Characterization of a Glucose-Repressed Pyruvate Kinase (Pyk2p) in Saccharomyces cerevisiae That Is Catalytically Insensitive to Fructose-1,6-Bisphosphate

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We have characterized the gene YOR347c of Saccharomyces cerevisiae and shown that it encodes a second functional pyruvate kinase isoenzyme, Pyk2p. Overexpression of the YOR347c/PYK2 gene on a multicopy vector restored growth on glucose of a yeast pyruvate kinase 1 (pyk1) mutant strain and could completely substitute for the PYK1-encoded enzymatic activity. PYK2 gene expression is subject to glucose repression. A pyk2 deletion mutant had no obvious growth phenotypes under various conditions, but the growth defects of a pyk1 pyk2 double-deletion strain were even more pronounced than those of a pyk1 single-mutation strain. Pyk2p is active without fructose-1,6-bisphosphate. However, overexpression of PYK2 during growth on ethanol did not cause any of the deleterious effects expected from a futile cycling between pyruvate and phosphoenolpyruvate. The results indicate that the PYK2-encoded pyruvate kinase may be used under conditions of very low glycolytic flux.

Pyruvate kinase is the last enzyme in the glycolytic pathway of sugar catabolism. It catalyzes the irreversible conversion of phosphoenolpyruvate (PEP) into pyruvate by the addition of a proton and the loss of a phosphate group, which is transferred to ADP. Pyruvate kinases from a wide range of organisms have been extensively studied, and much is known about their physical and catalytic properties (8, 35, 38, 39). Nearly all characterized eukaryotic pyruvate kinases are tightly regulated and are activated by fructose-1,6-bisphosphate (FBP). The mammalian muscle isoenzyme M1 is the only known pyruvate kinase that displays hyperbolic kinetics and lacks allosteric control (40). On the other hand, pyruvate kinases from prokaryotes can be activated by either FBP or other sugar phosphates (e.g., ribose phosphate), and those from trypanosomes can be activated by fructose-2,6-bisphosphate (35).

In the yeast Saccharomyces cerevisiae, pyruvate kinase has been thought to be encoded solely by the PYK1 gene. The gene was cloned and sequenced by Burke et al. (16), and part of the nucleotide sequence was revised by McNally et al. (36). PYK1 codes for a 54.5-kDa protein (Pyk1p) consisting of 500 amino acids. Mutants defective in the PYK1 gene fail to grow on fermentable carbon sources and are even inhibited by them (17, 18, 30). However, they grow normally on ethanol or other gluconeogenic carbon sources. Under those conditions, hexose phosphates are provided by the gluconeogenic pathway, which uses the enzymes FBP and PEP carboxykinase to bypass the 6-phosphofructo-1-kinase and pyruvate kinase reactions, respectively. The concentrations of glycolytic metabolites in pyk1 deletion mutants after growth on an ethanol-containing medium are similar to the wild-type levels, but after addition of glucose, a large increase in the amounts of PEP and phosphoglycerates can be observed (12, 17). These results suggested that Pyk1p is the main enzyme which catalyzes the conversion of PEP into pyruvate in S. cerevisiae.

The biochemical properties of yeast Pyk1p suggest that it plays a central regulatory role in carbon metabolism, undergoing changes in activity during the transitions between fermentation, when sugars are abundant, and gluconeogenesis, when ethanol is consumed. The other tightly regulated reaction is catalyzed by the hetero-octameric phosphofructokinase enzyme (see reference 27 and references cited therein). Yeast cells, like other organisms or cells which are able to carry out glycolysis and gluconeogenesis, have developed multiple regulatory mechanisms to avoid the simultaneous operation of these antagonistic metabolic pathways (7, 22, 42). One of these mechanisms is thought to be the activation of pyruvate kinase by FBP, which is the most potent and important activator of yeast Pyk1p. This glycolytic metabolite shifts the sigmoidal characteristics of a PEP saturation curve to a hyperbolic curve without affecting the maximum velocity of the enzyme but drastically lowers the K_m values for PEP and, to a lesser degree, ADP (2, 29, 56). Under conditions of glucose excess, the activity of pyruvate kinase should be high enough to provide sufficient amounts of ATP and pyruvate, which is a central metabolite involved in a variety of metabolic pathways. On the other hand, under gluconeogenic conditions, down-regulation of the Pyk1p-catalyzed reaction is necessary in order to avoid a futile cycling between pyruvate and PEP via the ATP-consuming reactions catalyzed by pyruvate carboxylase and PEP carboxykinase (15, 51, 53). Accordingly, the concentration of FBP is high in cells growing on fermentable carbon sources. During gluconeogenic growth, Pyk1p remains nearly inactive due to the low concentration of its allosteric activator, FBP (2, 3, 10, 23, 34). Thus, it is sometimes assumed that the positive control of Pyk1p by FBP provides the mechanism for switching between glycolysis and gluconeogenesis. Additionally, although the basal level of expression of the PYK1 gene is already very high, the amount of enzyme is still increased 4- to 20-fold in cells grown on fermentable sugars compared to those grown on gluconeogenic carbon sources (10, 33).

Unexpectedly, after completion of the yeast genome sequencing project, a new open reading frame (ORF) of S. cerevisiae, on chromosome XV, was identified which has a high...
degree of similarity to the PYK1 sequence (44). Here, we report the characterization of this gene, whose expression appears to be subject to glucose repression and which encodes a functional pyruvate kinase that is catalytically almost insensitive to FBP.

**MATERIALS AND METHODS**

**Yeast strains and growth conditions.** Unless otherwise stated, all yeast strains used in this work were derived from strain CEN.PK2-1C (MATα leu2-3,112 ura3-52 trp1-289 his3-D18 SUC2) or CEN.PK2-1D (MATα; otherwise identical to CEN.PK2-1C) or the corresponding diploid strain, CEN.PK2. These strains were generously provided by M. Rose and K.-D. Entian. FY1679 has been described by Winston et al. (54). Yeast cells were grown at 30°C in YEP medium (1% yeast extract, 2% Bacto Peptone), in synthetic minimal (SM) medium [0.17% Difco yeast nitrogen base without amino acids, 10 mM KCl, pH 7.0 (supplemented as necessary with amino acid, glucose, or adenine demands), or in synthetic complete (SC) medium supplemented with different carbon sources.

**Molecular biology techniques.** DNA and RNA were isolated and manipulated according to procedures previously described (47, 49). Plasmid transformations of yeast cells were carried out by the freeze method (19). Escherichia coli JM101, DH5α, and SURE (Strategene GmbH) were transformed by electroporation. pUC7, pUC18, pUC19, and pUC21, and the yeast-E. coli shuttle vectors from the series of Gietz and Sugino (25) served as vectors. All other experiments were performed as described previously (9).

**Construction of plasmids.** The PYK2 gene was cloned by PCR with a pair of primers designed to amplify a DNA fragment enclosing the complete gene as well as its 5′- and 3′-regulatory regions. One oligonucleotide (5′-GAGTCTAGACATGGGATCCGTGG-3′) is located from –574 to –552 bp in front of the ATG start codon, and it contains an Xhol restriction site at its 5′ end. The other oligonucleotide (5′-ATCCGGGTGATACCTAAAC-3′) is located from 745 to 765 bp behind the PstI site, just behind an NheI restriction site (at 741 bp). PCR with the Expand High Fidelity PCR System (Boehringer, Mannheim, Germany), with this oligonucleotide pair as primers and whole cells of strain FY1679 as the template (45 s at 95°C, 45 s at 50°C, 3.5 min at 68°C, 30 cycles), yielded a 2.8-kb DNA fragment. The fragment was cloned with Xhol and SacI and cloned into plasmids Yeplac195 and pUC19, resulting in plasmids Yeplac195-PYK2 and pUC-PYK2, respectively. To delete most parts of the ORF of PYK2, the strategy described in reference 20 was employed. This strategy takes advantage of PCR to amplify the upstream and downstream sequences of the region to be deleted along with the whole cloning vector, thereby replacing the ORF with a unique restriction site. One oligonucleotide (5′-CCGATCCCATCACCGTCTTGGTG-3′) was complementary to the sequence at positions –16 to –3 bp, next to the start codon of the PYK2 gene, with a BamHI restriction site at its 5′ end. The other oligonucleotide (5′-CCGGATCCCATCACCGTCTTGGTG-3′) was located at positions +1422 to +1438 bp, at the end of the ORF, and also contains a BamHI restriction site at its 5′ end. PCR with the Expand High Fidelity PCR System, with this oligonucleotide pair as primers and pUC-PYK2 as the template (45 s at 95°C, 30 s at 50°C, 6 min at 68°C, 25 cycles), yielded a 4.1-kb DNA fragment which was isolated, cleaved with BamHI, and self-ligated, resulting in pDPYK2. In this plasmid, the DNA sequence encoding amino acids 2 to 474 of the ORF is completely replaced by the fragment from pJJH85 containing the ORF, just behind a restriction site. The resulting plasmid was called pJJH85. In parallel, the BamHI site was replaced by a fragment (745 to 765 bp behind the PstI site, near the start codon of the PYK2 gene) from plasmid pPYK-1 (48) as a template and the oligonucleotide primers PpyK-5 and PpyK-3′ as primers and whole cells of strain FY118B (MATα pyk2Δ::URA3) were used in this work. PCR amplification of strain FY1679 and tetrad dissection resulted in strains EBY118A (MATα pyk2Δ::URA3) and EBY118B (MATα pyk2Δ::URA3). The correct replacements of the PYK2 gene were confirmed by PCR (data not shown). For construction of VVH3A (MATα pyk1Δ::HIS3 leu3-112 ura3-52 trp1-289 his3-D18 SUC2), pYKKdel2 was used as a template for PCR amplification with the same pair of primers (pPYK-5′ and pPYK-3′) and under the same conditions as described above. The 2.6-kb PCR fragment obtained was transformed into CEN.PK2-1C, and transformants were selected on SC medium with 1% glucose and 3% ethanol as carbon sources but without histidine. Five colonies were picked and shown to be unable to grow on media containing 2% glucose. One was selected and shown to carry a deletion in pYK1. EBY118B was crossed with strain VVH3A, the diploid strain was sporulated, and the tetrads were dissected, resulting in the pYK1 pyk2 double-deletion mutant strains EBY121A (MATα) and EBY121B (MATα).

**Determination of metabolic levels and enzyme activities.** Preparation of metabolite extracts was performed as previously described (10). The concentrations of metabolites and ATP were assayed enzymatically (6). Pyruvate kinase activities were determined in 50 mM imidazole buffer (pH 7.0) containing 10 mM magnesium chloride and 100 mM potassium chloride. Under standard conditions, substrates (ADP and PEP) were used at a 2 mM concentration and FBP was added as an activator at 1 mM. The reaction was coupled to NADH oxidation by addition of 1 U of lactate dehydrogenase per ml and 0.2 mM NADH. The kinetics were monitored at 30°C by measuring the increase in absorbance at 340 nm with a Beckman DU-7400 spectrophotometer. For determinations of kinetic parameters, pyruvate kinase from the strains indicated was partially purified by a two-step precipitation with polyethylene glycol (PEG) 4000. First, crude extracts were prepared from 20 ml of an overnight culture grown in YEP medium plus 2% glucose (YPED) to late logarithmic phase as described before (14), in a final volume of 0.2 ml each. They were mixed in a 2:1 ratio with a 7.5% PEG solution. After centrifugation at 15,000 x g for 15 min, the pellets were shown to contain less than 5% of the total pyruvate kinase activity and were discarded. The supernatants were mixed again in a 2:1 ratio with a 30% solution of PEG 4000. The time, more than 90% of the specific activity was retained in the pellets obtained by centrifugation at 15,000 x g, both for Pyk1p and Pyk2p. Each pellet was resuspended in 0.5 ml of imidazole buffer, mixed with an equal volume of glycerol, and stored at –20°C for further studies. Alternatively, they were each dialyzed against a total of 4 liters of imidazole buffer with continuous stirring for 70 h at 4°C. β-Galactosidase activities were measured as described previously (37). Protein was determined according to the microbiur method (57), with bovine serum albumin as a standard.

**RESULTS**

After completion of the *S. cerevisiae* genome sequencing project, we compared all the yeast ORFs with the amino acid sequence of the *S. cerevisiae* Pyk1p (Fig. 1). We found an ORF designated YOR347c which codes for a protein with 506 amino acids having nearly 71% amino acid identity to Pyk1p (Fig. 1) (44). The ORF was renamed PYK2. The DNA sequence identity between PYK1 and PYK2 is 68% within the coding regions of the two genes but does not extend into the 5′ and 3′ regulatory regions. However, in contrast to PYK1, whose sequence is specified by a highly restricted set of codons which points to a high level of expression (4) (codon bias, 0.965), the codon bias of the PYK2 gene is only 0.154. Furthermore, like Pyk1p, Pyk2p has distinct sequence similarities also to the mannamin tissue-specific pyruvate kinase isozymes (Fig. 1), whose activities either are not allosterically regulated (M1 type), are activated by FBP (M2 and R types), or are controlled by FBP and GTP (M3 type). (8, 48, 49). Physiological characterization of Pyk2p. The yeast PYK2 gene is located on chromosome XV between the PUT4 gene, which is encoded on the same DNA strand and whose ORF terminates 437 bp upstream of the PYK2 start codon, and the REV1 gene, which is encoded on the other DNA strand and whose ORF terminates 157 bp downstream of the PYK2 ORF.
The **PYK2** gene, including the complete promoter region (574 bp of the 5' region in front of the ATG codon) and 741 bp of the 3' noncoding region, was amplified by PCR using chromosomal DNA from wild-type strain FY1679. The 2.8-kb DNA fragment was cloned into the 2μm-based multicopy plasmid YEplac195 (25) and transformed into the wild-type yeast strain VW1A and three different **pyk1** deletion mutant strains (VWH3A [see Materials and Methods], EBY55 [12], and EBY56 [13]). The transformants were plated on a uracil-free medium (to select for plasmid uptake) which was supplemented with 3% ethanol and 0.1% galactose (to allow all transformants to grow). After 3 days, the colonies were replica plated onto the same basic medium containing 1% glucose as the sole carbon source. Not only the wild-type cells but also the **pyk1** deletion mutants containing the **PYK2** gene on the multicopy vector grew very well on the glucose medium, with similar growth rates. Transformants of the **pyk1** deletion mutant strains containing only the cloning vector YEplac195 without an insert did not grow on the glucose medium within 1 week.

In order to avoid false conclusions due to error-prone PCR amplification of the **PYK2** gene, the PCR-generated **PYK2** fragment was replaced with a chromosomal copy of **PYK2** by the gap repair method. Again, **pyk1** mutant cells transformed...
with a multicopy plasmid containing this genome-derived copy of PYK2 (YEpPYK2) grew very well on glucose media. The plasmid YEpPYK2 was reisolated from cells of the pyk1 mutant strain growing on glucose and was shown by restriction enzyme analysis to be identical to the originally transformed plasmid. After prolonged growth on nonselective YEP medium with 3% ethanol as the sole carbon source, several cells had lost the plasmid, as judged by their uracil auxotrophy, and concomitantly the ability to grow on a medium with glucose. Thus, overexpression of the PYK2 gene on a multicopy plasmid restores the ability of pyk1 deletion mutants to grow on fermentable carbon sources. This stands in contrast to a similar situation reported for isocitrate lyase, in which a homolog, ICL2, present in the yeast genome is under no circumstances able to complement an icl1 defect (28). The results reported above suggest that PYK2 encodes a functional pyruvate kinase.

A pyk2 deletion mutant showed growth properties identical to those of the wild-type strain under all conditions tested (see below). After crossing it with a pyk1 deletion mutant, the diploid strain was sporulated, and spores containing pyk1 as well as pyk2 deletion alleles were obtained. Cells of the pyk1 pyk2 double-deletion mutant were able to grow on YEP medium with 3% ethanol, like wild-type cells. However, like cells of a pyk1 single-deletion strain, they were not able to grow on a medium with 2% glucose. Growth of the pyk1 mutant strain (VWH3A), the pyk2 mutant strain (EBY118A), the pyk1 pyk2 double-mutant strain (EBY121), the pyk1 mutant strain containing plasmid YEpPYK2 (EBY120A), and a corresponding mutant strain (CEN.PK2-1C) was tested on various media with different carbon sources and under various conditions. The cells were plated on SM, SC, and YEP media containing either 2, 0.5, or 0.1% glucose, maltose, or galactose or 2% raffinose or sucrose, with or without 0.7 M NaCl or 1 M sorbitol and with either ammonium or proline as a nitrogen source, or on SC medium containing 3% ethanol, with or without 2, 1, or 0.5% glucose, and were incubated at 30 or 37°C. No significant growth differences among the wild-type strain, the pyk2 mutant strain, and the pyk1 mutant strain containing plasmid YEpPYK2 were found. The growth properties of the pyk1 deletion mutant and the pyk1 pyk2 double mutant were similar in that neither strain grew on SM or SC medium with 0.1 to 2% glucose or 0.1 to 2% galactose under any conditions. However, there were also distinct differences. For instance, the pyk1 mutant strain grew very slowly on SC medium with small amounts of maltose or with 2% raffinose, and the pyk1 pyk2 double mutant did not. Also, the growth-inhibiting effect of increasing concentrations of glucose added to an ethanol-based SC growth medium was more pronounced in the pyk1 pyk2 double-mutant strain, which, unlike the pyk1 mutant strain, did not grow at all on a mixture of 3% ethanol and >0.5% glucose.

Pyruvate kinase activities were determined in the different strains after growth on YEP medium with 3% glycerol–ethanol or 2% glucose (Table 1). As already shown previously (10), pyruvate kinase activity in wild-type cells is induced about fourfold on a glucose medium compared to an ethanol medium. The same was true for a pyk2 deletion strain, which did not differ from the wild-type strain. Only a very low specific activity close to the limit of detection could be determined in a pyk1 deletion strain, and the level was even lower in the pyk1 pyk2 double-mutant strain. Interestingly, pyruvate kinase activity in the pyk1 deletion strain containing plasmid YEpPYK2 on a glycerol-ethanol medium was much higher than in the wild-type strain and was decreased to wild-type levels on a glucose medium (Table 1).

The concentrations of some of the glycolytic metabolites were determined in the different strains during growth on YEP medium with 3% ethanol and 1 h after addition of 2% glucose to the cultures (Table 2). No significant differences among the wild-type strain, the pyk2 deletion strain, and the pyk1 deletion strain containing YEpPYK2 were observed in the concentrations of any of the various metabolites, including ATP, either during growth on ethanol or after addition of glucose. Moreover, no significant differences in metabolic concentrations were found between the pyk1 and the pyk1 pyk2 deletion strains. Both strains accumulated large amounts of glycolytic metabolites after addition of glucose, and their ATP levels were reduced, characteristic for a block in glucose catabolism. However, during growth on ethanol, the concentrations of metabolites in both strains were similar to those in the wild-type strain. The physiological data provide in vivo evidence that a single chromosomal copy of the PYK2 gene provides only very low levels of pyruvate kinase activity under these conditions but that overexpression from a multicopy vector can completely bypass the pyk1 defects.

**Biochemical characterization of Pyk2p.** The kinetic properties of partially purified Pyk1p and Pyk2p enzymes were compared (Table 3). Pyruvate kinase activities from the wild type and the pyk2 deletion strain were strongly dependent on the addition of FBP to the assay. Under standard conditions, FBP activated Pyk1p enzyme activity about 8-fold and lowered the \( K_m \) value for FEP about 30-fold. In contrast, the PYK2-encoded activity and its \( K_m \) value for FEP were only marginally affected by FBP, and the \( K_m \) value of Pyk2p for FEP, with or

### TABLE 1. Pyruvate kinase activities in different yeast strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Pyruvate kinase activity (μmol min⁻¹ mg of protein⁻¹) in</th>
<th>YEP + 3% ethanol–3% glycerol</th>
<th>YEP + 2% glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEN.PK2-1C</td>
<td>Wild type</td>
<td>1.212</td>
<td>4.914</td>
<td></td>
</tr>
<tr>
<td>VWH3A</td>
<td>pyk1Δ</td>
<td>0.038</td>
<td>NG (b)</td>
<td></td>
</tr>
<tr>
<td>EBY118A</td>
<td>pyk2Δ</td>
<td>1.280</td>
<td>4.704</td>
<td></td>
</tr>
<tr>
<td>EBY121A</td>
<td>pyk1Δ pyk2Δ</td>
<td>0.017</td>
<td>NG (b)</td>
<td></td>
</tr>
<tr>
<td>EBY120A</td>
<td>pyk1Δ YEpPyK2</td>
<td>7.763</td>
<td>4.928</td>
<td></td>
</tr>
</tbody>
</table>

*Activities are mean values of data from at least two independent experiments. Cells were grown overnight in the indicated medium to the mid-exponential growth phase.

*NG, no growth.

### TABLE 2. Concentrations of internal metabolites in various yeast strains

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>CEN.PK2-1C (wild type)</th>
<th>VWH3A (pyk1Δ)</th>
<th>EBY118A (pyk2Δ)</th>
<th>EBY121A (pyk1Δ pyk2Δ)</th>
<th>EBY120A (pyk1Δ YEpPYK2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glc-6-P</td>
<td>2.6/3.0</td>
<td>2.0/2.2</td>
<td>1.7/2.7</td>
<td>2.7/2.2</td>
<td>2.0/3.1</td>
</tr>
<tr>
<td>Fru-1,6-bP</td>
<td>0.5/0.9</td>
<td>0.9/1.8</td>
<td>0.5/8.0</td>
<td>0.9/20.8</td>
<td>0.7/9.9</td>
</tr>
<tr>
<td>3-P-glycerate</td>
<td>0.3/2.3</td>
<td>1.1/3.0</td>
<td>0.7/3.2</td>
<td>1.3/9.8</td>
<td>0.5/4.2</td>
</tr>
<tr>
<td>PEP</td>
<td>0.6/1.5</td>
<td>0.8/7.0</td>
<td>0.8/1.5</td>
<td>0.9/7.8</td>
<td>0.4/2.2</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.2/1.5</td>
<td>0.1/0.4</td>
<td>0.3/1.5</td>
<td>0.1/0.6</td>
<td>0.4/1.8</td>
</tr>
<tr>
<td>ATP</td>
<td>4.4/4.0</td>
<td>3.3/1.9</td>
<td>4.1/4.2</td>
<td>4.6/2.0</td>
<td>4.9/5.4</td>
</tr>
</tbody>
</table>

*Metabolites were determined in cells grown overnight in YEP medium supplemented with 3% ethanol (first value) and 1 h after the addition of 2% glucose to the cultures (second value). Mean values of data from three independent experiments are given.

*Abbreviations: Glc-6-P, glucose-6-phosphate; Fru-1,6-bP, fructose-1,6-bisphosphate; 3-P-glycerate, 3-phosphoglycerate.
addition of glucose (Fig. 2). A PYK2: lacZ fusion construct on a multicopy vector was introduced into the wild-type strain and used to determine the PYK2 promoter activity under different conditions (Table 4). Consistent with the Northern analysis, the PYK2 promoter was strongly repressed by high concentrations of glucose and maltose, but repression was relieved with low concentrations of these sugars, in cells grown on 2% glucose into the stationary phase or after growth on raffinose, which is hydrolyzed outside the cells, producing only small amounts of free sugars. Moreover, PYK2 expression was even induced by ethanol. β-Galactosidase activities were slightly higher during growth on 2% glucose when proline was used as a (poor) nitrogen source instead of ammonium sulfate. No change in promoter activity was seen after a heat shock. In contrast to the PYK2: lacZ fusion, which was repressed by glucose about 40-fold compared to ethanol (Table 4), Pyk2p enzyme activities were repressed no more than 2-fold (Table 1). This may be explained by the high selective pressure for the PYK2-containing plasmid in the pyk1 deletion mutant during growth on glucose but not on ethanol, which may lead to an increase in the copy number of the plasmid and a deregulation of PYK2 expression during growth on glucose. A similar observation has been made with overexpression of GDH2, encoding glutamate dehydrogenase, in a pg1 phosphoglucone isomerase mutant during growth on glucose (11). Interestingly, the Pyk2p enzyme activity adjusted exactly to wild-type pyruvate kinase levels under these conditions. In contrast, there is no selective pressure for the PYK2: lacZ fusion construct.

Transcriptional repression by glucose is a common mechanism in yeast cells (22, 24, 45). The glucose repression pathway triggers repression of genes that are dispensable or unimportant to cells growing on glucose. Phosphorylation of glucose by one of the two yeast hexokinases, Hxk1p and Hxk2p, has been proposed to be involved in the triggering reactions for glucose repression of the GAL, MAL, and SUC genes (21, 32, 46). However, the ability of the PYK2 promoter to respond to glucose in hsk1 hsk2 double mutants containing only the GLK1-
encoded glucokinase remained intact (Table 4), similar to the glucose-repressed glucogenetic genes \textit{FBP1} and \textit{PKC1} (55). Ethanol induction of the \textit{PYK2} promoter was not affected in a \textit{cat8} deletion mutant. The \textit{CAT8} (\textit{MSP8}) gene encodes a transcription factor required for derepression of gluconeogenic enzymes during growth on ethanol (26). Another central component of the glucose repression pathway is the \textit{GLC7}-encoded protein phosphatase 1, which is targeted by the Reg1p (Hex2p) protein to other proteins in the glucose repression regulatory pathway (52). Interestingly, glucose repression of the \textit{PYK2} promoter was relieved in a reg1 deletion mutant (Table 4). Altogether, our results suggest that the \textit{PYK2} gene is subject to the general mechanisms of glucose repression.

**DISCUSSION**

We have shown that the previously discovered ORF designated \textit{YOR347c/O6342} (44), which has a high level of sequence similarity to the \textit{PYK1} gene, encodes a second functional yeast pyruvate kinase isoenzyme. The unusual properties of \textit{PYK2} gene expression and of the encoded enzyme, namely, the low enzyme activity concomitant with a relatively high transcript level during growth on an ethanol medium, the strong repression of the gene by high concentrations of glucose, the lack of an obvious phenotype in a \textit{pyk2} deletion mutant, and the catalytic insensitivity to FBP, suggest that Pyk2p is used by the cells only under very specific conditions. Our results are compatible with the notion that Pyk2p activity may be used by the cells under conditions in which the level of glycolytic flux is very low. This is the case, for example, under starvation conditions, i.e., when the cells are consuming internal glucose derived from storage carbohydrates or when the external glucose supply is low. Under such conditions, the \textit{PYK2} gene is derepressed and the low levels of intracellular FBP may not be sufficient to activate the Pyk1p enzyme.

On the other hand, overexpression of the unregulated Pyk2p enzyme activity during growth on ethanol did not cause any deleterious effects on the cells, as might be expected if regulation of pyruvate kinase activity by FBP provides the mechanism for switching between glycolysis and gluconeogenesis. A similar observation was made by Maitra and Lobo (34), who isolated a yeast mutant with a Pyk1p enzyme which did not require FBP for activity. In contrast to our results, this mutant could grow on an ethanol medium even faster than the wild-type strain, only after a considerable lag period of 3 to 4 days without growth. Altogether, the results suggest that control parameters other than FBP-dependent regulation of pyruvate kinase, capable of maintaining a level of PEP sufficient for gluconeogenesis and of avoiding the energy-wasting cycling by the pyruvate kinase-pyruvate carboxylase-PEP carboxykinase enzyme reactions, must operate in the cells. They could be as-yet-unidentified allosteric effectors of pyruvate kinase activity. On the other hand, it is tempting to speculate that the gluconeogenic enzymes form specific enzyme complexes (1, 50) that allow channeling of intermediate metabolites, e.g., from oxaloacetate to glyceral-2-phosphate. In this case, PEP would not be accessible to pyruvate kinase during growth on ethanol.

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**REFERENCES**


**TABLE 4. Specific \boldsymbol{\beta}-galactosidase activities in different yeast strains containing a \textit{PYK2}:\textit{lacZ} fusion plasmid**

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>CEN.PK2-1C (wild type)</th>
<th>ENY.WA-1A (wild type)</th>
<th>WAY.gk1-1A (hsk1A/hsk2A)</th>
<th>WAY.7-2D-2A (hsk1A/hsk2D)</th>
<th>reg1Δ mutant</th>
<th>EBY152 (cat8A [reg8A])</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0% glucose</td>
<td>21</td>
<td>42</td>
<td>48</td>
<td>48</td>
<td>235</td>
<td>49</td>
</tr>
<tr>
<td>0.1% glucose</td>
<td>215</td>
<td>ND*</td>
<td>48</td>
<td>ND</td>
<td>235</td>
<td>ND</td>
</tr>
<tr>
<td>2.0% maltose</td>
<td>59</td>
<td>115</td>
<td>125</td>
<td>125</td>
<td>95</td>
<td>ND</td>
</tr>
<tr>
<td>0.1% maltose</td>
<td>300</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2.0% raffinose</td>
<td>210</td>
<td>435</td>
<td>335</td>
<td>335</td>
<td>595</td>
<td>205</td>
</tr>
<tr>
<td>2.0% ethanol</td>
<td>940</td>
<td>565</td>
<td>795</td>
<td>795</td>
<td>1,180</td>
<td>355</td>
</tr>
<tr>
<td>2.0% glucose-0.1% proline</td>
<td>80</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2% glucose (stationary phase)</td>
<td>205</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2.0% glucose (2 h, 42°C)</td>
<td>18</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Cells were grown overnight in SC medium lacking uracil and supplemented with different carbon sources to a density of 1 × 10^7 to 3 × 10^7 cells/ml. Mean values of data from at least two independent experiments are given.
* ND, not determined.
* The medium contained 0.1% proline instead of ammonium.
* The cells were grown in a SC medium with 2% glucose to stationary phase for 2 days.
* The cells were shifted to 42°C for 2 h before being harvested.