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Regulation of fitness in yeast overexpressing glycolytic enzymes: parameters of growth and viability

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Summary

Current models predict that large increases over wild-type in the activity of one enzyme will not alter an organism's fitness. This prediction is tested in *Saccharomyces cerevisiae* through the use of a high copy plasmid that bears one of the following: hexokinase B (*HEXB*), phosphoglucose isomerase (*PGI*), phosphofructokinase (*PFKA* and *PFKB*), or pyruvate kinase (*PYK*). Transformants containing these plasmids demonstrate a four to ten-fold increase in enzyme specific activity over either the parent strain or transformants containing the plasmid alone. Haploid and diploid transformants derived from independent backgrounds were grown on both fermentable and non-fermentable carbon sources and evaluated for several components of fitness. These include growth rate under non-limiting conditions, maximum stationary phase density, and viability in extended batch culture. Cell viability is not affected by overproduction of these enzymes. Growth rate and stationary phase density do not differ significantly among strains that overexpress *HEXB*, *PGI* or contain the vector alone. *PFKA, B* transformants show reduced growth rate on glucose in one background only. For these loci the current model is confirmed. By contrast, when grown on glucose, yeast overexpressing *PYK* demonstrate reduced growth rate and increased stationary phase density in both backgrounds. These effects are abolished in cells containing plasmids with a Tn5 disrupted copy of the *PYK* gene. Our results are consistent with reports that the *PYK* locus may exert control over the yeast cell cycle and suggest that it will be challenging to model relations between fitness and activity for multifunctional proteins.

1. Introduction

Research in physiological genetics has focused either on efforts to demonstrate adaptation at specific loci (e.g. DeJong & Scharloo, 1976; Place & Powers, 1979; Koehn *et al.* 1976; Laurie-Ahlberg *et al.* 1981; Burton & Feldman, 1983), or an attempt to understand the global regulation of metabolism (Savageau, 1972, 1976; Garfinkel *et al.* 1970; Kacser & Burns, 1973, 1979, 1981; Heinrich & Rappoport, 1974; Crabtree & Newsholme, 1985). In recent years one model of metabolic regulation, Metabolic Control Theory (Kacser & Burns, 1973, 1979, 1981) has gained favour among many population biologists (Dean *et al.* 1986; Dykhuizen *et al.* 1987; Watt, 1985; Carter & Watt, 1989; Clark, 1989). The model provides a relatively simple conceptual framework for predicting the response of a pathway to variation in enzyme activity, as well as for understanding diverse genetic phenomena such as dominance, epistasis (Kacser & Burns, 1979, 1981) and the diversification of enzymes during evolution (Kacser & Beeby, 1984).

Kacser & Burns (1973, 1979, 1981) argue against the idea that one enzyme is principally responsible for the control of flux through a metabolic pathway. They suggest that control is a systemic property which is more a function of an enzyme's kinetic environment than of any intrinsic factor such as its kinetic constants, its capacity to undergo covalent modification or its position on the metabolic map. From steady-state analysis of a simple, linear pathway their model predicts that: (1) on average, the control an enzyme exerts upon a given pathway is inversely proportional to the number of steps in the pathway, and (2) large increases in activity above wild-type will have disproportionately smaller effects on flux than large decreases. These predictions have been experimentally verified for specific pathways in *Drosophila melanogaster* (Middleton & Kacser, 1983), *E. coli* (Dean *et al.*

1986), and *Neurospora crassa* (Kacser & Burns, 1973; Flint *et al.* 1981; Stewart *et al.* 1986).

Based on this evidence Hartl, Dykhuizen & Dean (1985) have proposed a model for the evolution of selective neutrality (see also Dean *et al.* 1986; Dean, 1989). If it is assumed that flux and fitness positively covary and both are maximized, a plot of average fitness versus the activity of one enzyme will result in a saturating, hyperbolic function. Insofar as they have marginal effects of flux and fitness, most naturally occurring alleles will be found on the plateau of this curve. This relationship does not preclude the activity of natural selection. Indeed, alleles on the plateau are regarded as having 'latent selective potential' and may form the raw material for adaptive shifts when environmental change alters the shape of the fitness/activity function.

The Saturation Theory of Hartl, Dykhuizen & Dean (1985) can serve as a null model analogous to the Hardy–Weinberg Theory. It predicts that large increases over wild-type in the activity of a single enzyme should have negligible impact on fitness. As such it could provide a powerful tool for evaluating the selective potential of individual loci. If overexpression of a gene does promote fitness differences, then this locus should be the subject of further study to determine the physiological mechanism(s) by which those differences arise and the ecological variables to which it might be sensitive.

In this communication we describe an experimental system using *Saccharomyces cerevisiae* that uses the Saturation Model (Hartl *et al.* 1985; Dykhuizen *et al.* 1987) as a means of evaluating the selective potential of four loci in glycolysis. Using high-copy plasmid vectors we have overexpressed DNA fragments which complement metabolic lesions at the three so-called 'rate limiting' steps (hexokinase, phosphofructokinase, and pyruvate kinase) and, for comparison, one 'equilibrium' step (phosphoglucose isomerase). In this way, four- to ten-fold increases over wild-type in the maximum specific activity are obtained for each enzyme. Using haploid and diploid yeast from two genetic backgrounds we present data which describe the effect of these perturbations on three components of fitness: log-phase growth rate, maximum stationary phase density, and cell viability in older cultures. Elsewhere (Rosenzweig, 1991) we describe how these perturbations affect other population parameters such as heat-shock sensitivity and sporulation, as well as physiological and biochemical parameters such as carbon allocation and the pool size of key glycolytic intermediates.

2. Methods

(i) *Strains and plasmids*

The strains and genotypes of *Saccharomyces cerevisiae* used in these experiments are given in Table 1. Plasmids and their relevant characteristics are listed in Table 1. Partial restriction maps of these plasmids are provided in Fig. 1.

(ii) *Media and growth conditions*

Complex medium (YEPD), and synthetic minimal media with amino acids omitted as specified were prepared as described (Sherman, Fink & Hicks, 1986). Carbon sources were added in the following concentrations: glucose 2% w/v glucose, glycerol 2% v/v, and ethanol 3% v/v. Unless otherwise specified, cells were grown on solid media or in 10 ml cultures on a gyratory shaker at 30 °C.

(iii) *Cell count and size*

Cell densities for growth rate calculations were estimated using an electronic particle counter (Coulter Counter ZM; Hialeah, Florida). Prior to counting, cell samples were diluted in Isoton II buffer (Curtin-Matheson, Atlanta, Georgia) and sonicated 10 s at 40 W using a Braun 160 sonicator. The cell size parameter which we report, major peak diameter, was obtained from a size frequency histogram generated by pulse height analysis of the particle counter data (Coulter 256 Channelizer; Hialeah, Florida). We defined this parameter as the average of upper and lower size channels that bracket the major peak and include one-third the total count of particles between 2–8 μm.

(iv) *Cell viability and plasmid segregation*

Viability of cells in older cultures was estimated as the ratio of the number of colony forming units appearing on rich YEPD plates after standard dilution to the number of cells obtained by Coulter count of an equivalent dilution. Plasmid segregation was estimated from a ratio of the number of colonies obtained from plating standard dilutions onto minimal agar plates without leucine to the number of colonies which appeared after plating equivalent dilutions onto YEPD. For both parameters the values reported consist of the average of duplicate plate counts from each standard dilution.

(v) *DNA manipulations*

Plasmid DNA generously provided by Glenn Kawasaki (pHKB, pPGI, and pPK) and Jurgen Heinisch (pPFK1, 2) was transformed into *Escherichia coli* RR1 (Maniatis *et al.* 1982), amplified, and then isolated by CsCl gradient ultracentrifugation as described by Clewell & Helinski (1970). Yeast leucine auxotrophs were grown to mid-log phase in YEPD, transformed by the lithium acetate method (Ito *et al.* 1983), and selected for leucine prototrophy.

Isogenic diploids of haploid strain KTY348 were generated by switching mating type and back-crossing

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Table 1. *Yeast strains and plasmids used in this study*

Yeast strain		Genotype	Reference
DFY410	*a*	*leu2-1, pyk1*	Clifton *et al.* 1977
KTY90	*a*	*trp1, ura3, leu2*	Lab strain, K. Tatchell
KTY91	α	*trp1, ura3, leu2*	Lab strain, K. Tatchell
KTY92	*a*/a	*trp1, ura3, leu2* *trp1, ura3, leu2*	Lab strain, K. Tatchell
KTY348	*a*	*leu2-3, ura3-52, leu2-112*, can^r	Lab strain, K. Tatchell
RRY74	α	*leu2-3, ura3-52, leu2-112*, can^r	This study
RRY78	*a*/a	*leu2-3, ura3-52, leu2-112*, can^r *leu2-3, ura3-52, leu2-112*, can^r	This study

Plasmid	Relevant characteristics	Reference
YEp13	LEU-2, AMP^r, TET^r	
pHKB.1	Hexokinase B, LEU-2, AMP^r	Walsh *et al.* 1983
pPGI.1	Phosphoglucose isomerase, LEU-2, AMP^r	Kawasaki & Fraenkel, 1982
pPFK.1, 2	Phosphofructokinase, α and β subunits, LEU-2, AMP^r	Heinisch, 1986
pPK.1	Pyruvate kinase, LEU-2, AMP^r	Kawasaki & Fraenkel, 1982
pPK::Tn5	Pyruvate kinase disruption, KAN^r, LEU-2, AMP^r	This study

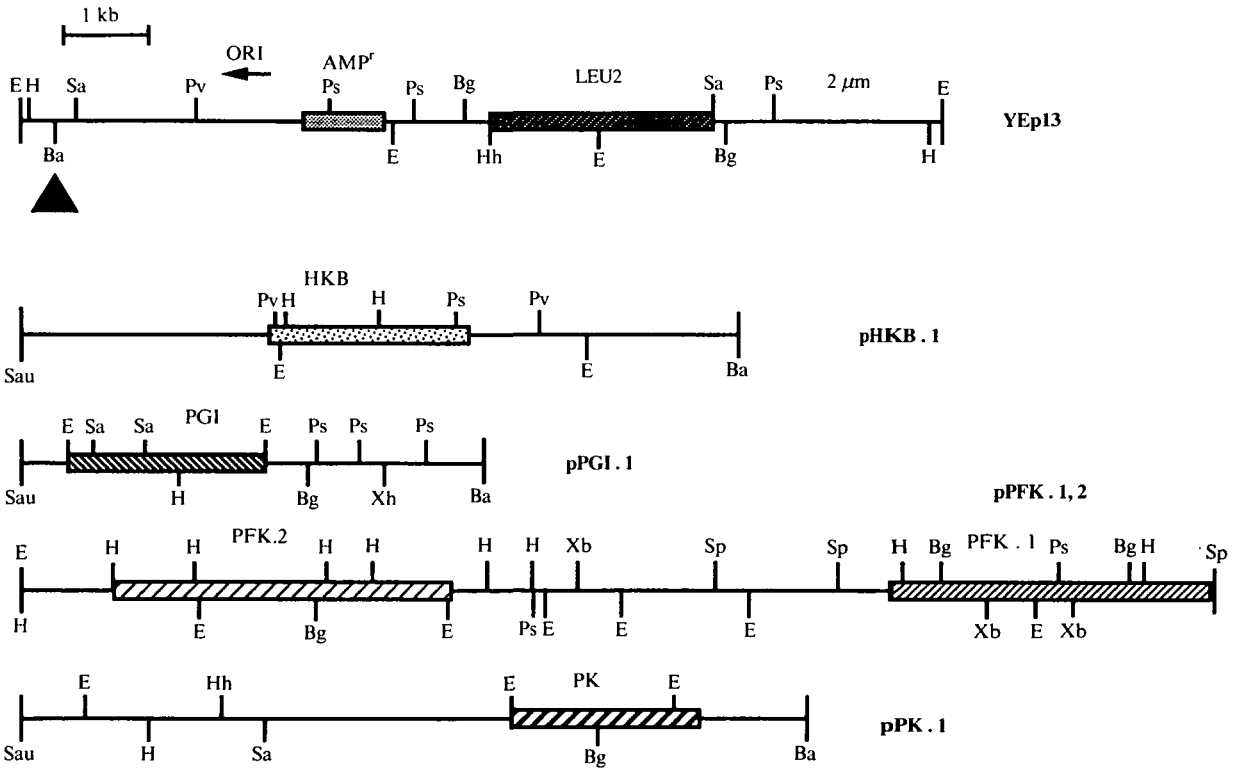

Fig. 1. Restriction maps of YEp13 and inserts used in this study. Genes coding for hexokinase, phosphoglucose isomerase, both phosphofructokinase subunits, and pyruvate kinase were isolated by complementation from a genomic library inserted in the *Bam*H I site of YEp13 (denoted by the black triangle) (Kawasaki & Fraenkel, 1982; Walsh *et al.* 1983; Heinisch, 1986). pPFK.1, 2 was constructed by ligating the 5·7 kb fragment isolated from a partial *Sph* I digest of the PFK1 complementing fragment to a 19·3 kb *Sph* I linearized plasmid which complemented PFK2 (Heinisch, 1986). Restriction endonucleases used: *Bam*H I (Ba), *Bgl* II (B), *Eco*R I (E), *Hin*d III (H), *Pst* I (Ps), *Pvu* II (Pv), *Sal* I (Sa), *Sau3A* (Sau), *Sph* I (Sp), *Xba* I (Xb) and *Xho* I (Xh).

the resulting haploids to the parent strain. Switching was accomplished by transforming the *ura*$^-$, *ho*$^-$, *a*-type parent, KTY348, with a derivative of the YCp50 plasmid that carries both the *URA*3 and *HO* genes. These transformants were screened for the α genotype. α-mating cells were then subcultured several times on complex agar (YEPD) to isolate plasmid-free cells (see Jensen *et al.* 1983). The resulting α, *ura*$^-$, *ho*$^-$, strain, RRY74, was mated to KTY348 to produce the diploid strain RRY78.

Transposon mutagenesis of plasmid-borne pyruvate kinase was performed by a modification of the

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procedure described by Yamamoto (1987). *Escherichia coli* strain DH1 previously transformed with pPk.1 was infected with λ::Tn5 in ten pools at 30 °C for 1 h; cells were then plated onto L agar (10 g tryptone, 5 g yeast extract, 5 g NaCl, 1 ml 10 M-NaOH) containing 50 μg/ml ampicillin and 50 μg/ml kanamycin. Ampicillin, kanamycin resistant colonies were collected from plates using a glass spreader and 5 ml cold 50 mM Tris-Hcl, 15 mM-EDTA, pH 8·0/20% sucrose. Plasmic DNA was isolated from a lysate of these cells by the method of Holmes & Quigley (1981) and used to transform *E. coli* strain DH1λ1. Colonies from these pools were screened for ampicillin and kanamycin resistance; individual amp^R/kan^R colonies were picked and grown overnight at 37 °C in 10 ml L broth containing 50 μg/ml kanamycin, 50 μg/ml ampicillin. Plasmid DNA isolated as described above was then used for subsequent restriction digests and transformations.

Physical evidence presented by Coleman *et al.* (1986) and Burke *et al.* (1983) suggests that most of the pyruvate kinase coding region lies within a 1·9 kb *Eco*R I fragment. Plasmid isolates whose *Eco*R I restriction pattern suggested disruption of the *PYK1* gene were transformed into yeast strain DFY410 (kindly provided by Dan Fraenkel and Drago Clifton), plated onto glycerol + ethanol minimal agar lacking leucine and incubated at 30 °C for 4–5 days. Colonies were then replica plated onto glucose minimal agar lacking leucine. Transformants which failed to grow on the fermentable carbon source were presumed to carry a *PYK* gene on the plasmid that had been disrupted by Tn5. Plasmids generating this phenotype were transformed into the wild-type diploid RRY78, grown in selective media and assayed for pyruvate kinase activity as described below.

(v) *Protein electrophoresis*

SDS-polyacrylamide electrophoresis was performed by the method of Laemmli (1970) as revised by Hames (1981). Extracts were prepared from cells harvested in late log phase. 10 ml cultures were centrifuged at 3 K, 3 min, washed once with ice-cold glass distilled water, and then extracted at a concentration of 200 mg/ml in 50 mM-K_2HPO_4, 15 mM-EDTA, 2 mM-β-mercaptoethanol, 2 mM-PMSF pH 7·4 by glass bead disruption. Protein was determined by Biorad (Richmond, California) microassay based upon the method of Bradford (1976) using bovine serum albumin as standard. Samples containing 50 μg protein were suspended in 100 μl 2% SDS/20% glycerol/0·01% bromophenol blue/2 mM-β-mercaptoethanol and boiled at 100 °C, 5 min. These were loaded onto a 7·5% polyacrylamide gel and run under denaturing conditions at 120 V for 8 h along with protein standards ranging from 29 to 200 kDal. Gels were fixed, stained with Coomassie brilliant blue R and destained as described by Hames (1981).

(vi) *Enzyme assays*

Cell extracts were prepared according to the method of Clifton *et al.* (1977). Cells harvested in late log phase were washed successively in distilled water, cold 150 mM-KCl, and cold 50 mM-K_2HPO_4/2 mM-EDTA/2 mM-β-mercaptoethanol/2 mM-PMSF, pH 7·4. Pellets were then stored at −20 °C. Prior to assay these pellets were resuspended in phosphate buffer to a concentration of 200 mg wet weight cells/ml buffer. Cells were disrupted by vortexing with chilled glass beads at high speed for four 30 s intervals interspersed by 30 s on ice. The resulting extract was centrifuged for 10 min at 10000 RPM in a Sorvall RC5B centrifuge at 4 °C and the supernatant assayed.

Enzyme assays for hexokinase, phosphoglucose isomerase, and pyruvate kinase were performed as in Maitra & Lobo (1971) by measuring changes in absorbance at 340 nm for NAD/NADH and NADP/NADPH coupled reactions with a Beckmann DU-8 spectrophotometer. Phosphofructokinase specific activity was measured as in Breitenbach-Schmitt *et al.* (1984) after Banuelos *et al.* (1977). Total soluble protein was quantitated by Biorad (Richmond, California) microassay based upon the method of Bradford (1976) using bovine serum albumin as standard.

(vii) *Analyses*

Growth rate was determined by regression analysis of that portion of the growth plot where change in $\log_e$ transformed density was linear with time. Estimates were obtained from data which included at least three doublings. For this and other parameters data were compiled from at least three replicate experiments. Comparisons of means were made using the Tukey–Kramer method (Sokal & Rohlf, 1981). These comparisons are presented as ranked, multiple range tests. Each experiment was initiated from a single colony originating from an independent transformation. Since no heterogeneity was observed, we concluded that the transformation procedure had no detectable mutagenic effect. Means of stationary phase densities and viabilities were also compared by the Tukey–Kramer method. As with growth rate experiments, no heterogeneity was observed among replicates originating from independent transformations.

3. RESULTS

(i) *Overexpression of glycolytic enzymes*

Protein extracts from cells harvested in late exponential phase were assayed spectrophotometrically to determine maximum specific activity. Figure 2 shows that the activities of HKB, PFK, and PK are elevated four- to five-fold and that of PGI approximately ten-fold over the untransformed parent strain grown in nonselective glucose minimal media. Compared to the

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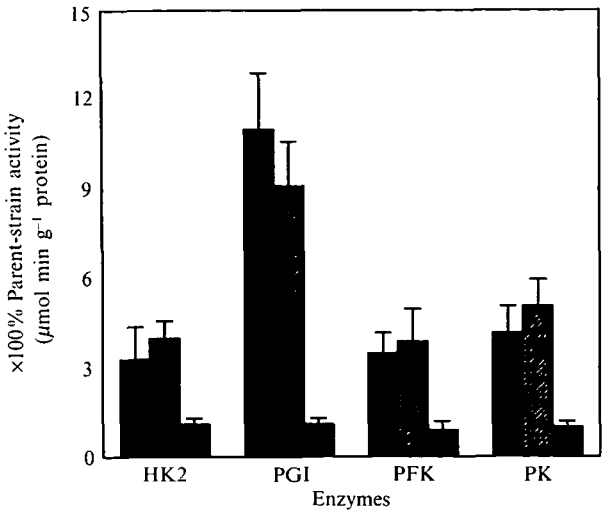

Fig. 2. Transformation of yeast strains KTY90 and KTY348 with high copyplasmids bearing glycolytic genes increases *In vitro* specific activities of these gene products. Transformation with the vector (YEp13) alone does not significantly alter these activities.

Table 2. *Growth of haploid strains KTY90, the isogenic diploid KTY92 and their derivatives on glucose minimal media*

Strain	*N*	Growth rate (hr^{-1}) ± S.E.M.
KTY90	9	0·448 ± 0·004
KTY90 YEp13	8	0·399 ± 0·007
KTY90 HKB	9	0·388 ± 0·006
KTY90 PGI	6	0·380 ± 0·013
KTY90 PFK1, 2	6	0·326 ± 0·11
KTY90 PK	9	0·320 ± 0·005
KTY90 YEp13 HKB PGI PFK PK		
KTY92	6	0·450 ± 0·007
KTY92 YEp13	3	0·388 ± 0·004
KTY92 HKB	3	0·367 ± 0·013
KTY92 PGI	3	0·366 ± 0·024
KTY92 PFK1, 2	3	0·329 ± 0·018
KTY92 PK	3	0·332 ± 0·005
KTY90 YEp13 HKB PGI PFK PK		

Horizontal lines indicate non-significant differences between strains as calculated using the Tukey–Kramer multiple range test ($\alpha = 0{\cdot}05$) (Sokal & Rohlf, 1981).

parent strain, YEp13 transformants demonstrate no significant difference in the activity of these enzymes.

(ii) *Log-phase growth of cells overexpressing glycolytic enzymes*

Growth rates under non-limiting conditions were estimated for haploids and diploids of two independent backgrounds growing in glucose minimal media. In all experiments we employed at least two controls: (1) the untransformed parent grown in nonselective (leucine-plus) minimal media; (2) transformants bearing the vector only (i.e. the vector containing no glycolytic gene) grown in selective

Table 3. *Growth of haploid yeast KTY348, the isogenic diploid RRY78 and derivatives on glucose minimal media*

Strain	*N*	Growth rate (hr^{-1}) ± S.E.M.
KTY348	9	0·419 ± 0·007
KTY348 YEp13	6	0·388 ± 0·010
KTY348 HKB	6	0·397 ± 0·005
KTY348 PGI	9	0·379 ± 0·004
KTY348 PFK1, 2	6	0·327 ± 0·023
KTY348 PK	6	0·290 ± 0·016
KTY348 HKB YEp13 PGI PFK PK		
RRY78	9	0·372 ± 0·005
RRY78 YEp13	9	0·359 ± 0·006
RRY78 HKB	6	0·346 ± 0·007
RRY78 PGI	6	0·346 ± 0·008
RRY78 PFK1, 2	6	0·343 ± 0·004
RRY78 PK	9	0·294 ± 0·008
RRY78 YEp13 YEp13 HKB PGI PFK PK		

Horizontal lines indicate non-significant differences between strains as calculated using the Tukey–Kramer multiple range test ($\alpha = 0{\cdot}05$) (Sokal & Rohlf, 1981).

(leucine-minus) minimal media. Where significant differences were observed a third control was used: (3) transformants bearing a plasmid containing a Tn5 disrupted copy of the gene appearing to promote those differences.

Several patterns emerge from these experiments (Tables 2, 3). First, growth rate is reduced among all transformants. This result is consistent with a large body of evidence suggesting that among microbes, plasmid maintenance can restrict the rate of cell division (Zund & Lebek, 1980; Cheah *et al.* 1987). However, for yeast overexpressing glycolytic enzymes, that cost is not detectable either as changes in flux to ethanol or in steady-state ATP levels during unrestricted growth on glucose (Schaaf *et al.* 1989; Rosenzweig, 1991). This reduction in growth rate was found to be background dependent. In KTY90 and its isogenic diploid, KTY92, transformation *per se* accounts for over twice the total among-strain variance observed in KTY348 and its isogenic diploid RRY78 (analyses not presented). Since KTY348 transformants most closely approximated the parent strain grown under no selection, we chose this background for subsequent experiments.

Yeast transformed with an 'empty' vector, i.e. with Yep13 alone, generally show higher growth rates than glycolytic transformants, but no statistically significant difference can be distinguished between such transformants and strains overexpressing hexokinase B or phosphoglucose isomerase. By contrast, the overexpression of phosphofructokinase and pyruvate kinase consistently depresses growth rate in both backgrounds. The effect of pPFK1, 2 varies with background and ploidy: phosphofructokinase trans-

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Table 4. *Growth of haploid KTY348, the isogenic diploid RRY78, and derivatives on glycerol+ethanol minimal media*

Strain	*N*	Growth rate (hr^{-1}) ± S.E.M.
KTY348	6	0·101 ± 4·23E-3
KTY348 YEp13	6	0·76 ± 4·14E-3
KTY348 HKB	6	0·085 ± 2·14E-3
KTY348 PGI	6	0·071 ± 1·57E-3
KTY348 PFK1, 2	6	0·075 ± 4·01E-3
KTY348 PK	6	0·079 ± 9·43E-3
KTY348 HKB PK YEp13 PFK PGI		
RRY78	3	0·099 ± 1·04E-3
RRY78 YEp13	3	0·094 ± 1·72E-3
RRY78 HKB	3	0·096 ± 1·70E-3
RRY78 PGI	3	0·088 ± 1·03E-3
RRY78 PFK1, 2	3	0·099 ± 5·55E-3
RRY78 PK	3	0·100 ± 2·59E-3
PK RRY78 PFK HKB YEp13 PGI		

Horizontal lines indicate non-significant differences between strains as calculated using the Tukey–Kramer multiple range test ($\alpha = 0{\cdot}05$) (Sokal & Rohlf, 1981).

formants significantly differ from both controls in three out of four ploidy/genotype combinations, but in less than half of all pairwise comparisons with other transformants. However, highly significant differences are observed between pPK.1 transformants and both controls in all four background/ploidy combinations and between pPK.1 and all treatments except pPFK1,2 in three out of four combinations.

(iii) *Log-phase phase growth on alternate carbon sources: glycerol+ethanol*

In yeast the shift from fermentative to respiratory metabolism is characterized by partial repression of key glycolytic enzymes and the derepression of synthesis of enzymes involved in aerobic metabolism (Entian & Zimmermann, 1980; Entian, 1988; Gancedo & Gancedo, 1986). Hexokinase, phosphofructokinase, and pyruvate kinase are all to some extent repressible, either by transcriptional regulation (Maitra & Lobo, 1971; Clifton & Fraenkel, 1981) or allosteric modulation (Hunsley & Sueltor, 1969; Banuelos *et al.* 1977). We might expect that overproducing such enzymes under conditions when they would normally be either repressed or inactivated could be detrimental to the cell.

Although it is constitutively expressed, phosphoglucose isomerase functions in a novel context during respiratory growth. In the gluconeogenic pathway, phosphoglucomutase and fructose-1,6 bisphosphatase replace phosphofructokinase and hexokinase as its principal kinetic linkages. However, in this context, as in glycolysis, PGI catalyses an equilibrium reaction, thus we expected that pPGI transformants would behave identically to the controls.

The results of growth experiments using glycerol+ethanol minimal media are summarized in Table 4. Among haploid transformants derived from KTY348 no significant differences in growth rates could be detected. Indeed, 84% of the variance among strains can be attributed to the substantially higher growth rate demonstrated by the untransformed parent strain (analysis not presented). No significant differences in growth rate could be detected among the isogenic diploid RRY78 and its transformed derivatives. Contrary to our expectations, cells overproducing PGI showed a consistent, but not statistically significant, reduction in growth rate compared to all other transformants.

(iv) *Maximum stationary phase densities in older cultures*

When examining the effects of genetically altered enzyme activity, microbial evolutionists (e.g. Dykhuizen & Hartl, 1981, 1983; Dean *et al.* 1989) have typically equated fitness with maximum growth rate or growth under limiting substrate conditions. However, another component of fitness which may be no less important is the efficiency with which a variant converts resource into biomass or cell number. We therefore determined saturation density among older cultures of yeast growing on both fermentable and non-fermentable carbon sources.

Among haploids in the KTY348 background, significant differences in density were observed after cells had been cultured on glucose for 7 days (Table 5). Those treatments with lowest growth rates (i.e. pyruvate kinase transformants) achieved stationary phase densities which were consistently and significantly higher than all other treatments and controls. No significant differences were observed among strains transformed with pHKB, pPGI, pPFK1,2 and the vector-only control. Among strains cultured on the non-fermentable carbon sources, glycerol+ethanol, this effect was considerably reduced. Significant differences among strains as tested by Analysis of Variance was principally due to differences between pPK.1 and both the parent strain and pPGI transformants (Rosenzweig, 1991). All other pairwise comparisons revealed no significant differences.

Similar patterns were observed among diploids (Table 5). On a fermentable carbon source the parent strain attains significantly lower densities and pyruvate kinase transformants significantly higher densities in every pairwise comparison. All other treatments are statistically indistinguishable. On non-fermentable carbon sources, only pPFK1,2 and pPGI.1 differ at $P < 0{\cdot}05$. It is noteworthy that pPGI transformants, which consistently grew slowest on this media, also show a pattern of reduced stationary phase density among both haploids and diploids. Unfortunately, within-strain variance comprises nearly two thirds of the total variance for both haploids and diploids and

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Table 5. *7 day stationary phase densities of haploid strain KTY348, the isogenic diploid RRY78, and transformants*

	Carbon source			
	Glucose		Glycerol + EtOH	
Strain	*N*	Density ± S.E.M. (10^{-6} cells/ml)	N	Density ± S.E.M. (10^{-6} cells/ml)
KTY348	3	136·2 ± 3·7	6	141·8 ± 4·0
KTY348 YEp13	3	181·6 ± 5·2	5	177·6 ± 3·3
KTY348 HKB	3	187·0 ± 10·8	5	163·1 ± 6·5
KTY348 PGI	3	199·6 ± 7·5	6	142·4 ± 16·4
KTY348 PFK1, 2	3	219·9 ± 10·1	6	166·8 ± 22·6
KTY348 PK	3	277·4 ± 24·7	6	210·4 ± 16·6
PK PFK PGI HKB YEp13 348			PK YEP13 PFK HKB	PFK HKB PGI 348
RRY78	8	87·8 ± 1·1	6	87·6 ± 2·6
RRY78 YEp13	9	120·1 ± 1·4	6	90·5 ± 5·1
RRY78 HKB	9	122·0 ± 4·1	6	113·7 ± 9·7
RRY78 PGI	9	116·5 ± 3·5	6	81·7 ± 6·3
RRY78 PFK1, 2	8	132·0 ± 3·5	6	130·1 ± 14·8
RRY78 PK	9	179·5 ± 11·4	6	120·4 ± 17·6
PK PFK HKB YEp13 PGI 78			PFK PK HKB YEp13	78 PGI

Horizontal lines indicate non-significant differences between strains as calculated using the Tukey–Kramer multiple range test ($\alpha = 0{\cdot}05$) (Sokal & Rohlf, 1981).

Table 6. *Viability and plasmic segregation in stationary phase cultures*

	Glucose			Glycerol + Ethanol		
Strain	Colony forming units ± S.E.M.	(%) Viability	(%) + LEU	Colony forming units	(%) Viability	(%) + LEU
KTY348	99·7 ± 6·0	73·2 ± 4·6	...	N.T.	N.T.	...
KTY348 YEp13	147·3 ± 13·3	80·9 ± 4·9	N.T.	N.T.	N.T.	N.T.
KTY348 HKB	125·0 ± 2·3	67·4 ± 4·5	N.T.	N.T.	N.T.	N.T.
KTY348 PGI	121·7 ± 9·3	61·1 ± 4·7	N.T.	N.T.	N.T.	N.T.
KTY348 PFK	164·7 ± 10·5	75·0 ± 4·7	N.T.	N.T.	N.T.	N.T.
KTY348 PK	216·7 ± 21·5	78·0 ± 1·9	N.T.	N.T.	N.T.	N.T.
(%) Viability	YEp13 PK PFK 348 HKB PGI					
RRY78	56·5 ± 4·2	64·2 ± 4·8	...	46·1 ± 3·9	56·2 ± 3·1	...
RRY78 YEp13	95·6 ± 4·0	79·2 ± 2·7	77·5 ± 3·8	50·8 ± 6·7	57·8 ± 3·9	86·9 ± 3·4
RRY78 HKB	84·7 ± 2·6	71·4 ± 3·4	71·4 ± 3·1	50·8 ± 6·7	57·8 ± 3·9	86·9 ± 3·4
RRY78 PGI	89·0 ± 5·0	73·1 ± 3·6	65·2 ± 2·3	55·7 ± 3·6	60·7 ± 5·3	68·1 ± 3·6
RRY78 PFK	87·7 ± 8·2	71·1 ± 2·9	74·3 ± 7·4	71·4 ± 3·0	59·0 ± 3·8	71·6 ± 3·3
RRY78 PK	154·0 ± 9·1	74·6 ± 2·6	74·6 ± 2·5	72·5 ± 12·3	61·6 ± 5·3	80·0 ± 5·0
(%) Viability	YEp13 PK PGI HKB 78			PK PGI PFK YEp13 HKB 78		
(%) + LEU	YEp13 PK PFK HKB PGI			YEp13 HKB PK PFK PGI		

Horizontal lines indicate non-significant differences between strains as calculated using the Tukey–Kramer multiple range test $\alpha = 0{\cdot}05$) (Sokal & Rohlf, 1981). N.T. = not tested.

may obscure any real differences in performance among strains.

(v) *Viability and plasmid segregation in older cultures*

It is not unreasonable to assume 100% viability among cells whose growth rate is not limited by external substrate concentrations. However, it is not certain that cell number, as determined by a particle counter, represents all viable cells in older cultures. If density is to be regarded as a component of fitness it must correlate with viability. Moreover, some strains might convert primary resources into fewer offspring, but leave them more amply provisioned or somehow better equipped physiologically to deal with the stationary phase environment.

Densities estimated from 7-day-old cultures of both

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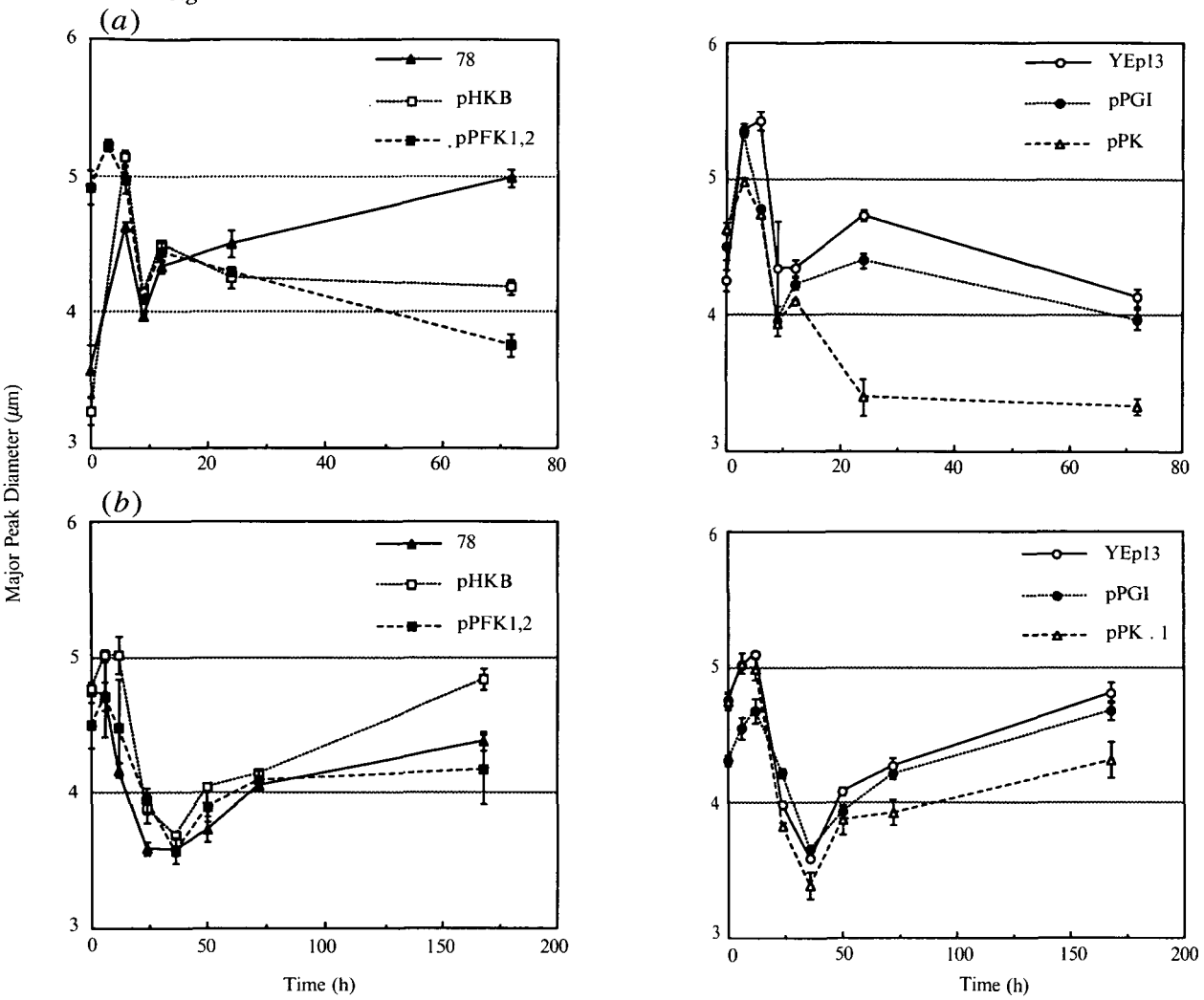

Fig. 3. (*a*) Changes in cell size of RRY78 and transformed derivatives during batch culture on glucose minimal media (mean peak diameter ± S.E.M.). (*b*) Changes in cell size of RRY78 and transformed derivatives during batch culture on glycerol + ethanol minimal media (mean peak diameter ± S.E.M.).

haploids and diploids consistently predict the number of colony forming units (Table 6). Older cultures of transformants bearing the vector-only, pHKB, pPGI, or pPFK1,2 are statistically equivalent for density and number of colonies formed, regardless of the original carbon source. Consistent with their estimated densities in glucose minimal media, the parent strain and pPK transformants produce, respectively, the fewest and greatest numbers of colonies. As a consequence, no significant among-strain differences manifest in cell viability regardless of ploidy or carbon source. Such data indicate that pPK transformants growing on glucose show greater efficiency in converting primary resources into cells.

Since episomal plasmids are unstable, we also estimated in strain RRY78 the proportion of viable cells which still carried the plasmid, i.e. still grew on leucine-minus media agar. Significant differences in this parameter could be interpreted to indicate selection for or against the presence of a particular plasmid (and specific plasmid-borne genes). The results of these experiments are also summarized in Table 6.

Plasmid segregation was largely invariant among strains. Among glucose-grown diploids no significant differences can be demonstrated in the proportion of an older cultures' population which retains the plasmid. For diploids growing on glycerol + ethanol significant ($P < 0{\cdot}05$) among-strain variance is generated by differences between the vector-only control and pPGI transformants (for analyses see Rosenzweig, 1991). Altogether, these data suggest that there is no advantage associated with carrying one plasmid as opposed to another.

(iv) *Change in cell size associated with batch culture of diploids*

No significant differences in cell size among RRY78 and its derivatives were observed in either exponential phase or early stationary phase cultures (Fig. 3*a*, *b*). On glycerol + ethanol minimal media this pattern continues through late stationary phase. However, diploids growing on glucose differ markedly. At 24 h the parent and all transformants except pPK.1 are within one standard error of 4·5 μm. Vector-only, pHKB, pPGI and pPFK1,2 transformants diminish gradually in size as stationary phase progresses, but as

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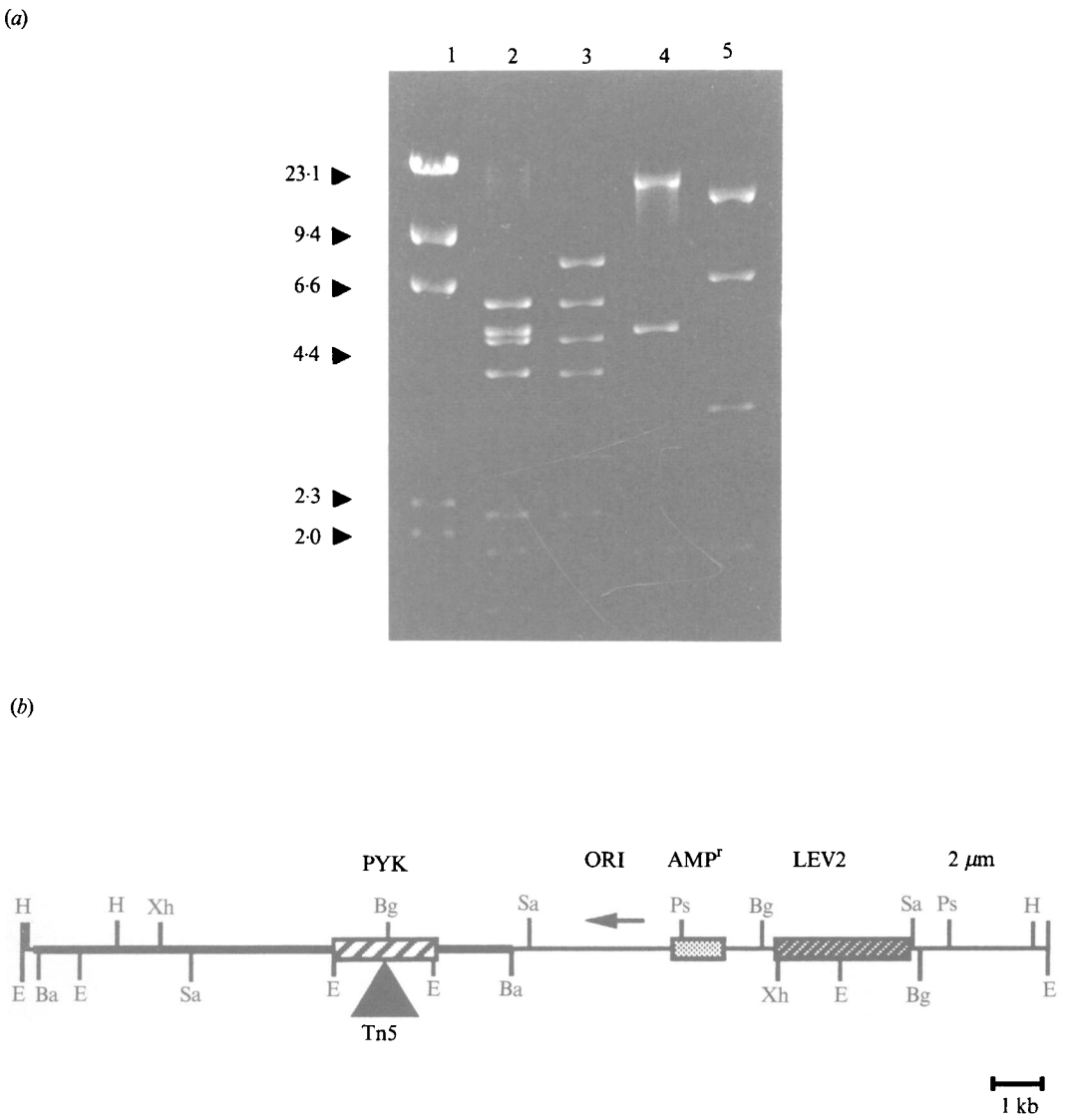

Fig. 4. (*a*) 0·7% agarose gel electrophoresis of pPK.1 and pPK::Tn5 digests. Lane 1, *Hin*d III digested lambda DNA, lane 2, pPK digested with *Eco*R I, lane 3, pPK::Tn5 digested with *Eco*R I, lane 4, pPK digested with *Hin*d III, and lane 5, pPK::Tn5 digested with *Hin*d III. (*b*) *Hin*d III restriction fragment size data locates the Tn5 insertion approximately 5·4 kb downstream of the second *Hin*d III site in pPK.1. Restriction endonucleases used: *Bam*H I (Ba), *Bg1* II (Bg), *Eco*R I (E), *Hin*d III (H), *Pst* I (Ps), *Sa1* I (Sa), and *Xho* I (Xh).

a group they are not distinguished by any significant differences. The parent, RRY78, attains maximum density at approximately 24 h and with no further divisions size remains constant. Elsewhere, we demonstrate that the parent strain's patterns of changes in cell size and density during growth on glucose can be made identical to these treatments by *periodic*, as opposed to initial, supplements of leucine to the media (Rosenzweig, 1991).

By contrast, yeast overexpressing pyruvate kinase are already 25% smaller than other treatments at 24 h and become significantly smaller with the progress of stationary phase. This suggests that on a fermentable carbon source a large fraction of pPK transformants are able to undergo an additional round of division through an alteration in the minimum cell size requirement for cell division. This phenomenon might explain their increased saturation density.

(vii) *Growth parameters of diploids transformed with pPK::Tn5*

Transformation with pPK.1 confers an unusual phenotype for many of the parameters which we have estimated. Pyruvate kinase transformants show depressed growth rate in both backgrounds when grown in glucose minimal media. They attain higher stationary phase densities on a fermentable carbon source and demonstrate reduced cell size in older cultures. These phenotypes may be a direct consequence of overexpressing pyruvate kinase; however, they could arise as a consequence of overexpressing other genes which might be on the pPK.1 plasmid.

The *PYK* locus was originally mapped to chromosome I as a temperature-sensitive cell division cycle mutation, *cdc19* (Hartwell, 1973). Several transcripts arise from regions adjacent to *PYK1/CDC19*

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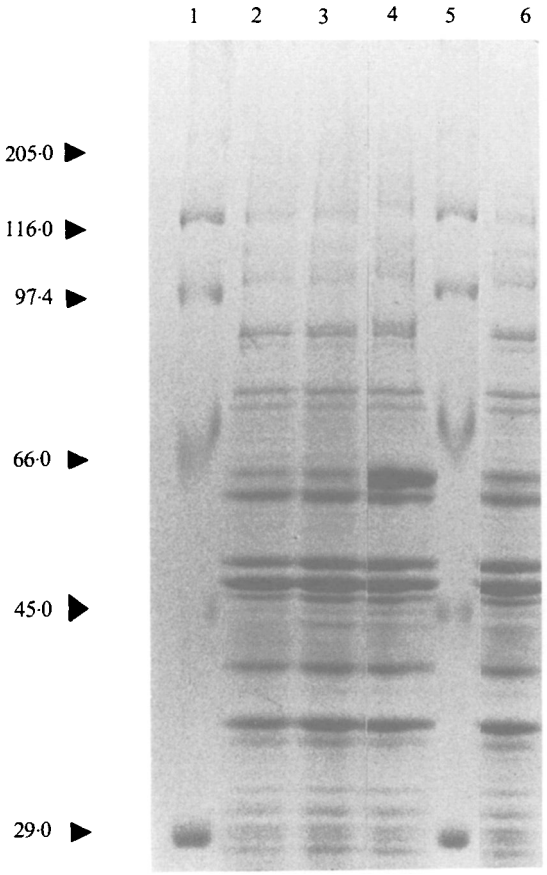

Fig. 5. SDS-polyacrylamide gel electrophoresis (7·5%) of protein from crude extracts of parent strain RRY78 and transformed derivatives. Lanes 1 and 5 contain the following molecular weight standards: myosin (200000 MWU), β-galactosidase (116000), phosphorylase B (97400), bovine serum albumin (66000), egg albumin (45000) and carbonic anhydrase (29000). Lane 2 contains the parent strain RRY78, lane 3, YEp13, lanes 4 and 6, extracts from pPK and pPK::Tn5 transformants, respectively. Overexpression of pyruvate kinase increases band intensity at 60 K. This corresponds to that enzyme's published subunit molecular weight estimate (Burke *et al.* 1983). Tn5 disruption of pPK reestablishes a normal band pattern.

Table 7. *Growth parameters are dependent on levels of pyruvate kinase expression*

Strain	*N*	Growth rate (hr^{-1}) ± S.E.M.	7 day density 10 ~ 6 cells/ml
RRY78 YEp13	9	0·359 ± 0·06	127·8 ± 4·3
RRY78 PK	9	0·294 ± 0·008	207·9 ± 6·2
RRY78 PK::Tn5	8	0·401 ± 0·010	143·5 ± 7·1
YEp13 PK::Tn5 PK			YEp13 PK::Tn5 PK

Horizontal lines indicate non-significant differences between strains as calculated using the Tukey–Kramer multiple range test $\alpha = 0{\cdot}05$) (Sokal & Rohlf, 1981).

(Coleman *et al.* 1986). Most or all of one of these, *FUN10*, is present on plasmid pPK.1. The *FUN10* (renamed *CLN3*) gene product appears to be a cyclin homolog, a dominant mutation in which alters the minimum size requirement for cell division (Carter & Sudbury, 1980; Cross, 1988; Nash *et al.* 1988, Wittenburg *et al.* 1990). To determine which, if any, of the observed phenotypes were specific to pyruvate kinase overexpression the plasmid-borne gene was disrupted by Tn5 mutagenesis. The disruption was mapped by restriction analysis (Fig. 4) and confirmed by protein electrophoresis (Fig. 5). RRY78 yeast transformed with this plasmid demonstrate pyruvate kinase activity equivalent to vector-only transformants and the parent (data not shown).

Transformation of diploid strain RRY78 with pPK::Tn5 corrects the defect in growth rate observed among pPK.1 trains on glucose minimal media (Table 7). Indeed, pPK::Tn5 transformants' growth rate exceeds that of the vector-only control and approximates that of the parent itself (see Table 3). In addition, transformation with pPK::Tn5 abolishes the high stationary phase density phenotype observed among pPK.1 transformants.

The reduced stationary phase cell size displayed by pPK.1 transformants grown on glucose is not completely altered by disrupting plasmid borne PYK. pPK::Tn5 transformants demonstrate the same steep decline in size upon entry into stationary phase as pPK.1 transformants (data not shown). However, the mean diameter of the most abundant size classes in older cultures demonstrate values intermediate between pPK.1 and the vector-only control (see Rosenzweig, 1991). We conclude that while *CLN3* overexpression may confer a detectable phenotype, pyruvate kinase overexpression is primarily responsible for the altered growth parameters which we report.

4. Discussion

In nature, large increases in enzyme specific activity over wild-type may be achieved by a variety of mechanisms. Mutations can arise that extend protein half-life, amplify production, enhance catalytic efficiency, or alter the regulated state. The dominant mutation *WHI-1* (at the *CLN3* locus) may provide an example of the first mechanism: deletion of PEST signal sequences necessary for proteolysis appears to promote accumulation of gene product (Nash *et al.* 1988). In *Drosophila*, structural changes which enhance thermostability appear to extend the half-life of certain *ADH* alleles (McDonald, 1983); such differences may underlie the latitudinal cline in their frequencies.

Overproduction of an enzyme may be achieved by enhanced transcription, improved translation or increased gene dosage. The *cis*-dominant mutation *CYC7-H3*, which causes a 20-fold overproduction of yeast iso-2-cytochrome *c*, arises as a consequence of a deletion fusing the structural portion of the locus to a novel regulatory region (McKnight *et al.* 1981). Transposition can alter the kinetics of transcription;

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Ty1 elements have been shown to enhance gene expression at the *CYC7*, *URA2*, and *ADH2* loci in yeast (Errede, 1980; Williamson *et al.* 1983; Bach, 1984). Segregation frequency data suggest that gene duplications have elevated the activities of glycyl-tRNA synthetase in *Escherichia coli* (Folk & Berg, 1971) and ribitol dehydrogenase in *Klebsiella aerogenes* (Thompson & Krawiec, 1983).

Elevated activity can also arise from structural changes that enhance catalytic efficiency. Such an example has been reported for experimentally evolved *Klebsiella* ribitol dehydrogenase having xylitol affinity (Lin *et al.* 1976). Hall (1981) demonstrated several classes of structural mutations among *lacZ* deletion strains of *E. coli* with evolved β-galactosidase activity. V_{max}/K_m values for lactose among these mutants varied over two orders of magnitude.

Under appropriate physiological conditions elevated activity could be associated with changes in an enzyme's regulated state. This could be mediated either by constitutive activation of the enzyme itself or modification of protein(s) that regulate its activity. Schmidheini *et al.* (1989) describe three alleles in the yeast *ARO7* gene coding for chorismate mutase that exhibit 10-fold increases over basal activity. A single base pair substitution renders the gene product insensitive to two key covalent modifiers, leaving the enzyme permanently 'on'.

Many sugar fermentation enzymes such as invertase (Gascon *et al.* 1968) and galactokinase (Adams, 1972), as well as enzymes of gluconeogenesis (Polakis & Bartley, 1965), and the TCA cycle (Perlman & Mahler, 1974) are normally subject to catabolite repression/derepression. A variety of regulatory mutants have been isolated which derepress these enzymes under normally repressing conditions. Carlson *et al.* (1984) reported a *snf1* suppressor *ssn6* which induces constitutive synthesis of invertase and other repressible enzymes. *and*$^-$ (arginase derepression) mutations derepress asparaginase synthesis in yeast growing on nitrogen rich media producing, thereby, 25- to 200-fold increases in activity (Kamerud & Roon, 1986). The *glr-1* mutant in *Saccharomyces carlsbergensis* demonstrates 3- to 10-fold increases in levels of galactokinase, cytochrome *c*, and cytochrome *c* oxidase, and 20-fold increases in maltase under glucose repressing conditions (Michels & Romanowski, 1980).

Clearly, natural selection may have many opportunities to test the relative fitness of alleles that produce much higher than wild-type levels of 'housekeeping' enzymes. Wild-type alleles may occupy the plateau of a fitness-activity function; however, the means by which that plateau is established could involve either positive or negative adjustments in activity. It is, therefore, biologically reasonable and logically imperative to consider the potential selective advantages and disadvantages associated with variants overexpressing these enzymes.

Four- to five-fold increases in hexokinase B and phosphofructokinase, and ten-fold increases in phosphoglucose isomerase have negligible impact on unrestricted growth rate, stationary phase density, and viability of cells grown on either glucose or glycerol + ethanol. Insofar as these parameters measure organismal fitness, increases in these enzymes would appear to confer little or no selective advantage or disadvantage. Where measured quantitatively, generation time also appears to be unaffected among catabolite repression insensitive mutants growing under repressing conditions (e.g. Michels & Romanowski, 1980; Kamerud & Roon, 1986). These data are highly consistent with the Hartl, Dean & Dykhuizen (1985) Saturation Model which postulates that intrinsic properties of metabolic networks constrain the relationship between fitness and single step variation in enzyme activity to a hyperbolic, saturating function.

However, it could also be that no selective potential was realized because our treatments did not concurrently enhance hexose transport capacity. Becker (1972) and others (Bisson & Fraenkel, 1982; Does & Bisson, 1989) have suggested that sugar transport is the rate-limiting step in yeast fermentation. To the extent that flux is controlled at the level of transport and that transport capacity is unimproved, any potential gain in fermentative ability from overexpressing a 'limiting' enzyme will not be realized. In this context, we note that Dean (1989) demonstrated significantly higher control (and, hence, selection) coefficients among permease than β-galactosidase variants in *Escherichia coli*.

The model does not appear to hold for large increases in the activity of pyruvate kinase. Five-fold increases in the activity of this enzyme depress maximum specific growth rate on glucose in haploids and diploids from independent genetic backgrounds. Our results are consistent with the findings of Schaaf *et al.* (1989) and Moore *et al.* (1990) who also present evidence indicating that pyruvate kinase overproduction reduces growth rate. This reduction is not correlated with significant changes in ethanol production or ATP levels in log-phase cultures (Schaaf *et al.* 1989; Rosenzweig, 1991). Insofar as these physiological parameters are measures of pathway flux, we cannot use Metabolic Control Theory to explain this phenomenon. In addition, we observed that pyruvate kinase transformation increases saturation density among cultures grown on a fermentable carbon source. Elsewhere (Rosenzweig, 1991), we present evidence that this treatment provokes an abnormal response to starvation conditions resulting in decreased flux to the storage carbohydrate glycogen. Changes in carbon allocation may partly explain how pyruvate kinase transformants are able to achieve higher saturation densities.

The pyruvate kinase locus was originally isolated as the temperature-sensitive cell division cycle mutation,

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cdc19 (Hartwell, 1973). Like *cdc25*, *cdc29*, and *cdc35*, *cdc19* mutants arrest in G_1 under restrictive conditions. Subsequent work by Sprague (1977) and Sinha & Maitra (1977) mapped the *pyk* lesion to the same location on Chromosome I. The characteristic defects among *pyk1/cdc19* mutants manifest only on fermentable carbon sources (Fraenkel, 1982, 1986). Our results are consistent with these observations insofar as they suggest that the *pyk1/cdc19* gene product may have both regulatory and catalytic functions and that the regulatory function is pronounced on fermentable carbon sources.

There is mounting evidence that attributes regulatory or structural roles to proteins long believed to be solely catalysts. For example, lactate dehydrogenase forms much of the crystalline matrix in the avian eye (Wistow & Piatorgsky, 1988). Mammalian phosphoglucose isomerase is essentially homologous with the growth factor neuroleukin-2 (Chaput *et al.* 1988). And in yeast, the hexokinase B isoenzyme has been implicated in the mechanism by which external glucose concentration modulates the relative activities of glycolytic and TCA cycle enzymes (Entian *et al.* 1984; Frohlich & Entian, 1984). In *Saccharomyces carlsbergensis* the *glr1* (glucose repression) mutant has been shown to be allelic to hexokinase B (Michels *et al.* 1983). And more recently, HKB has been demonstrated to have protein kinase activity (Herrero *et al.* 1989).

Our results suggest that pyruvate kinase may have regulatory as well as catalytic activity. This suggestion is reinforced precisely because modulation of the locus has effects not predicted by Hartl, Dean and Dykhuizen's Saturation Model (Hartl *et al.* 1985; Dykhuizen *et al.* 1987). Since their model is predicated on a kinetic analysis of pathways composed of enzymes with narrow substrate specificities, the authors themselves recognize that it may be difficult to predict relations between fitness and single locus activity changes of multifunctional enzymes (Hartl *et al.* 1985). However, this very limitation makes the model a useful tool for discovering the loci to whose variable expression an organism might be most sensitive.

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