they are proteolytically degraded when glucose is added to a derepressed yeast (8–10), a process known as catabolite inactivation (11). In addition, yeast FbPase is inhibited by Fru-2,6-P_2 (12) and by AMP (6). The existence of a multilayered regulation of the gluconeogenic enzymes through catabolite repression, catabolite inactivation, and, at least for FbPase, allosteric control suggests that the different mechanisms have been selected to avoid the operation of futile cycles in yeast growing on glucose. A yeast expressing FbPase under the control of the *PHO5* promoter is able to grow on glucose (13), but no attempts have been made to determine whether cycling takes place when some of the controls on FbPase or PEPCK are removed and how this cycling affects the growth of the yeast.

To investigate the physiological relevance of catabolite repression and catabolite inactivation of the gluconeogenic enzymes, we have put the genes encoding FbPase and PEPCK under the control of a promoter not repressed by glucose. We found that the expression of the gluconeogenic enzymes under glycolytic conditions has no striking consequences on yeast survival.

MATERIALS AND METHODS

Materials. ^{13}C-labeled glucose (99.9% ^{13}C) and $^{2}H_2O$ (99.9% ^{2}H) were obtained from ISOTEC (Miamisburg, OH).

Plasmids, Strains, and Culture Conditions. The plasmids used in this work are shown in Fig. 1. To construct pAN5, the *PCK1* gene (encoding PEPCK) was inserted into pAAH5 (14); the *PCK1* gene was taken from pMV7 (15) after replacement of the *Eco*RI site by a *Hin*dIII site and introduction, by site-directed mutagenesis, of a new *Hin*dIII site at position −37 with respect to the first ATG of the coding sequence. To construct pAN10, the *Bam*HI–*Bam*HI fragment from pAAH5, which contains the promoter and terminator of *ADC1*, was introduced into the *Bam*HI site of YEp352 (16) after elimination of the *Hin*dIII site from the multiple cloning site of YEp352. To construct pAN11, the *Nde* I–*Xba* I fragment carrying the *FBP1* gene (encoding FbPase) was taken from pRG6 (13, 17), *Hin*dIII linkers were added to the blunted ends, and the resulting DNA was inserted into the *Hin*dIII site of pAN10.

Escherichia coli strains HB101 and TG1 (18) were used for plasmid propagation and isolation. All yeast strains were derived from *S. cerevisiae* CJM152 (*MAT***a** *leu2-3,112 ura3-251,328,373*) by transformation with two plasmids, as follows: transformation with pAAH5 and pAN10 gave strain

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Abbreviations: FbPase, fructose-1,6-bisphosphatase; Fru-2,6-P_2, fructose 2,6-bisphosphate; G6PDH, glucose-6-phosphate dehydrogenase; PEPCK, phospho*enol*pyruvate carboxykinase; YNB, yeast nitrogen base with ammonium sulfate.

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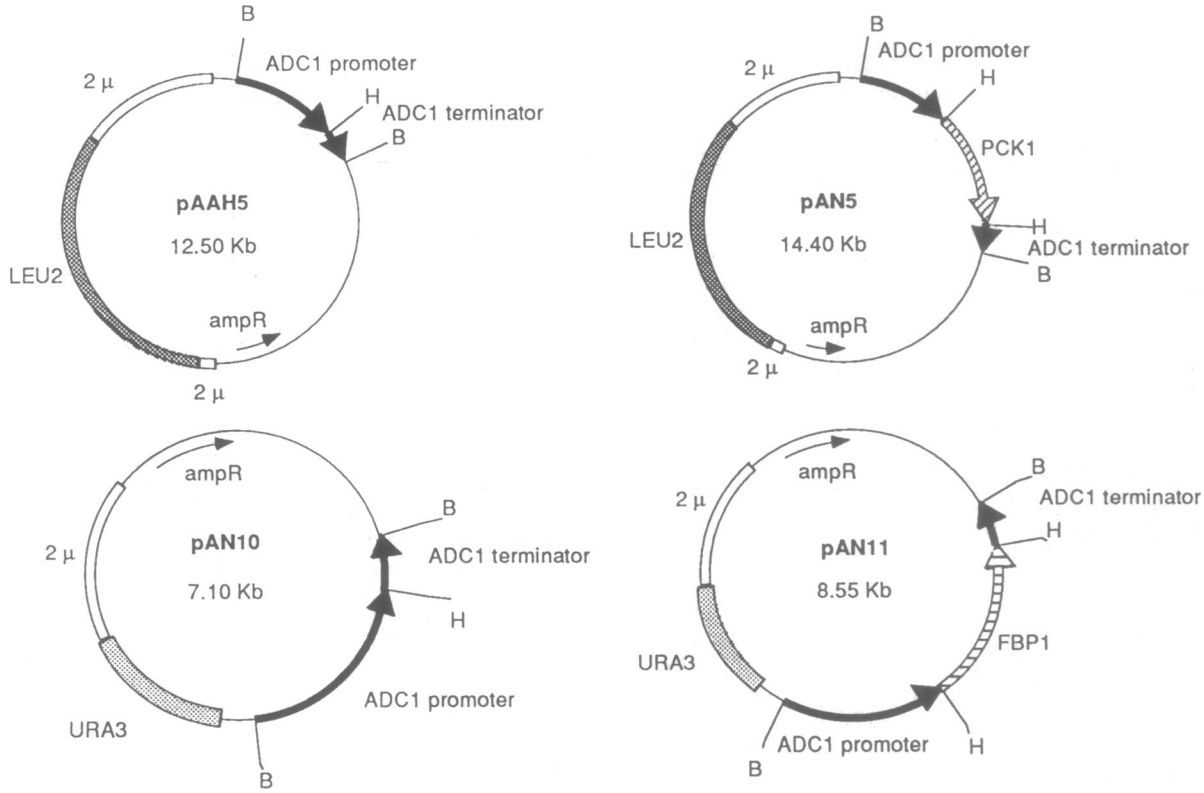

FIG. 1. Plasmids used in this work. Construction details are given in *Materials and Methods*. B, *Bam*HI; H, *Hin*dIII; ampR, ampicillin-resistance gene; 2 μ, DNA from the yeast 2-μm plasmid.

CJM189, with pAN5 and pAN10, strain CJM190, with pAAH5 and pAN11, strain CJM191, and with pAN5 and pAN11, strain CJM186 (see Fig. 1).

Yeasts were grown with shaking at 30°C in 0.7% Difco yeast nitrogen base with ammonium sulfate (YNB) and 1% or 2% glucose. Generation times were measured by following the OD_{660}. To measure growth yields, culture samples were filtered through Whatman GF/C glass-fiber filters and dried at 80°C to constant weight.

DNA Manipulations. Recombinant DNA manipulations were done by standard techniques (18). Yeast transformation was carried out as described (19). Site-directed mutagenesis to introduce a *Hin*dIII site in the *PCK1* promoter was performed with the Amersham kit, using the synthetic oligonucleotide 5′-AAACCAAGCTTACGCAA-3′.

Measurement of Enzyme Activities, Fermentation, and Respiration. Extracts were prepared with glass beads (8) in 20 mM imidazole (pH 7). FbPase, PEPCK, and glucose-6-phosphate dehydrogenase (G6PDH) were measured spectrophotometrically (8, 20, 21). One unit is the amount of enzyme which transforms 1 μmol of substrate per minute at 30°C. Protein was assayed according to Lowry *et al.* (22). Rates of fermentation and respiration were measured in a Warburg respirometer at 30°C, with yeast suspensions [7 mg (wet weight)/ml] in 50 mM phosphate buffer (pH 6) containing 2% glucose.

Determination of Metabolites. Samples were obtained and treated (23) and metabolites were assayed (24) as described. Fru-2,6-P_2 was measured in alkaline extracts (25). To measure ethanol production, yeast suspensions in buffer (see above) were incubated in flasks with tightly fitting rubber caps and sampled with a hypodermic needle. Ethanol and glucose in the medium were measured by using alcohol dehydrogenase and hexokinase/G6PDH, respectively (24).

Measurement of Futile Cycles by ^{13}C NMR. Yeast cells grown on YNB with glucose were collected during the logarithmic phase of growth and resuspended at 20 mg (wet weight)/ml in fresh medium containing 2% [6-^{13}C]glucose. The yeast suspension was incubated with shaking at 30°C for 1 hr. Then two aliquots of 12.5 ml were filtered and the yeast cells were extracted with trichloroacetic acid (26). A sample of the medium was also taken and analyzed by ^{13}C NMR after addition of 10% ^2H_2O. After trichloroacetic acid was removed with ethyl ether, the extracts were freeze-dried and resuspended in 3 ml of ^2H_2O. Similar experiments were performed with [1-^{13}C]- or unlabeled glucose, the latter as control to assess the contribution of natural-abundance ^{13}C. High-resolution ^{13}C NMR analysis of extracts and medium was performed at 8.4 T on a Bruker AM-360 NMR spectrometer. Broad-band proton-decoupled ^{13}C spectra (22°C, pH 4.7) were obtained at 90.55 MHz by using a WALTZ-16 decoupling sequence gated only during the acquisition (0.3-W average forward power). ^{13}C NMR conditions were as follows: 55° pulses, 200 ppm sweep width, 64-kiloword data table (1.57-sec acquisition time), and 6.0-sec total recycle time. Usually 8000 scans, representing 13 hr of accumulation time, were collected. Free induction decays were zero-filled to 128 kilowords prior to Fourier transformation. Chemical shifts were referred to the signal of a 10% dioxane solution (67.4 ppm) placed in a concentric capillary.

RESULTS

Strains with Unregulated Gluconeogenic Enzymes. To obtain strains expressing the gluconeogenic enzymes during growth on glucose, we constructed plasmids in which the *FBP1* and *PCK1* genes were placed under the control of the promoter of *ADC1*, the gene coding for alcohol dehydrogenase I, which is not repressed by glucose (Fig. 1). Yeast cells, wild-type for *FBP1* and *PCK1*, transformed with one of these plasmids or with both together were viable on glucose. The gluconeogenic enzymes were present during growth on glu-

cose (Table 1). The activity of FbPase was 10 times higher than in the wild-type yeast under gluconeogenic conditions, PEPCK was 3 times higher, and G6PDH showed the same activity in all strains. No significant differences with the wild-type, in generation time or in growth yield, were found for strains expressing only one of the gluconeogenic enzymes (Table 2). An increase in generation time of about 20% and a decrease in yield of about 30% were observed in the strain expressing both enzymes. There was no marked increase in lag time before resumption of growth when the yeasts were transferred from a pyruvate medium to a glucose medium.

Futile cycles would be expected to cause a decrease in intracellular ATP. However, no differences in the intracellular concentration of adenine nucleotides were found between the different strains (Table 3). These results suggest that the cycles are not operating *in vivo*, or operating only at very low rates.

Estimation of FbPase Activity in the Transformed Strains Growing on Glucose. To estimate FbPase activity *in vivo* during growth on glucose, we measured AMP and Fru-2,6-P_2 in these conditions and found a concentration of 0.2 mM for AMP (Table 3) and of 8 μM for Fru-2,6-P_2. When FbPase was assayed at these concentrations of AMP and Fru-2,6-P_2 it was 95% inhibited; the remaining activity, about 20 milliunits per mg of protein, would not support a high rate of cycling. However, as regulatory effects observed *in vitro* do not always reflect the situation *in vivo* (27), we measured the activity of the cycles *in vivo* by using ^{13}C NMR techniques.

Measurement of Futile Cycles. Yeast cells grown on glucose were transferred to a fresh medium containing [6-^{13}C]- or [1-^{13}C]glucose. After 1 hr, when glucose was still present in the medium, the distribution of label within the different carbons of the ^{13}C NMR detectable metabolites was measured. If glucose labeled in C1 or C6 is used, glucose 6-phosphate and metabolites directly derived from it, such as trehalose, should be labeled exclusively in C1 or C6, unless cycling occurs (28). The NMR spectrum of an extract from yeast expressing the gluconeogenic enzymes and incubated with [6-^{13}C]glucose is shown in Fig. 2. The region corresponding to the labeled carbons of fructose 1,6-bisphosphate is shown enlarged. Doublets from the β C6 (resonance 22) and β C1 (resonance 23) carbons, derived from the scalar coupling to ^{31}P ($^2J_{CP}$ = 5.1 Hz) were clearly detected. Peak 18 corresponds to C6 of trehalose; no signal could be seen at the C1 position of trehalose, around 94 ppm. The isotopic distribution in selected positions of relevant metabolites is shown in Table 4. The high degree of equilibration between C1 and C6 from fructose 1,6-bisphosphate indicates a high activity of aldolase and triose-phosphate isomerase. The lack of significant labeling at C1 of trehalose indicates that recycling at the level of phosphofructokinase/FbPase is <2%. It was not possible to measure the recycling at the pyruvate kinase/PEPCK level, due to the poor equilibration of label between C2 and C3 of oxaloacetate, as indicated by the distribution of label in aspartate. With the low label present at C2 from aspartate, at least 20% cycling would have to occur to allow observation of significant labeling in C1 from ethanol. Therefore, we can conclude only that the recycling at this level is <20%.

To try to circumvent the problem of the poor equilibration of oxaloacetate, we added [3-^{13}C]pyruvate as a marker to the yeast growing on glucose; however, pyruvate uptake was too low in these conditions to be useful.

Glucose Fermentation in Resting Yeast. Glucose consumption and CO_2 and ethanol production were measured in yeasts suspended in a medium lacking a nitrogen source (Table 5). For all strains the rate of glucose consumption was markedly lower than that observed during growth, as already reported for untransformed strains by Lagunas *et al.* (29). Glucose utilization increased 10–20% in the transformants with a single gluconeogenic enzyme and up to 45% in the double transformant. The fermentation rate and ethanol production increased also, although not exactly in parallel to the glucose consumption. Respiration was low in all the strains, accounting for no more than 2% of the glucose utilized.

Table 1. Levels of gluconeogenic enzymes in *S. cerevisiae* strains during growth on glucose

Strain	Activity, nmol/min per mg of protein		
	FbPase	PEPCK	G6PDH
CJM189	<2	<5	220
CJM190	<2	464 ± 68	210
CJM191	325 ± 97	<5	250
CJM186	399 ± 85	510 ± 56	250

Yeast cells were grown on YNB medium with glucose and collected during the logarithmic phase of growth. In derepressed cells of CJM189, levels of FbPase and PEPCK were 40 and 180 nmol/min per mg of protein, respectively.

Table 2. Characteristics of *S. cerevisiae* strains overexpressing *FBP1*, *PCK1*, or both during growth on glucose

Gene expressed	Generation time, min	Growth yield
—	154 ± 5	0.13 ± 0.03
FBP1	160 ± 5	0.12 ± 0.04
PCK1	156 ± 7	0.14 ± 0.04
FBP1, *PCK1*	183 ± 7	0.09 ± 0.01

Yeast cells were grown on YNB with glucose. Growth yield is expressed as g of yeast (dry weight) produced per g of glucose consumed during the exponential phase of growth.

DISCUSSION

^{13}C NMR techniques provide a powerful tool for evaluating the contribution of alternative pathways to the metabolism of a labeled compound (28). In particular, the relative flow through FbPase can been calculated from the reshuffling of the ^{13}C label from [1-^{13}C]glucose or [6-^{13}C]glucose into the C6 and C1 positions of trehalose, respectively (30). In the experiments reported in ref. 30 the results obtained with the two labeled substrates were similar, although in the case of [1-^{13}C]glucose, the transaldolase reaction could also produce [6-^{13}C]glucose derivatives. In the strains described here and in the metabolic conditions used, the reshuffling of the carbon atoms due to the transaldolase reaction was considerable (results not shown), and therefore the experiments had to be performed with [6-^{13}C]glucose. To estimate the flux through PEPCK it would be necessary that this enzyme catalyze the synthesis of phospho*enol*pyruvate molecules labeled at a carbon position different from that of the phospho*enol*pyruvate formed from glucose. This would be possible if a good equilibration of label between C2 and C3 of oxaloacetate were reached or, alternatively, if labeled oxaloacetate could be formed from externally added labeled pyruvate. As none of these conditions applies for yeast growing on glucose, due

Table 3. Intracellular adenine nucleotides in *S. cerevisiae* strains overexpressing *FBP1*, *PCK1*, or both during growth on glucose

Gene expressed	ATP, mM	ADP, mM	AMP, mM
—	1.5	1.5	0.2
FBP1	1.4	1.3	0.2
PCK1	1.3	1.4	0.2
FBP1, *PCK1*	1.5	1.2	0.2

Adenine nucleotides were measured spectrophotometrically (24) in duplicate samples of yeast actively growing on YNB with glucose.

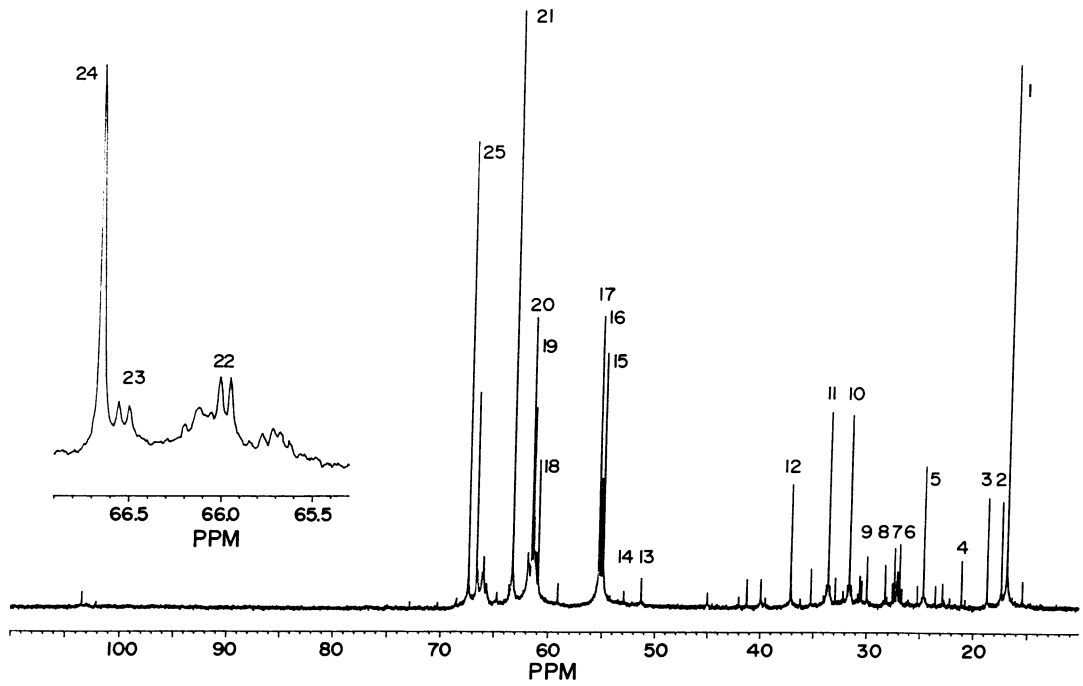

FIG. 2. ^{13}C NMR spectrum (90.55 MHz) of an extract prepared from strain CJM186 (overexpressing *FBP1* and *PCK1*) grown on [6-^{13}C]glucose. Acquisition conditions were described in *Materials and Methods*. The free induction decay was multiplied before Fourier transformation by an exponential function resulting in 0.5-Hz artificial line broadening. Peak assignments: 1 and 13, alanine C3 and C2, respectively; 2, ethanol C2; 3, unassigned; 4, lactate C3; 5 and 8, arginine C4 and C3, respectively; 7, 11, and 17, glutamate C3, C4, and C2, respectively; 6, 10, and 15, glutamine C3, C4, and C2, respectively; 9, unassigned; 12 and 14, aspartate C3 and C2, respectively; 16, arginine C2; 18, trehalose C6; 19 and 20, glucose C6, α and β, respectively; 21, glycerol C1 plus C3; 22 and 23, fructose-1,6-bisphosphate β C6 and β C1, respectively; 24, threonine C2; 25, dioxane.

to catabolite repression of fumarase and malic dehydrogenase (31) and lack of induction of pyruvate transport (32), we can conclude only that cycling is <20% at the level of pyruvate kinase/PEPCK.

The absence of activity of FbPase *in vivo* could be due to its strong inhibition by Fru-2,6-P_2. At a glycolytic flux of 35 μmol/min per g (Table 5) and an activity of FbPase at the physiological concentrations of AMP and Fru-2,6-P_2 of 20 milliunits/mg of protein (equivalent to 1 μmol/min per g of yeast), the expected rate of cycling would be 3%, a value in the range of that measured. A higher level of cycling has been observed by Campbell-Burk *et al.* (33) in yeast with derepressed gluconeogenic enzymes. This difference may be accounted for by the different metabolic conditions used, since in those experiments the glucose concentration was very low and presumably the concentration of Fru-2,6-P_2 was much lower than that measured in our experiments. It should be noted that no significant cycling was observed by Bañuelos and Fraenkel (34) in growing yeasts in which a moderate amount of FbPase was present.

With respect to PEPCK, no effectors have been described which could control its activity. We have tested possible candidates like fructose-1,6-bisphosphate, Fru-2,6-P_2, or malate, with negative results. The low cycling at the pyruvate kinase/PEPCK level could be due to the very low intracellular concentration of oxaloacetate (35), at least an order of magnitude lower than the K_m value (0.5 mM) reported for yeast PEPCK (36).

The high levels of FbPase and PEPCK measured in yeast growing on glucose indicate that the system of catabolite

Table 4. ^{13}C NMR measurements of relative isotopic distribution in relevant carbons of fructose-1,6-bisphosphate (Fru-1,6-P_2), trehalose, aspartate, and ethanol formed from [6-^{13}C]glucose by *S. cerevisiae* strains CJM186 (overexpressing *FBP1* and *PCK1*) and CJM191 (overexpressing *FBP1*)

		CJM186		CJM191	
Metabolite	Carbon	Signal*	Ratio	Signal*	Ratio
Fru-1,6-P_2	β C1	11.6	0.7	11.4	0.9
	β C6	16.4		12.6	
Trehalose	C1	<0.5	<0.02	<0.6	<0.03
	C6	24.2		20.5	
Aspartate	C2	2.3	0.10	2.1	0.09
	C3	21.3		24.4	
Ethanol	C1	<56	<0.01	37	0.005
	C2	4265		6780	

Yeast cells were grown on YNB with glucose and transferred to fresh medium containing 2% [6-^{13}C]glucose. After 1 hr of growth, at which time glucose was still present in the medium at ≈60 mM, acid extracts were prepared and analyzed by ^{13}C NMR.

*Relative intensity of the individual ^{13}C resonance to the intensity of the dioxane external reference, arbitrarily taken as 100. ^{13}C labeling in ethanol was determined in the yeast growth medium.

Table 5. Glucose utilization and ethanol production in *S. cerevisiae* strains overexpressing *FBP1*, *PCK1*, or both during growth on glucose

	Resting yeast			Glucose consumption in growing yeast
Gene expressed	Glucose consumption	CO_2 production	Ethanol production	
—	22.5 ± 2.2	39 ± 2	41.3 ± 6.4	38
FBP1	26.8 ± 2.5	43 ± 7	47 ± 4	40
PCK1	25 ± 2	37 ± 4	41.5 ± 2.1	35
FBP1, *PCK1*	32.4 ± 3.9	45 ± 5	50.5 ± 5	47

Yeast cells grown on YNB with glucose were resuspended in phosphate buffer, and CO_2 and ethanol production were measured. Figures for growing yeast were calculated from Table 2. Rates of consumption or production are expressed in μmol/min per g of yeast (wet weight). Results for resting yeast are the mean ± SD of three separate experiments.

inactivation does not operate during prolonged growth on glucose, or at least that its capacity is limited (13, 15).

Although futile cycling is below the limits of detection, the presence of the gluconeogenic enzymes has a significant effect on the rate of glucose consumption. A decrease in the ATP level and subsequent activation of phosphofructokinase does not seem to be the cause, as ATP levels do not vary between the different strains. Even in resting yeast we did not find consistent changes in the ATP concentrations. Other glycolytic intermediates, including hexose phosphates, fructose-1,6-bisphosphate, and phospho*enol*pyruvate, did not show significant differences either (results not shown). The possibility remains that some unidentified metabolite has a role in regulating the glycolytic flux and that the concentration of this metabolite changes in the presence of the gluconeogenic enzymes.

The fact that in the transformed strains, CO_2 and ethanol production do not increase in parallel with glucose consumption suggests that changes in glucose metabolism occur. An NMR spectrum of the growth medium showed only ethanol and a small amount of glycerol. This indicates that the possible modifications in glucose metabolism would lead to an increased formation of internal end products.

We have shown that the presence of the gluconeogenic enzymes during growth on glucose, even at levels much higher than those which would be present in a yeast lacking catabolite inactivation, has no major consequences for a yeast cell. Why, then, did *S. cerevisiae* develop such an elaborate system of regulation? Inactivation of the gluconeogenic enzymes could be superfluous for vegetative cells but could present an advantage in other conditions. We observed no effect of the presence of the gluconeogenic enzymes during germination in YNB glucose, but we cannot exclude that it would be harmful in other circumstances. Alternatively, since the nitrogen sources available might be limiting for growth, catabolite inactivation could have been selected to recycle amino acids from proteins which are not in an active conformation and are therefore dispensable. Finally, a very small decrease in growth rate which does not appear significant in the laboratory would be sufficient in natural conditions to wipe out an unregulated strain in competition with the regulated wild type.

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