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Expression of heterologous phosphofructokinase genes in yeast

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Genes encoding phosphofructokinases (PFK) from *Escherichia coli* and from the human muscle were expressed in PFK-deficient strains of *Saccharomyces cerevisiae* under the control of an inducible *GAL1* promoter. They restored PFK activity under inducing conditions and complemented the galactose-negative growth phenotype of the recipient strains. The PFK enzymes expressed appear to be stable in yeast. The human muscle enzyme crossreacts with specific antibodies and shows the expected subunit size. As expected, its activity can be activated by fructose-2,6-bisphosphate, in contrast to the bacterial enzyme.

Heterologous gene expression, Glycolysis; *Saccharomyces cerevisiae*; Galactose induction

1. INTRODUCTION

Glycolysis is a major pathway for carbohydrate utilization in most organisms and is especially well established in the yeast *Saccharomyces cerevisiae* (see [1] for a recent review). The phosphofructokinase (PFK) reaction, converting fructose-6-phosphate with ATP consumption to fructose-1,6-bisphosphate, is generally assumed to be a key controlling step in this pathway [2].

In yeast, PFK is a heterooctameric enzyme, composed of 4α- and 4β-subunits [3], which are encoded by *PFK1* and *PFK2*, respectively [4]. PFK activity is allosterically controlled by a variety of effectors (21 listed in [5]). The most potent activator of eukaryotic PFK's is fructose-2,6-biphosphate discovered by van Schaftingen et al. [6] and shown to be also an activator of yeast PFK [7]. Genetic characterization in yeast gave the puzzling result that nullmutants in any one of the *PFK* genes do not show in vitro detectable PFK activity but do grow on and ferment glucose [4]. Only double mutants (*pfk1 pfk2*) are glucose-negative. Of the different hypotheses to explain this phenotype (summarized in [8]) only two remain valid: (i) The PFK-subunits serve a yeast-specific function in a 'bypass' reaction to glycolysis, or (ii) each of the subunits catalyzes the PFK-reaction in vivo by an activity that escapes detection in vitro. The latter could be due to an unstability of complexes formed by the remaining subunits upon dilution in the preparation of crude extracts.

In this context, we have started an investigation of heterologous expression of *PFK*-genes from other organisms in *S. cerevisiae*. We have already expressed the two genes encoding the PFK subunits of the closely related yeast *Kluyveromyces lactis* [9]. There, the enzyme is also functional only in the heteromeric state (i.e. mutations in any one of the genes leads to a loss of detectable PFK activity). Each of the genes is able to complement a *S. cerevisiae pfk*-double mutant for growth on glucose but does not restore in vitro measurable enzymatic activity. However, a yeast-specific function of PFK subunits cannot be excluded from these data, as the deduced amino acid sequences are more than 70% identical and could have retained such a function.

In most organisms studied, PFK is active as a homotetrameric enzyme. Thus, *Escerichia coli* contains two homotetrameric isoenzymes, with PFKA constituting about 90% of the cellular activity [10]. The PFK subunits of both enzymes are about half the size of those of mammalian and yeast PFK, leading to the hypothesis of an evolution by gene duplication events [11,12]. The PFK enzymes of *E. coli* and *Bacillus stearothermophilus* are comparatively well studied in terms of X-ray crystallographic analysis [13] and in vitro mutagenesis of crucial amino acid residues [14–16]. Amino acid residues determined as functional in these studies are generally conserved in eukaryotic PFK's sequenced so far [9,11].

Mammalian PFK has drawn major attention because glycogen storage disease VII (Tarui's disease) was found to be associated with a lack of muscle PFK activity [17]. Three kinds of subunits have been reported for mammalian PFK, with homo- and heterotetrameric forms being active and being present in different amounts in different tissues [18,19]. cDNA clones of human muscle PFK (hmPFK) have been isolated [20,21] and the genetic defect has been attributed in two cases to point mutations leading to defective splicing products [22,23].

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In this work I describe the expression of *E. coli* PFKA and hmPFK in a *pfk1 pfk2* double-null mutant of *S. cerevisiae*. The data are used to address the question of a specific function of the yeast PFK-subunits (see above). In addition, this work provides the basis for the investigation of human (defective) PFK genes in a simple, genetically well defined yeast system.

2. EXPERIMENTAL

2.1. *Strains and media*

The yeast strains HD100-2A (*MATα pfk1 HIS3 pfk2 HIS3 his3-11,15 ura3-52 trp1 GAL*) and HD114-16A (*MATa pfk1 HIS3 pfk2 HIS3 his3-11,15 ura3-52 leu2-3,112 MAL SUC GAL*) were used as recipient strains for heterologous *PFK* gene expression. Both yeast strains were constructed during this work. HD100-2A derived from a number of crosses starting with the strains AMW-13C$^+$ (originally a gift from M. Whiteway) and SMC-19A (from F.K. Zimmermann). HD114-16A is a haploid strain and, except for the deletions in *pfk* genes, isogenically derived from the diploid strain MC99 (kindly provided by M. Ciriacy). They were grown in rich media (1% yeast extract, 2% bacto peptone, 2% glycerol and 2% ethanol; YEPGE) for the preparation of competent cells. Synthetic minimal media were based on 0.67% yeast nitrogen base w/o amino acids, supplemented with amino acids and adenine as described in [24]. As carbon sources 2% glucose (SCD), 2% galactose (SCGal) or 2% glycerol plus 2% ethanol (SCGE) were added. Solid media were prepared by the addition of 2% oxoid agar. Cells were always incubated at 30°C, with constant shaking where liquid media were employed.

2.2. *Genetic manipulations, sequencing and plasmids used*

Standard molecular procedures were used in handling of DNA [25]. Yeasts were transformed with a modification of the freeze-method described by Klebe et al. [26,27]. *E. coli* strain DH5αF′ (Gibco/BRL) was used throughout and competent cells were prepared according to [28]. Plasmid DNA was prepared from *E. coli* with the kit of Quiagen (Düsseldorf) according to the instructions of the manufacturer.

Sequencing was based on the dideoxy-chain-termination method of Sanger et al. [29] using the USB sequenase kit according to the instructions of the manufacturer. The custom-made oligonucleotide 5′-CTGCATAACCACTTTAAC-3′ was used as a sequencing primer to determine the sequence around the *GAL1* fusion points.

For expression in yeast, plasmid pBM272 was used. It is a derivative of pBM150 [30], where a *Hin*dIII-linker was inserted into the unique *Bam*HI site, without destroying the latter. Pchpfkm1, carrying a cDNA clone of the human muscle *PFK* in pUC13, was kindly provided by A. McLachlan (La Jolla/California). The *Eco*RV fragment from this vector carrying the entire gene and some polylinker sequences, was subcloned into pUK1921 [31] to yield pJJH50. From there, the *Bam*HI/*Sph*I fragment was isolated and cloned into pBM272 opened with the same restriction enzymes to give pJJH54. For construction of a fusion directly at the ATG translation start codon, the oligonucleotide 5′-GAGTGGATCCATGACCCATGAAGAGC-3′ was used in conjunction with the reverse sequencing primer to amplify the *hmPFK* gene from pJJH50 by PCR according to the protocoll of Perkin-Elmer. The resulting fragment was isolated from an agarose gel and digested with *Bam*HI and cloned into pBM272 linearized with *Bam*HI to give pJJH55. The clone with the *E. coli PFKA* gene contained within a *Bam*HI fragment, pHL1, was a gift from P. Evans (Cambridge, UK).

2.3. *Enzymatic and immunological analysis*

For determination of specific PFK activities, crude extracts were prepared with glass beads as described earlier [32]. Protein concentrations in crude extracts were determined by the method of Zamenhof [33], using bovine serum albumin as a standard. For routine determinations of PFK activities, 0.1 M potassium phosphate buffer at pH 7.0 was used. The testmix contained 5 mM magnesium chloride, 1 mM ATP, 0.3 mM ADP, 0.1 mM AMP, 0.25 mM NADH and 3 mM fructose-6-phosphate at final concentrations. The decrease in absorption at 340 nm was followed for 3 min prior to the addition of substrate and for 15 min in the presence of fructose-6-phosphate. The tests were carried out in a Beckmann DU7000 spectrophotometer at a constant temperature of 30°C.

To determine the activatory effect of fructose-2,6-bisphosphate, 50 mM MES-buffer at pH 6.4 was used, ADP and AMP were omitted from the testmix, the ATP concentration was raised to 3 mM and the fructose-6-phosphate concentration was lowered to 1.5 mM. 100 μM fructose-2,6-bisphosphate was added where indicated.

For immunoblot analysis, samples containing 30 μg of crude extract protein were applied to 7.5% SDS-polyacrylamide gels. Western blots were carried out as described by Zachariae et al. [34] using a 1:200 dilution of mammalian PFK-antiserum obtained from rabbits kindly provided by G. Dunaway (Springfield, Illinois) and a 1:400 dilution of an actin-antibody raised in mouse, purchased from Boehringer, Mannheim. Anti-rabbit and anti-mouse secondary antibodies coupled to alkaline phosphatase were purchased from Sigma.

3. RESULTS AND DISCUSSION

3.1. *Construction of expression plasmids*

Heterologous *PFK*-genes may not be expressed in yeast under the control of their own promoters. In addition, some proteins may have a deleterious effect when expressed in the yeast cell in high amounts. Therefore, the gene from *E. coli* (*EcPFKA*; from pHL1 described in [35]) and a cDNA-clone containing the human muscle *PFK* gene (*hmPFK*; [21]) were cloned into the vector pBM272 (see section 2) under the control of the *GAL1*-promoter as shown in Fig. 1. For this purpose, the *Bam*HI fragment of pHL1 was inserted into the single *Bam*HI site of pBM272 to yield pJJH40. The correct orientation was verified by restriction and sequence analyses. Only 5 bp of the original 5′-noncoding sequence of *EcPFKA* remained in front of the ATG translation start codon.

For subcloning of *hmPFK*, first convenient *Eco*RV sites of the clone provided were used (see section 2) to yield pJJH54. This clone contains 76 bp of the 5′-noncoding region of the originally isolated cDNA clone as well as some polylinker sequences between the *GAL1* promoter and the translation start codon. As this region seems to interfere with expression in yeast (see below), a fragment starting at the ATG codon was amplified by a PCR approach (see section 2) and cloned into pBM272 (pJJH55; Fig. 1).

3.2. *Phenotypes upon expression in a yeast* pfk1 pfk2 *double-null mutant*

The three plasmids constructed as well as the vector without a *PFK* insertion were transformed into the strains HD100-2A and HD114-16A, carrying deletions in the two yeast *PFK* genes. The recipient strains are unable to grow on glucose or galactose as sole carbon sources, due to the block in the glycolytic pathway. Consequently, transformants were selected on SCGE lacking uracil. Three clones obtained from each plasmid

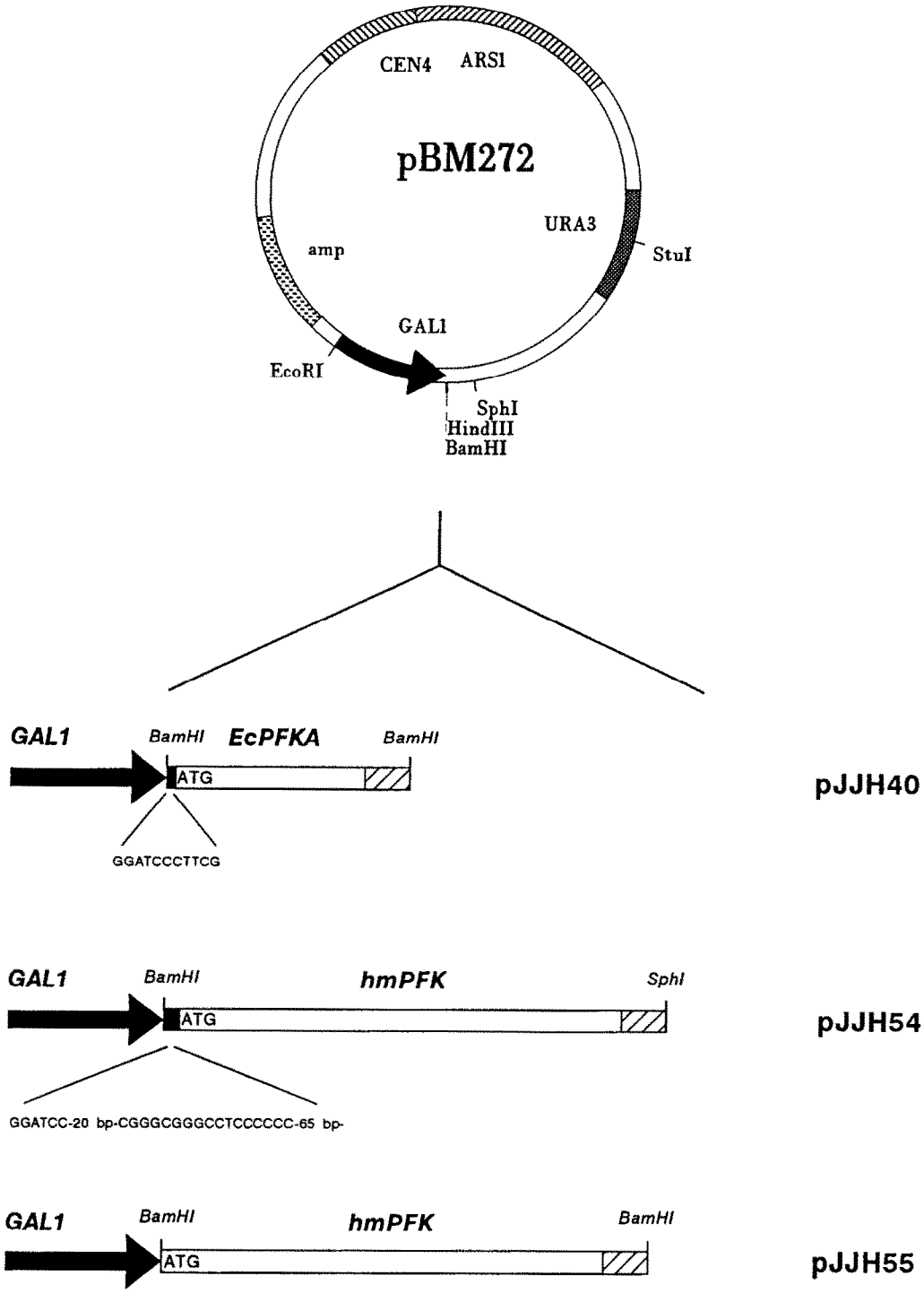

Fig. 1. Expression constructs for heterologous PFK genes The genes were cloned into convenient restriction sites of the vector pBM272 shown in the upper part (see text for details). In the lower part of the figure, the black arrows indicate the *GAL1* promoter (816 bp), the black boxes 5'-noncoding sequences of the heterologous *PFK* genes retained in cloning (with sequences indicated below). Open boxes starting with the ATG translation start codon designate the open reading frames and boxes with a right slant show the 3'-noncoding sequences retained from the original clones.

were picked and tested for their ability to grow on media with glucose or galactose as sole carbon sources (Fig. 2). As expected, all of the transformants were glucose-negative. On galactose, only transformants with pJJH40 (*EcPFKA*) and pJJH55 (*hmPFK*) were able to grow. In liquid medium without uracil and 2% galactose they grew with generation times of 2.5 to 3 h as compared to 2 h of an isogenic, untransformed strain being

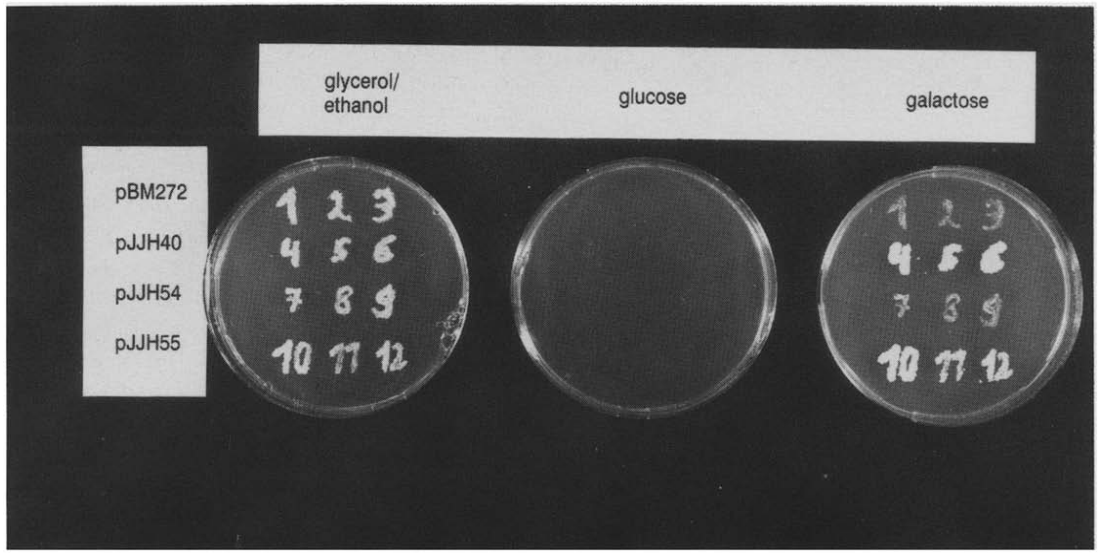

Fig. 2. Growth of transformants on different carbon sources Three independent transformants of strain HD100-2A (*pfk1··HIS3 pfk2 ·HIS3*) with the plasmids indicated (compare Fig. 1) were streaked onto SCGE, left to grow and replica-plated onto synthetic media with the indicated carbon sources lacking uracil They were incubated for 3 days at 30°C. Residual growth on galactose medium of transformants with pBM272 and pJJH54 can be attributed to less stringent carbon catabolite repression exerted by this carbon source as compared to glucose. No residual growth can be observed upon plating again on galactose or streaking out for single colonies.

wild-type for the yeast *PFK* genes with the addition of uracil. A single *pfk2* deletion mutant grows with a generation time of 3 h under these conditions.

From this growth behaviour, one can conclude that both *EcPFKA* and *hmPFK* can be expressed in yeast to the extent of complementing the galactose-negative phenotype. Furthermore, the 5′-noncoding sequences in pJJH54 originating from the human cDNA clone and polylinker sequences seem to prevent a level of expression necessary for complementation. This may be attributed to an extremely GC-rich region from −65 to −81 bp relative to the translation start codon (Fig. 1) that may interfere with efficient translation [36].

Transformants with pJJH40 (*EcPFKA*) and pJJH55 (*hmPFK*) had restored PFK activity in crude extracts (Fig. 3A and B). Thus, PFK activities were about 50% higher in transformants with pJJH40 (*EcPFKA*; 480 mU/mg protein) as compared to those of an isogenic strain being wild-type for the yeast *PFK* genes (305 mU/mg protein). On the other hand, transformants with pJJH55 (*hmPFK*; 140 mU/mg protein) showed less than 50% of the activity of the wildtype control. These data indicate, that both heterologous *PFK* genes can be functionally expressed in yeast.

Further evidence that the PFK activities observed are due to the expression from the transformed plasmids was obtained by shifting cells from galactose to glucose media (Fig. 3A). Upon such a shift, specific PFK activities decreased to less than 10% within 24 h of incubation. This reduction can be largely attributed to the dilution of intracellular PFK during growth, rather than to degradation of the protein. In cultures left to grow to stationary phase on galactose medium, more than 60% of the initial PFK activity was retained in both types of transformants in the same period. The reduction after 24 h of incubation can be attributed to the consumption of galactose from the medium and the consequent decrease in *GAL1* promoter activity. When glucose was added to stationary phase cells expressing the heterologous PFK's, more than 60% of the initial PFK-activity was retained even after 24 h of incubation, indicating that the proteins are very stable. Vice versa, galactose addition to cells pregrown well into stationary phase on galactose and shifted overnight onto SCGE lacking uracil resulted in induction of PFK activity (Fig. 3B). For transformants with pJJH55 (*hmPFK*) the increase in PFK-activity was shown to be accompanied by an increase in the amount of protein in a Western blot (Fig. 3C). The crossreactivity with the antiserum and the size of the band indicate that the correct subunits are produced in yeast.

3.3. *Effect of fructose-2,6-bisphosphate*

Eukaryotic PFK enzymes are allosterically activated by fructose-2,6-bisphosphate, whereas prokaryotic enzymes do not respond to the presence of this compound [37]. This holds true for the enzymes expressed in *S. cerevisiae*. Whereas the specific PFK activities in crude extracts from transformants with pJJH40 (*EcPFK*) did not vary significantly upon addition of the activator (247 mU/mg protein and 265 mU/mg protein without and with fructose-2,6-bisphosphate, respectively), they increased by a factor of 3–4 in those from transformants with pJJH55 (*hmPFK*; 22 mU/mg protein and 70 mU/

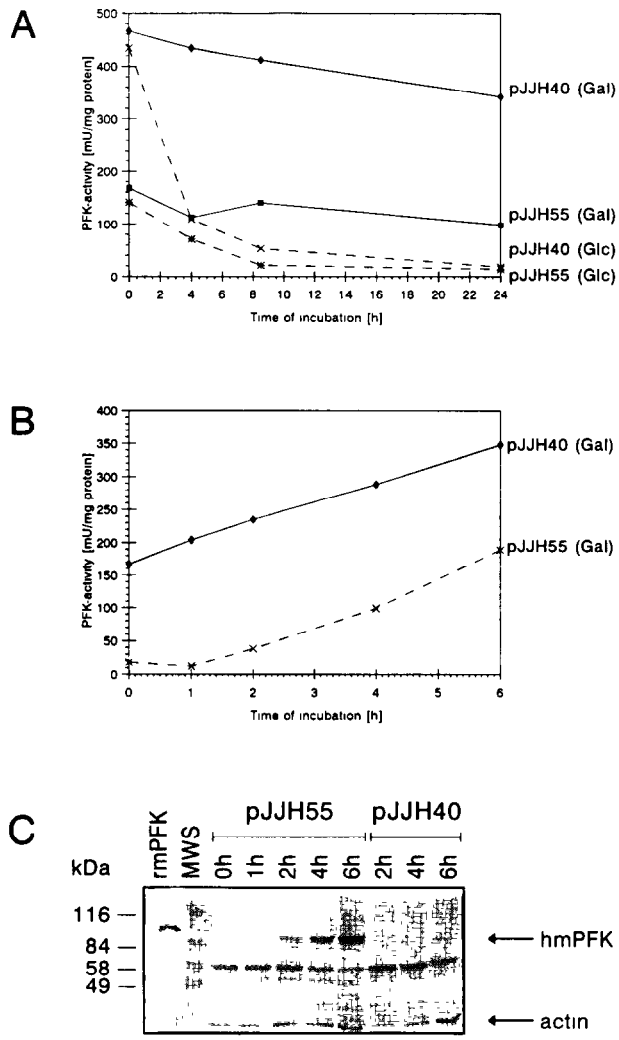

Fig. 3. Control of PFK-expression by the carbon source. Strain HD114-16A (*pfk1::HIS3 pfk2::HIS3*) transformed with the plasmids indicated (compare Fig. 1) was used to prepare crude extracts after the following incubations. (A) Cells were grown in 50 ml SCGal to early stationary phase, washed once with sterile water and inoculated in fresh SCGal (Gal) and SCD (Glc) at about half the original cell density at time 0. At the time points indicated, samples of 8 ml were taken, washed twice and the cell pellet was frozen at −20°C until used for preparation of crude extracts and determination of specific PFK activities. (B) Cells were left to grow to stationary phase for 3 days in 100 ml SCGal. 30 ml of each culture were used to inoculate 100 ml of fresh SCGE and incubated overnight At time 0 galactose was added to a final concentration of 2% and samples were taken and treated as described above. (C) Western blot with 30 μg of protein of the crude extracts prepared in (B) loaded in each lane. The apparent molecular weights of the size standard are indicated. As a control, rabbit muscle PFK obtained from Boehringer-Mannheim (rmPFK) was loaded in the leftmost lane. Transformants with pJJH40 (*EcPFKA*) were used as a negative control where the human PFK is not expressed. As a loading control, the blot was also probed with anti-actin antibodies as indicated. A band at about 58 kDa is not PFK-specific and represents a yeast protein crossreacting with the mammalian antiserum.

mg protein without and with the activator, respectively). Thus, the latter enzyme is not only folded correctly in its catalytic domains, but also retains its allosteric properties.

As both a prokaryotic and a eukaryotic *PFK* gene can be functionally expressed in the yeast *S. cerevisiae*, this work provides the basis for the study of mutant genes and their products in a simple eukaryotic system. In addition, it confirms that PFK functions are highly conserved through evolution. From the fact, that the *pfk* defect of the recipient strain can be phenotypically complemented, one can conclude that either the yeast PFK subunits do not serve a second function distinct from catalysis in hexose fermentation or that the heterologous PFK proteins would also carry out such a function. Taking into account that the *E. coli*-PFK subunits are less than half-size of those of yeast and that the bacterial enzyme is in many ways differently regulated by effectors [38], the latter explanation seems highly improbable.

Transformants with the *E. coli PFKA* gene did not show a significantly impaired growth defect on galactose medium as compared to wild-type yeast strains. The main allosteric effectors of yeast PFK activity under physiological conditions are ATP (inhibition) and fructose-2,6-bisphosphate (activation). Both compounds do not affect the activity of *E. coli* PFK [37,38]. Thus, the lack of these allosteric control mechanisms in the respective transformants does not seem to have a major phenotypic effect on the yeast cells. However, substitution by ohter regulatory factors (i.e. inhibition by elevated PEP concentrations and activation by ADP and ATP), to which the *E. coli* PFK is liable [38] cannot be excluded. Also, the regulatory differences between the yeast and the *E. coli* PFK could be relevant under other experimental conditions. In this context, we are in the process of subcloning the heterologous *PFK* genes under the control of a constitutive promoter to allow for growth on hexoses other than galactose.

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