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The Regulatory Characteristics of Yeast Fructose-1,6-Bisphosphatase Confer Only a Small Selective Advantage

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The question of how the loss of regulatory mechanisms for a metabolic enzyme would affect the fitness of the corresponding organism has been addressed. For this, the fructose-1,6-bisphosphatase (FbPase) from *Saccharomyces cerevisiae* has been taken as a model. Yeast strains in which different controls on FbPase (catabolite repression and inactivation; inhibition by fructose-2,6-bisphosphate and AMP) have been removed have been constructed. These strains express during growth on glucose either the native yeast FbPase, the *Escherichia coli* FbPase which is insensitive to inhibition by fructose-2,6-bisphosphate, or a mutated *E. coli* FbPase with low sensitivity to AMP. Expression of the heterologous FbPases increases the fermentation rate of the yeast and its generation time, while it decreases its growth yield. In the strain containing high levels of an unregulated bacterial FbPase, cycling between fructose-6-phosphate and fructose-1,6-bisphosphate reaches 14%. It is shown that the regulatory mechanisms of FbPase provide a slight but definite competitive advantage during growth in mixed cultures.

Many metabolic enzymes present regulatory features which should facilitate the coordinate operation of different metabolic pathways in response to changes in the environment. It has therefore been assumed that the acquisition by enzymes of specific regulatory mechanisms confers benefits to the organisms in which they are found. However, a quantitation of the selective advantage provided by a given regulatory feature has been rarely attempted. As the fructose-1,6-bisphosphatase (FbPase) from *Saccharomyces cerevisiae* is subject to multiple regulatory mechanisms—catabolite repression, catabolite inactivation, and inhibition by AMP and fructose-2,6-bisphosphate (F-2,6-P_2) (8, 10, 11)—this enzyme could be used to evaluate the physiological significance of a particular regulatory mechanism. The presence of the gluconeogenic enzyme FbPase in a glucose-growing yeast strain could allow the operation of futile cycles (13) and therefore decrease the fitness of the yeast. However, an engineered yeast strain which can synthesize FbPase during growth on glucose did not differ markedly from a wild-type yeast strain either in its generation time or in its growth yield (13). A possible explanation for this observation was that the FbPase was inhibited by AMP and F-2,6-P_2 and therefore had little activity in vivo. It appeared then worthwhile to remove different controls on FbPase and to examine the characteristics of the corresponding yeast strains.

To bypass catabolite repression, the coding region of the corresponding gene can be fused to a promoter not repressed by glucose (13); to overcome other regulatory mechanisms, the structure of the yeast FbPase itself should be modified. Site-directed mutagenesis cannot be used to obtain a deregulated yeast FbPase, as there is not enough information available about the residues which are important for the sensitivity to catabolite inactivation or for inhibition by F-2,6-P_2. However, use can be made of the FbPase from *Escherichia coli*, which is not affected by catabolite inactivation (9) and is insensitive to F-2,6-P_2 (2). Although the bacterial FbPase is inhibited by AMP (7), a mutated enzyme with low sensitivity to AMP has been reported previously (15). The *E. coli fbp* gene, under the control of the yeast *FBP1* promoter, fully complements a yeast *fbp1* mutant for gluconeogenic growth (9). The *fbp-5* allele from *E. coli*, when expressed in yeast cells, specifies an enzyme with low sensitivity to AMP (K_i around 700 μM against values of 20 and 50 μM for the wild-type bacterial and yeast FbPases) (unpublished data). It is therefore possible to construct yeast strains expressing during growth on glucose either the native bacterial enzyme or the enzyme with low sensitivity to AMP, encoded by allele *fbp-5*.

MATERIAL AND METHODS

Construction of plasmids and yeast strains. The plasmids used, all multicopy, carried different selective markers as follows: *LEU2* for pAAH5 (1); *URA3* for pAN10 (13), pAN15, and pAN35; and both *URA3* and *leu2-d* for pDP34 (12), pJM78, pJM79, and pJM80, as *leu2-d* ensures a high copy number of the plasmid (5). The construction of plasmids was as follows. Plasmid pJS54 (16) was used as the source for the *E. coli* gene *fbp* (see reference 9 for details of the procedure). The coding region of *fbp* was subsequently taken from pAN31 (9) as a *Stu*I-*Hin*dIII fragment. After addition of *Hin*dIII linkers the gene was placed under the control of the yeast *ADH1* promoter by inserting it in the unique *Hin*dIII site of pAN10, yielding plasmid pAN15. By the same strategy the *E. coli* gene *fbp-5* was taken from plasmid pJS53 (15) to yield plasmid pAN35. To construct plasmids pJM78 and pJM79, a *Bam*HI-*Bam*HI fragment containing the bacterial *fbp* or *fbp-5* gene flanked by the promoter and terminator of yeast *ADH1* was taken from pAN15 or pAN35, respectively, and inserted in the single *Bam*HI site of pDP34. For pJM80, a *Sac*I-*Sal*I fragment containing the yeast *FBP1* gene flanked by the promoter and terminator of yeast *ADH1* was taken from pAN11 (13) and inserted between the single *Sac*I and *Sal*I sites of pDP34.

Two series of yeast strains were used in this work. All of them carry the yeast *FBP1* gene, which is not expressed during growth on glucose. One series was derived from *S. cerevisiae* CJM152 (*MAT*α *leu2 ura3*) (13) transformed either with two plasmids (one *LEU2* and one *URA3*) or with a plasmid containing both *URA3* and *leu2-d*, so as to be able to grow in a minimal medium without supplements. Specifically, CJM189 carries pAAH5 (1) and pAN10 (13) and is a control strain, CJM237 carries pAAH5 and pAN15 and expresses the wild-type *E. coli* FbPase, and CJM238 carries pAAH5 and pAN35 and expresses the mutated bacterial enzyme with a reduced sensitivity to AMP. CJM239 carries pDP34 and is a control for the yeast strains with a high copy number of plasmids, CJM240 and CJM241, which carry plasmids pJM78 and pJM79, respectively, and express either the wild-type or mutated *E. coli* FbPase. The other series of strains was derived from *S. cerevisiae* W303-1A (*MAT***a** *ade2 ura3 leu2 trp1 his3 can1*) (17). To allow the individual identification of strains in mixed cultures, the defective *trp1* gene was replaced by the wild-type *TRP1* gene in strains CJM242,

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TABLE 1. Characteristics of *S. cerevisiae* strains expressing a heterologous FbPase during growth on glucose[a]

Strain[b]	Gene expressed[c]	FbPase activity[d]	Doubling time (min)	Growth yield[e]	Fermentation rate[f]	Respiration rate[f]
CJM189	—	<2	144/152	0.122/0.147	30 ± 1 (3)	2.4/3.1
CJM237	*fbp*	79/116	167/176	0.100/0.106	37/39	1.5/2.5
CJM238	*fbp-5*	79/91	158/175	0.104/0.102	38/43	2.4/3.4
CJM239	—	<2	251 ± 38 (4)	0.125 ± 0.012 (3)	30 ± 4 (3)	1.3/2.6
CJM240	*fbp*	196/210	274 ± 43 (3)	ND	ND	ND
CJM241	*fbp-5*	210 ± 67 (4)	299 ± 42 (5)	0.074 ± 0.004 (4)	42/60	4.0/5.7

[a] The yeasts were grown on YNB-glucose and collected during the exponential phase of growth. Values presented are averages followed by the standard deviation and then the number of experiments in parentheses. When only two experiments were done, the actual figures are given. ND, not determined.
[b] CJM189 is the control strain for CJM237 and CJM238, which carry the heterologous FbPase genes in a plasmid with the *URA3* gene as the marker. CJM239 is the control strain for CJM240 and CJM241, which carry the heterologous FbPase genes in a plasmid with both the *URA3* and *leu2-d* markers, the latter causing an increase in the number of copies of the plasmid (5).
[c] The *fbp* gene encodes a wild-type *E. coli* FbPase; the *fbp-5* gene encodes an FbPase with a decreased sensitivity to inhibition by AMP. —, no FbPase gene expressed.
[d] The activity is given as nanomoles per minute per milligram of protein.
[e] Growth yield is expressed as gram of yeast (dry weight) produced per gram of glucose consumed during the exponential phase of growth.
[f] The rates are expressed as micromoles of glucose fermented (or respired) per minute per gram of yeast (wet weight).

CJM244, CJM245, and CJM246, while in strain CJM243 the defective *his3* was replaced by *HIS3*. The one-step replacement procedure (14) was used in both cases. Besides, strains CJM242 and CJM243 carry the control plasmid pDP34 while strains CJM244, CJM245, and CJM246 carry plasmids pJM80, pJM78, and pJM79, which express yeast FbPase, the wild-type *E. coli* FbPase, and the mutated bacterial enzyme, respectively.

Culture conditions and determination of doubling times and growth yields. Yeasts were grown with shaking at 30°C in 0.7% Difco yeast nitrogen base with ammonium sulfate (YNB) and 1 or 2% glucose, with the addition of the necessar'y supplements (at a final concentration of 20 μg/ml [except for tryptophan, which was used at 40 μg/ml]). For solid media, agar was added to a final concentration of 2%. Antimycin-containing plates were prepared by adding the drug dissolved in ethanol, after allowing the medium to cool to about 60°C. The corresponding control plates contained ethanol at the same final concentration of 1%. Doubling times were measured by monitoring the A_{660}s of liquid cultures. In the competition experiments (see Fig. 2), the doubling time value for strain CJM243 (b) in mixed culture was obtained at the end of the growth of the mixed cultures CJM243 and CJM244 (b + c) and CJM243 and CJM246 (b + e). The values obtained were 114 and 118 min respectively, and therefore, the same mean value, 116, was used for strain b in all four mixed cultures. The doubling time (T) of the other strain in the mixture was calculated from the change of the ratio between the number of faster and more slowly growing cells (A/B) along time (t), taking into account that A/B equals $A_0 2^{t/T_A}/B_0 2^{t/T_B}$ and therefore log (A/B) equals log $(A_0/B_0) + [(T_B - T_A)/T_A T_B](\log 2)t$. To measure growth yields, culture samples were filtered through Whatman GF/C glass fiber filters and dried at 80°C to constant weight. The level of glucose in the medium was determined with hexokinase and glucose-6-phosphate dehydrogenase (4).

Measurements of FbPase activity, fermentation, and respiration. FbPase activity was measured spectrophotometrically in cell extracts prepared with glass beads as described elsewhere (8). 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 1% glucose.

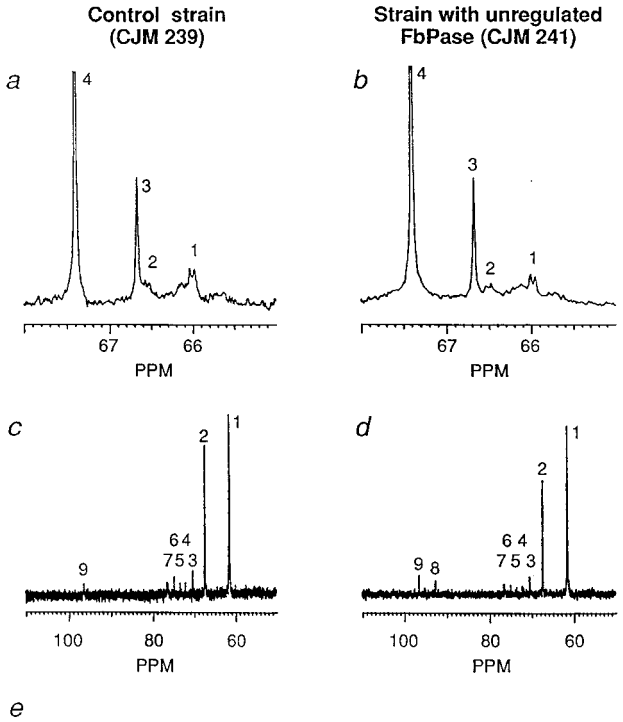
Measurements of futile cycles by ^{13}C nuclear magnetic resonance. Yeasts were incubated in [6-^{13}C]glucose as described previously (13). Duplicate samples were used to extract intracellular metabolites with trichloroacetic acid (13) and glycogen with KOH at 100°C (3). The extracted glycogen was hydrolyzed with HCl at 100°C as described elsewhere (3). The extracts were freeze-dried and resuspended in 3 ml of ^2H_2O. High-resolution ^{13}C nuclear magnetic resonance analysis of the extracts was performed as reported previously (13).

RESULTS AND DISCUSSION

Expression of heterologous unregulated FbPases in a yeast strain during growth on glucose caused a marked increase in fermentation rate, an increase in doubling time, and a decrease in growth yield (Table 1, strains CJM189, CJM237, and CJM238). The last effects, however, are relatively modest, and all of them appear to be independent of the sensitivity of the FbPase to AMP inhibition. No significant changes in the respiration rate were observed. To increase the amount of the bacterial FbPase in the yeast, we placed the corresponding fusion genes within a plasmid containing the *leu2-d* marker, since this plasmid has to be present in a high number of copies to complement a *leu2* mutation (5). Yeasts transformed with this plasmid, even without insertion, grew at a lower rate than did the yeast transformed with a plasmid containing the normal *LEU2* gene, but the presence of high levels of an unregulated FbPase increased the doubling time markedly and decreased the growth yield (Table 1, strains CJM239, CJM240, and CJM241). These results indicate that a futile cycle could be operating under these conditions. In the severely unregulated strain, marked increases in both the fermentation and the respiration rates were observed.

To measure the extent of cycling in the strain containing high levels of the unregulated bacterial FbPase, we incubated the yeast in [6-^{13}C]glucose, extracted its glycogen, and measured the distribution of label within the carbons of the glucose derived from this glycogen (Fig. 1). From the data, we calculated that about 14% of the F-1,6-P_2 formed by phosphofructokinase was recycled back to fructose 6-phosphate. No recycling took place in the control strain. Contrary to expectations, the level of ATP did not decrease, nor did those of ADP and AMP increase, in the strain with a futile cycle (results not shown); this could be due to an increased production of ATP caused by the increase in glucose respiration (Table 1). In fact, respiration was critical for the growth on glucose of the strain with an unregulated FbPase, as shown by its very poor growth on glucose in the presence of the respiratory inhibitor antimycin A compared with that of the strains expressing either the bacterial AMP-regulated FbPase or no FbPase at all under these conditions.

To determine the possible advantage conferred to yeasts by the presence of the different regulatory mechanisms for FbPase, we have grown in mixed cultures a yeast strain with only its native FbPase and a yeast strain in which an unregulated FbPase has been introduced. To allow an easy counting of the competing yeasts, when present in a mixture, we used a strain requiring histidine and another one requiring tryptophan; the two strains were otherwise isogenic. Results of such competition experiments are shown in Fig. 2. As a control we compared the growth of the parental strains, requiring histidine or tryptophan, transformed with the multicopy plasmid without an FbPase gene insert. These yeasts show a very small difference in growth rate (Fig. 2), which could be due to external tryptophan being less effective in sustaining growth than is internally produced tryptophan. The doubling times of the different strains were calculated from the competition experiments, with the results shown in the inset of Fig. 2. It can be seen that during growth in a glucose medium, the yeast expressing the wild-type *E. coli* FbPase at 200 nmol/min/mg of



e

		Strain CJM 239		Strain CJM 241	
Metabolite	Carbon	Signal	Distribution	Signal	Distribution
Fructose-1,6P_2	ß C1	18.6	38%	10.6	43%
	ß C6	30.2	62%	13.8	57%
Glucose	C1	<2	<1%	12.7	6%
	C6	197	100%	195	94%

FIG. 1. ^{13}C nuclear magnetic resonance spectra of extracts from strains CJM239 and CJM241 grown in [6-^{13}C]glucose and relative isotopic distribution of the label. (a and b) Yeasts were incubated in [6-^{13}C]glucose and acid extracts were prepared as described earlier (13). (c and d) In parallel experiments glycogen was extracted and hydrolyzed to yield glucose as described in Materials and Methods. (a and b) Peak assignments: 1 and 2, βC-6 and βC-1 carbons of F-1,6-P_2; 3, threonine C-2; 4, dioxane. (c and d) Peak assignments for glucose: 1, αC-6 plus βC-6; 3, αC-4 plus βC-4; 4, αC-2 plus αC-5; 5, αC-3; 6, βC-2; 7, βC-3 plus βC-5; 8, αC-1; 9, βC-1; 2, dioxane. (e) Signals are given as the area of the corresponding peak relative to that of the dioxane external reference, taken arbitrarily as 100. Values have been corrected for natural abundance of ^{13}C. To calculate cycling, it is assumed that there is a single glucose 6-phosphate pool, equilibrated with fructose 6-phosphate, and that glycogen is synthesized from it. The rate of phosphorylation of fructose 6-phosphate to F-1,6-P_2 is taken as 100, and the rate of hydrolysis of F-1,6-P_2 is called x. Then, the proportion of label in the C-1 position of the glucose derived from glycogen will be the product of x/100 by the proportion of label found in the C-1 position of F-1,6-P_2. From the data shown, it can be calculated that 14% of the F-1,6-P_2 formed by phosphofructokinase is recycled back to fructose 6-phosphate.

protein, strain d (CJM245), had a doubling time that was only slightly longer (118 min) than the 115-min doubling time of the control, strain a (CJM242). The presence of the unregulated bacterial FbPase, also 200 nmol/min/mg of protein, in strain e (CJM246) had a more pronounced effect, increasing the doubling time to 122 min. When present at very high levels, the native yeast FbPase itself, at up to 2,000 nmol/min/mg of protein, in strain c (CJM244) increased the doubling time to 126 min. If the doubling times shown in Fig. 2 are compared with those given in Table 1 for strains CJM239, CJM240, and CJM241, it can be observed that they are very different. Apparently the use of the pDP34 plasmid to correct a *leu2* mutation has a more harmful effect in the genetic background of strain CJM152 than in the W303-1A background, and perhaps as a consequence, the deleterious effect produced by an unregulated FbPase is also greater. It can also be seen in Table 1 that doubling times show relatively large fluctuations between experiments. Growth in mixed cultures avoids the problems associated with fortuitous variations in the growth conditions and allows the detection of small differences in fitness.

From the measurements performed, it is clear that the suppression of regulatory mechanisms does not necessarily cause strong alterations in yeast metabolism. A further example is the recent observation that the replacement of the multimodulated phosphofructokinase from yeasts by the unregulated enzyme from *Dictyostelium discoideum* does not affect mark-

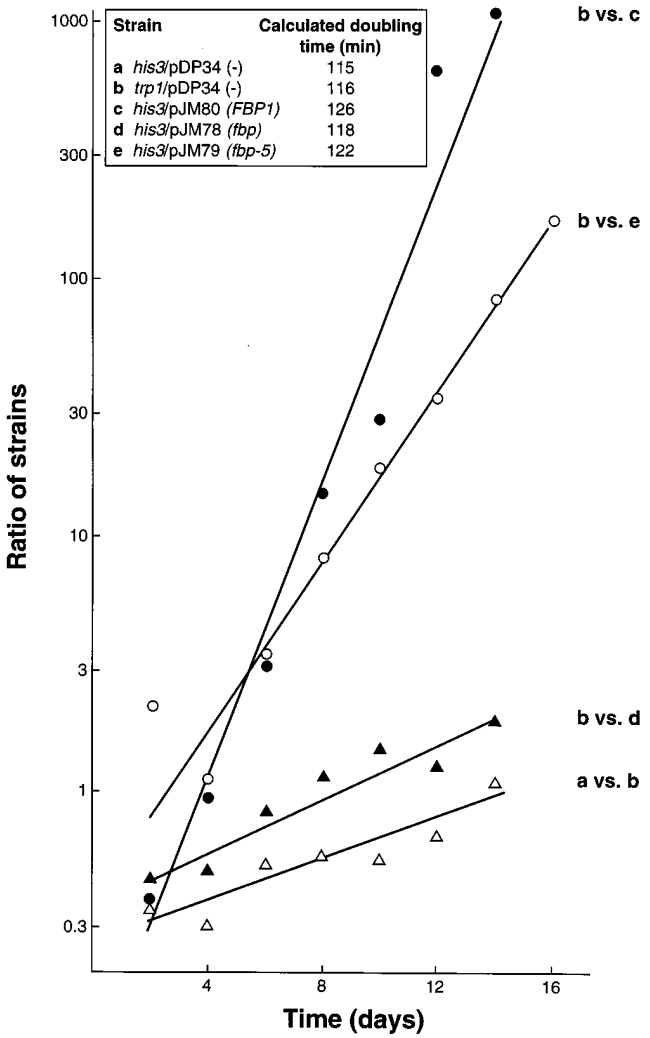


FIG. 2. Competition between yeast strains growing in mixed cultures. The strains used, CJM242 (a), CJM243 (b), CJM244 (c), CJM245 (d), and CJM246 (e), are described in Materials and Methods: as shown in the inset, strains a and b carried a control plasmid, strain c expressed yeast FbPase, and strains d and e expressed the wild-type *E. coli* FbPase and a mutated form of the enzyme, less sensitive to AMP, respectively. Media containing YNB-glucose supplemented with adenine, tryptophan, and histidine were inoculated with ca. 10^4 cells of the two strains to be compared per ml; when the cultures reached late logarithmic phase, up to 6×10^7 cells per ml, and before the glucose from the medium was exhausted, they were reinoculated at ca. 10^4 cells per ml in fresh medium. At intervals, samples were taken and the number of cells of each type was determined by counting colonies on selective plates. The ratio between the numbers of faster and more slowly growing cells is represented in logarithmic scale against time. Doubling times are shown in the inset.

edly the rate of growth of the yeast on glucose (6). It would be wrong, however, to conclude that the corresponding regulatory features are useless. Small differences in fitness may be difficult to measure unless direct competition experiments are performed. In such experiments we have detected differences in the growth rate of up 10%. These may not seem important in a laboratory setting but will be decisive in natural conditions to give a competitive advantage to the cell possessing a more tightly regulated enzyme. Thus, it can be concluded that although the acquisition by an enzyme of some new regulatory feature may not cause a large increase in fitness, it would be retained in evolution whenever it provides a selective advantage, however small.

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