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Regulation of fitness in yeast overexpressing glycolytic enzymes: responses to heat shock and nitrogen starvation

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Summary

Current models based on the analysis of linear metabolic pathways at steady-state predict that large increases over wild type in the activity of one enzyme will not alter an organism's fitness. This prediction is tested at steps in a highly branched pathway under two conditions known to alter steady-state: heat shock and nitrogen starvation. *Saccharomyces cerevisiae* transformants overproducing 1 of 4 enzymes in glycolysis (hexokinase B, phosphoglucose isomerase, phosphofructokinase, or pyruvate kinase) were subjected to heat shock in both exponential and stationary phases of growth. In neither phase does enzyme overexpression alter heat shock sensitivity. When starved for nitrogen in acetate medium, transformants overproducing hexokinase, phosphoglucose isomerase, and phosphofructokinase sporulate at the same rate and with the same frequency as cells harbouring only the plasmid vector. Current models therefore correctly predict the relationship between activity and components of fitness for 3 of 4 enzymes. By contrast, cells overexpressing pyruvate kinase sporulate poorly. This defect is not observed among cells transformed with a plasmid containing a Tn5 disrupted copy of the *PYK* gene. These findings are consistent with reports that implicate the *PYK* locus in yeast cell cycle control and suggest that it may be challenging to model relations between fitness and activity for multifunctional proteins.

1. Introduction

An organism's reproductive success is frequently determined by its ability to meet the challenges of a deteriorating environment. Individual variability in response to stress can produce differential mortality within natural populations, thus heritable components of these responses become subject to evolutionary change. It is therefore of interest to examine genetic variation at loci involved in physiological adaptations to stress, since catalytic differences among allozymes may promote differences in metabolic flux. Such differences could prove important determinants of fitness in stressful environments. Polymorphisms studied in this regard include: alcohol dehydrogenase (van Delden *et al.* 1978; van Delden, 1982; Middleton & Kacser, 1983) and α-glycerophosphate dehydrogenase (Curtsinger & Laurie-Ahlberg, 1981) in *Drosophila melanogaster*, lactate dehydrogenase in *Fundulus heteroclitus* (Place & Powers, 1979; DiMichele & Powers, 1982; DiMichele *et al.* 1991), glutamate pyruvate transferase in *Tigriopus californicus* (Burton & Feldman, 1983), and leucine aminopeptidase in *Mytilus edulis* (Hilbush & Koehn, 1985).

Several studies suggest that catalytic differences among allozymes can be physiologically significant. When osmoconforming marine invertebrates undergo osmotic stress, cell volume is regulated by modulation of the intracellular pool of free amino acids. In both the copepod, *Tigriopus californicus* (Burton & Feldman, 1983) and the mussel, *Mytilus edulis*, (Hilbush *et al.* 1982) differences in the specific activity of allozymes at steps leading into this pool correlate with significant pool size differences during the response to hyperosmotic shock. In the killifish, *Fundulus heteroclitus*, biochemical parameters such as blood pH, plasma lactate concentration, and intracellular ATP levels vary predictably with the relative catalytic efficiency of LDH allozymes at different temperatures (Place & Powers, 1979). These parameters are consistent with temperature-dependent, genotypic differences in swimming endurance and hatching success (DiMichele *et al.* 1982; Powers *et al.* 1983), and may thus explain a latitudinal cline in the frequencies of LDH alleles.

Such results would appear to conflict with a widely accepted model of metabolic regulation, Metabolic

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Control Theory (Kacser & Burns, 1973, 1979, 1981; Kacser, 1985). This theory holds that the control of flux is a systemic property, and is more a function of an enzyme's kinetic environment than any intrinsic property such as its kinetic constants. From steady-state analysis of a linear pathway the model predicts that (1) the flux response to variation in the activity of any one step is inversely proportional to the number of steps in the pathway, and that (2) large increases in activity will have, on average, negligible effects on flux compared to large decreases. Using these predictions, Hartl and co-workers (Hartl *et al.* 1985; Dean *et al.* 1986; Dykhuizen *et al.* 1987; Dean, 1989) have argued that if flux is held to co-vary with fitness and both are maximized, a plot of average fitness versus the activity of a single enzyme will result in a saturating, hyperbolic function. Most naturally occurring alleles will be found on the plateau of this curve, and would therefore be selectively neutral.

The evidence which supports this model has been obtained under conditions that satisfy its principal assumptions (Hartl *et al.* 1985, Dykhuizen *et al.* 1987; Dean, 1989). These include the use of chemostats to ensure steady-state, and the modulation of the lactose permease/β-galactosidase pathway in *Escherichia coli* to ensure a simple, linear pathway. Moreover, in these experiments the maximum enzyme activity possible has been defined as the fully induced wild type.

Proponents recognize several limitations to the Saturation Theory. The model may not accurately predict the effects of single step activity changes at a metabolic branch point, for a multifunctional enzyme, or for a locus exhibiting overdominance (Hartl *et al.* 1985). An additional limitation may lie in the assumption of steady-state. Because survivorship can critically depend on adaptive responses to stress, and because such responses necessarily remove the organism from steady-state, a general fitness/activity function produced by analyses of flux at steady-state could prove inaccurate. However, these very limitations permit the use of Saturation Theory as a null model by which we may identify loci to whose variable expression an organism is most sensitive.

In this communication we take advantage of this feature of the model and describe an experimental system using *Saccharomyces cerevisiae* which complements earlier studies (Hartl *et al.* 1985; Dean *et al.* 1986; Dykhuizen *et al.* 1987; Dean, 1989). The fitness effects of overexpressing enzymes in a branched pathway are evaluated under non steady-state conditions. We have transformed a diploid strain with a high-copy expression vector containing 1 of 4 yeast DNA fragments which complement specific metabolic lesions in glycolysis. Thereby, we obtain constituitively high activity at each step. We have targeted the three putative 'control' points in glycolysis: hexokinase, phosphofructokinase and pyruvate kinase, and for comparison, one 'equilibrium' step, phosphoglucose isomerase. We report the effects of these perturbations on viability and sporulation under conditions that alter cellular steady-state: heat shock and nitrogen starvation.

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 also listed in Table 1. Partial restriction maps of these plasmids are provided in Rosenzweig (1992).

(ii) *Media and growth conditions*

Complex growth medium YEPD, and synthetic minimal media with amino acids omitted as specified were prepared as described (Sherman *et al.* 1986). Prior to either heat shock or sporulation, yeast were maintained in exponential phase for at least 36 h by serial dilution in glucose minimal media. These 10 ml cultures were agitated by gyratory shaker at 30 °C. For heat-shock experiments, 25 ml of glucose minimal media in 125 ml Ehrlenmeyer flasks were inoculated to an initial density of 2×10^5 cells/ml and grown at 30 °C with constant agitation at 150 rpm. For sporulation experiments, 25 ml of sporulation media (nitrogen-free, 1% potassium acetate, pH 7·0) in 250 ml flasks were inoculated to an initial density of 2×10^7 cells/ml and maintained at 30 °C with constant agitation at 200 rpm.

(iii) *Cell count*

Cell densities 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.

(iv) *Genetic procedures*

Plasmid DNA generously provided by Glenn Kawasaki (pHKB, pPG1.1, and pPK.1) and Jurgen Heinisch (pPFK1, 2) was transformed into *Escherichia coli* strain RR1 (Maniatis *et al.* 1983), and isolated 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.

Transposon mutagenesis of pyruvate kinase and phosphoglucose isomerase were performed by a modification of the procedure described by Yamamoto (1987). *E. coli* strain DH1 (kindly provided by Don Higgins) 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/l tryptone, 5 g/l NaCl, 5 g/l yeast extract, 1 ml/l 10 N NaOH,

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

Yeast strain		Genotype	Reference
DFY 409	**a**	*leu 2–1, pgi 1*	Clifton *et al.* (1977)
DFY 410	**a**	*leu 2–1, pyk1*	Clifton *et al.* (1977)
RRY 78	**a**/α	*leu 2–3, ura 3–52, leu 2–112, can*^{r} *leu 2–3 ura 3–52 leu 2–112 can*^{r}	Rosenzweig (1992)

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)
pPGI::Tn5	Phosphoglucose isomerase disruption, KAN^r, LEU-2, AMP^r	This study
pPFK.1,2	Phosphofructokinase, α & 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

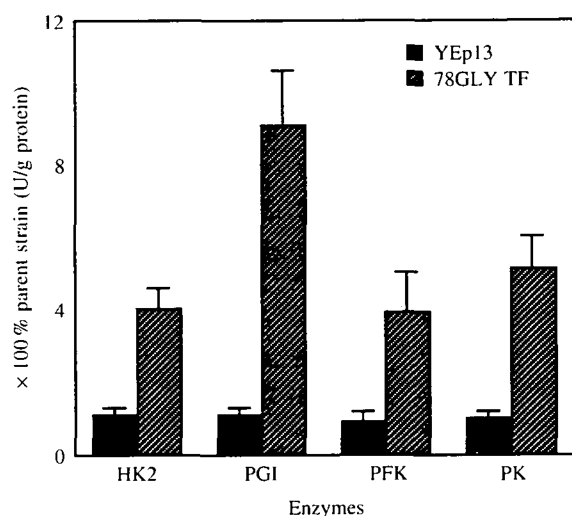

Fig. 1. Transformation of yeast strain RRY78 with a high copy plasmid bearing either of four glycolytic genes increases *in vitro* enzyme specific activity. Transformation with the vector (YEp13) alone does not significantly alter these activities.

15 g/l Bacto-agar) 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 ice-cold 10 mM-Tris, 1 mM-EDTA, 20% w/v sucrose pH 8·0. Plasmid 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 1 fragment of pPK.1 (see Fig. 5). Plasmid isolates whose *Eco*R 1 restriction pattern suggested disruption of the PYK1 gene were transformed into the *leu2, pyk1* yeast strain, DFY410 (kindly provided by D. Fraenkel and D. Clifton), plated onto glycerol + ethanol minimal agar lacking leucine, and incubated at 30 °C for 4–5 days. These colonies were then restreaked 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 diploid yeast strain RRY78, grown in selective media and assayed for pyruvate kinase activity as described below.

Evidence presented by Aguilera & Zimmerman (1986), as well as Green *et al.* (1988) indicates that the coding region for phosphoglucose isomerase is wholly contained within a 2·0 kb *Eco*R 1 fragment of pPG·1 (see Fig. 3). Plasmids with Tn5 insertions in this fragment were transformed into the *leu2, pgi1* yeast strain, DFY409 (also provided by Fraenkel and Clifton), plated onto fructose minimal agar, and incubated at 30 °C for 4–5 days. Colonies were restreaked onto glucose minimal media lacking leucine. Transformants failing to grow on glucose were presumed to carry a Tn5 disruption of the gene of interest on the plasmid. These plasmids were transformed into strain RRY78. Transformants were cultured in glucose minimal media lacking leucine and assayed for phosphoglucose isomerase 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. Ten-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

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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 kDa. 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, ice-cold 150 mM KCl, and ice-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/ml. Cells were disrupted by vortexing with glass beads at high speed for four thirty second intervals interspersed by thirty seconds on ice. The resulting extract was centrifuged at 10000 rpm, 10 min 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 spectrophometer. Phosphofructokinase was assayed as in Breitenbach-Schmitt *et al.* (1984) after Banuelos *et al.* (1977). Protein was assayed by the Biorad microassay based upon the method of Bradford (1976). Bovine serum albumin was used as standard.

(vi) *Heat shock*

Cells were grown at 30 °C in batch cultures to either mid-log or late stationary phases. At t_0, two 1 ml aliquots of cells were withdrawn. The control aliquot was placed in a test tube containing a 1 ml of cold minimal media and kept on ice, and the test aliquot was placed in a tube containing 1 ml minimal media which had been preincubated for 5 min in a water bath set at the shock temperature (48 °C for log phase, 54 °C for late stationary phase cultures). Cells were agitated at 150 rpm in a shaking water batch (New Brunswick Scientific, Edison, NJ) at either 48 or 54 °C. At specified intervals 200 μl aliquots were withdrawn, serially diluted in minimal media, then plated in duplicate onto YPD agar to a density of 50–200 cells. After incubation for 2–3 days at 30 °C colony-forming units (c.f.u.) were counted. Population viability at each time point was determined relative to the count obtained after dilution and plating of the unshocked sample at t_0. Each experiment was performed in triplicate using clones from independent transformations. Since little heterogeneity was observed we concluded that the transformation procedure had no detectable mutagenic effect.

(vii) *Sporulation*

Late exponential phase yeast growing in glucose minimal media were used to inoculate nitrogen free sporulation media to a density of 2×10^7 cells/ml. Cultures were maintained at 30 °C with vigorous agitation. At specified intervals, 10 μl of culture were withdrawn and visually scored for the number of asci observed in a population of 500 cells. Adopting the convention of Croes (1967), we scored only 3- and 4-spored asci. Data was pooled from two experiments, each conducted in triplicate. As with the heat shock experiments each replicate was initiated with colonies from an independent transformation. Little heterogeneity was observed among replicates.

3. Results

(i) *Overexpression of glycolytic enzymes*

DNA fragments complementing null alleles at hexokinase B (HKB), phosphoglucose isomerase (PGI), both subunits of phosphofructokinase (PFK), and pyruvate kinase (PK) were obtained as subclones in the high copy plasmid vector YEp13 (see Rosenzweig, 1992), and transformed into the diploid strain RRY78. Extracts from cells harvested in late exponential phase were then assayed spectrophotometrically to determine maximum specific activity. Fig. 1 shows that the activities of HKB, PFK, and PK are elevated four- to six-fold and that of PGI approximately ten-fold over the untransformed parent strain grown in non-selective minimal media. The level of activities measured among yeast transformed with the vector alone does not differ significantly from those of the parent strain.

(ii) *Heat-shock sensitivity of log phase cells overexpressing glycolytic genes*

The acquisition of thermotolerance in yeast has been shown to be influenced by a variety of factors, including preincubation temperature (McAlister & Finkelstein, 1980), cell-cycle position (Walton *et al.* 1979), and exposure to ethanol (Plesset *et al.* 1982). Brief exposure to an elevated, but non-lethal temperature elicits the rapid and preferential expression of a well-defined group of peptides termed heat-shock proteins (HSPs). This response has been observed across many phyla and the HSPs themselves demonstrate considerable interspecific homology (Bond & Schlesinger, 1987).

In *Saccharomyces cerevisiae*, at least two enzymes of the glycolytic pathway are subject to heat-shock activation. The mRNA and protein levels of both enolase (Lada & Yahara, 1985) and phosphoglycerate kinase (Piper *et al.* 1986) rise dramatically when

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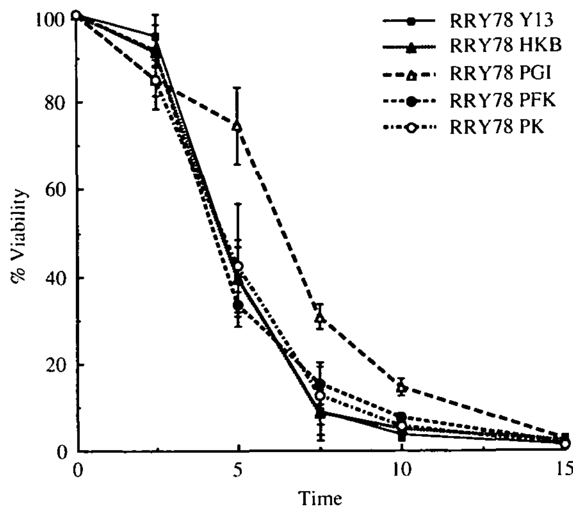

Fig. 2. Heat-shock response of exponential phase cells challenged for 15 min at 48 °C. pPGI transformants demonstrate transient heat-shock resistance.

fermentative log-phase cells are shifted to supra-optimal temperatures. These workers suggest that maintenance of ionic balance during conditions when membrane integrity is compromised places severe energy demands on cells. One element of yeast's adaptive response may be to ensure an intact glycolytic pathway through the overproduction of key enzymes. Accordingly, we tested the hypothesis that over-expression of either hexokinaseB (HKB), phospho-glucose isomerase (PGI), phosphofructokinase (PFK), or pyruvate kinase (PK) might confer thermo-tolerance.

Exponential phase cells grown in glucose minimal media at 30 °C were challenged for 15 min at 48 °C. RRY78 transformants overexpressing HKB, PFK, and PK demonstrate killing curves indistinguishable from RRY78 transformed with only the vector, YEp13 (Fig. 2). However, transformants overexpressing PGI show heat shock resistance through $t_3 = 7{\cdot}5$ min.

The phosphoglucose isomerase gene amplified in these experiments was isolated from a genomic library inserted into the *Bam*H I site of YEp13 (see Rosenzweig, 1992). Physical and genetic evidence (Aguilera & Zimmerman, 1986; Green *et al.* 1988) suggests that the PGI coding region is confined within two *Eco*R I sites on the 4·3 kb insert. To eliminate the possibility that heat shock resistance was conferred by other transcripts arising from this fragment, we disrupted the plasmic-borne copy of PGI with the transposon, Tn5. The disruption was mapped by restriction analysis (Fig. 3) and confirmed by SDS–polyacrylamide gel electrophoresis (Fig. 4).

When pPGI::Tn5 transformants were grown to late exponential phase and transferred to 48 °C they displayed a killing curve indistinguishable from that of transformants overexpressing phosphoglucose isomerase (data not shown). We concluded that PGI overexpression *per se* probably does not confer thermotolerance. Additional gene(s) encoded on the fragment most likely confer this phenotype. It is also possible that overexpression of a truncated, catalytically inactive form of the PGI1 gene product somehow mediates thermotolerance in exponentially growing cells.

(ii) *Heat-shock sensitivity of stationary phase cells overexpressing glycolytic genes*

Heat-shock resistance in yeast has also been shown to depend on the age of a batch culture (Parry *et al.* 1976; Welch *et al.* 1979). *Saccharomyces* grown at any permissive temperature are more thermotolerant in stationary than in exponential phase. This observation has led some workers to postulate adaptive responses that are independent of HSP expression (Henle *et al.* 1982; Hottiger *et al.* 1987). These hypotheses are strengthened by the finding that acquired thermo-tolerance in yeast may be independent of protein

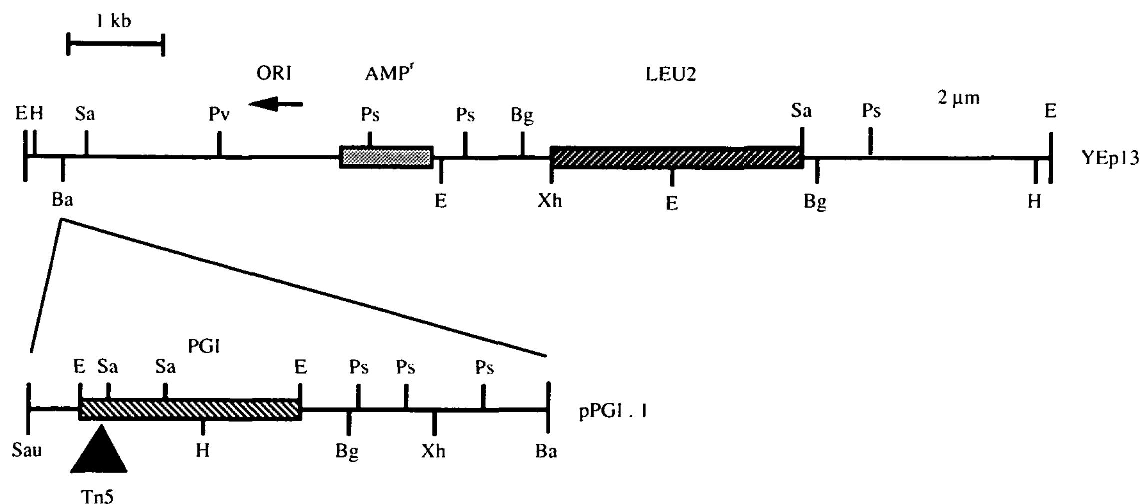

Fig. 3. Analysis of restriction fragment data locates the insertion of the transposon Tn5 approximately 1·1 kb upstream of the unique *Hind* III site in the *PGI* coding region. Restriction endonucleases used include: *Eco*R I (E), *Hind* III (H), *Sau*3A (Sau), *Sal* I (Sa), *Pvu* II (Pv), *Pst* I (Ps), *Bgl* II (Bg), *Bam*H I (Ba), and *Xho* I (Xh).

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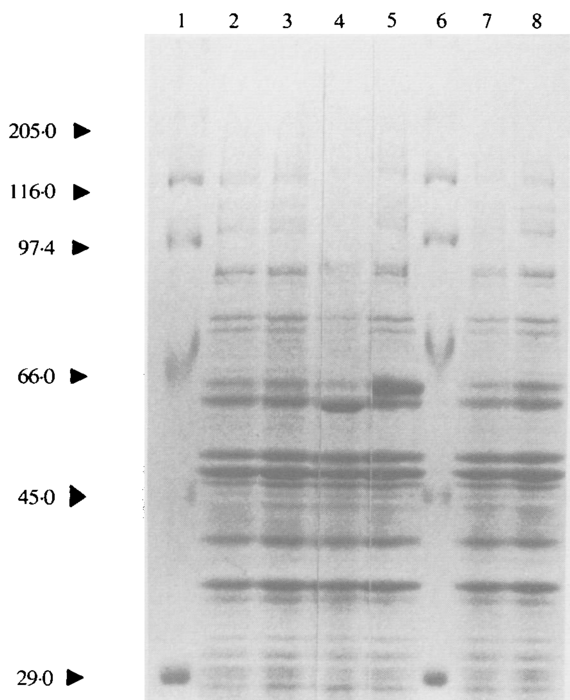

Fig. 4. SDS-polyacrylamide gel electrophoresis (7·5%) of protein from crude extracts of parent strain RRY78 and transformed derivatives. Lanes 1 and 6 contain the following molecular weight standards: myosin (200000 MWU), β-galactosidase (116000), phosphorylase B (97400)), bovine serum albumin (66000), egg albumin (45000), and carbonic anhydrase (29000). Lane 2 contains crude extracts from the parent strain, lane 3, from RRY78 transformed with YEp13, lanes 4 and 7, from pPGI and pPGI::Tn5 transformants, respectively, and lanes 5 and 8, from pPK and pPK::Tn5 transformants, respectively. The subunit molecular-weight estimates for phosphoglucose isomerase and pyruvate kinase are approximately 60000 (Kawasaki & Fraenkel, 1982). Overexpression of these enzymes results in increased band intensity near the BSA standard. In both cases, Tn5 disruption of the plasmid borne gene reestablishes banding patterns similar to those of the parent strain and the vector-only transformants.

synthesis (Hall, 1983; but see McAlister & Finkelstein, 1980), and that disruption of at least one yeast heat-shock gene, HSP26, has no effect on the acquisition of thermotolerance (Petko & Lindquist, 1986).

The resistance to heat shock observed in older cultures may be related to their carbohydrate content. Restriction of growth in *Saccharomyces* is normally attended by the accumulation of glycogen and trehalose (Lillie & Pringle, 1980; Fraenkel, 1982). These pools have been shown to be interconvertible (Grba *et al.* 1975; François *et al.* 1987). Significantly, Hottiger *et al.* (1987*a*) have demonstrated that heat shock induces hyperaccumulation of trehalose, and that thermotolerance is directly proportional to trehalose content. Trehalose is known to stabilize the structural and functional integrity of cell membranes to both heat and desiccation (Crowe *et al.* 1984), and it may also provide an emergency 'sink' to accommodate the rapid increase in intracellular glucose consequent to heat shock. This 'sink' could prevent damage to nucleic acids and proteins due to non-enzymatic reactions with glucose (Ceraki, 1986; Hottiger *et al.* 1987*b*).

Elsewhere (Rosenzweig, 1991) we have presented evidence that overexpression of glycolytic enzymes can perturb carbohydrate accumulation in stationary phase yeast. Overexpression of pyruvate kinase impairs cells' ability to accumulate glycogen, while overexpression of hexokinaseB can elevate levels of trehalose. We predicted, therefore, that such differences would produce distinct heat shock phenotypes among late stationary phase cultures.

Yeast cultures were left in stationary phase for 3 days at 30 °C, then challenged at 54 °C for 90 min as described in the Methods. Under these conditions heat killing reduces population viability by > 80%. Transformed strains are equally heat-shock resistant (data not shown). Even modest differences attributable to transformation with pPFK1,2 are not statistically significant at any time point. Although trehalose levels vary over four-fold among stationary phase transformants (Rosenzweig, 1991), such differences do not result in well defined heat shock phenotypes. Either variation of this magnitude is not physiologically relevant in the context of heat shock or the experimental conditions are not sufficiently stringent to reveal them.

(iii) *Sporulation kinetics of cells overexpressing glycolytic genes*

When starved for essential nutrients diploid yeast normally initiate a response culminating in four haploid spores that are metabolically quiescent and resistant to heat and desiccation. Sporulation encompasses the transition from the vegetative state through meiosis and the formation of ascospores (Esposito & Klapholz, 1981). This transition is initiated by an external stimulus which either signals or mimics the signal for conditions unfavourable to vegetative growth. For most strains this stimulus consists of nitrogen starvation on a non-fermentable carbon source (Croes, 1967; Dickenson, 1988). However, a variety of starvation conditions including phosphate, sulphate or guanine deprivation on low concentrations of non-fermentable carbon can trigger sporulation in some strains, albeit with reduced efficiency (Freese *et al.* 1982; Olempska-Beer & Freese, 1987). During early sporulation, sporogenous **a**/α diploids and isogenic asporogenous **a**/**a** and α/α strains demonstrate similar profiles of RNA transcription (Kurtz & Lindquist, 1984), protein expression (Hopper *et al.* 1974; Betz & Weiser, 1976; Trew *et al.* 1979; Kurtz & Lindquist, 1984) and carbohydrate accumulation (Kane & Roth, 1974). Thus, the initial phase of the process may be regarded as a general starvation response. Since natural populations of yeast inevitably confront cycles of nutrient abundance and limitation, their ability to respond quickly and appropriately to

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environmental signals comprises a significant component of fitness. In this context, we evaluated the sporulation response of nitrogen-starved cells overexpressing different glycolytic enzymes.

The physiology of sporulation suggests several mechanisms by which overexpression of glycolytic enzymes could alter this response. First, during the initial starvation response, reserve carbohydrates must be accumulated from sugar phosphates generated gluconeogenically (Dickenson & Wiliams, 1986). Both the structural components for spore wall formation and the energy required to complete meiosis and ascus development are thought to be derived from these reserves (Esposito & Klapholz, 1981). Overexpression of one gluconeogenic enzyme, phosphoglucose isomerase, has previously been shown to enhance glycogen accumulation in late exponential phase cells grown on glucose (Rosenzweig, 1991). We reasoned that PGI overexpression might also enhance carbohydrate assimilation during sporulation and, thereby, increase either the rate of asci formation or the final yield of asci.

Second, gluconeogenesis requires the bypass of phosphofructokinase and pyruvate kinase. This is usually accomplished by repression of activities at these steps and derepression of fructose-1,6-bisphophatase and phosphoenolpyruvate carboxykinase. Overproduction of the glycolytic enzymes could promote futile cycling at these two 'control' points, and reduce normal flux to storage carbohydrate during early sporulation. A reduction in the storage carbohydrate pool might be expected to reduce sporulation efficiency.

Finally, hexokinaseB has been implicated as the agent mediating (glucose) catabolite repression of TCA cycle enzymes (Entian & Frolich, 1984; Entian *et al.* 1984; Entian, 1986). A functional TCA cycle has been determined to be an absolute requirement for sporulation (Ogur *et al.* 1965; Dickenson & Williams, 1986). If overproduction of this enzyme could maintain repression and reduce cells' aerobic capacity, we could expect spore formation to be inhibited.

Table 2. *Frequency of asci formed among RRY78 derivatives grown in nitrogen-free 1% potassium acetate*

Strain	*N*	Frequency of Asci (± S.E.M.)
RRY78 YEp13	6	0·099 ± 0·010
RRY78 HKB	6	0·105 ± 0·009
RRY78 PGI	6	0·126 ± 0·010
RRY78 PFK1,2	6	0·116 ± 0·007
RRY78 PK	6	0·052 ± 0·009
PGI PFK HKB	Y13	PK
RRY78 PK::Tn5	6	0·094 ± 0·010
RRY78 PK	6	0·030 ± 0·050
PGI PFK HKB	Y13 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).

Diploid yeast were grown in glucose minimal media and then resuspended in sporulation media as described in the Methods. Cultures were monitored over the course of seven days for ascospore formation. The final yield of asci are presented in Table 2. Yield of asci was uniformly poor (< 20%) for all treatments and the vector-only control, however, such results are not unusual for cells grown in minimal media prior to sporulation (Vezinhet *et al.* 1979; Freese *et al.* 1982). In our experiments, it was necessary to use minimal media in order to maintain (leucine) selection on the plasmid.

The results were otherwise notable in two respects. Neither the rate of asci formation (data not shown)

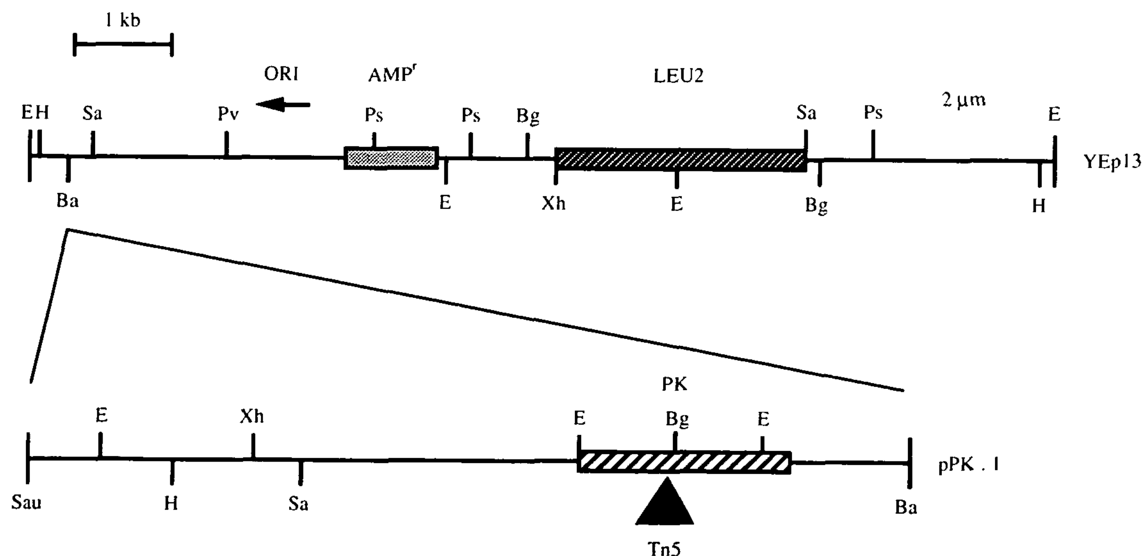

Fig. 5. Analysis of restriction fragment data locates the insertion of the transposon Tn5 approximately 5·4 kb downstream of the second *Hin*d III site in pPK·1. Restriction endonucleases used include: *Eco*R I (E), *Hin*d III (H), *Sau*3A (Sau), *Sal* I (Sa), *Pvu* II (Pv), *Pst* I (Ps), *Bgl* II (Bg), *Bam*H I (Ba), and *Xho* I (Xh).

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nor the final yield differed among the control and the pHKB, pPGI, and pPFK1,2 transformants. We conclude that for these enzymes the system is effectively buffered against such physiological effects as we hypothesized. However, both the kinetics of ascospore formation (data not shown) and the final yield of asci among pPK transformants were significantly reduced.

The *pyk1⁻* complementing fragment used in these experiments has been shown to contain additional coding sequences (Coleman *et al.* 1987), including the cyclin homologue *CLN3*. A hyperactive, dominant mutation at this locus alters the cell-size requirement for division and abbreviates G1 (Cross, 1988; Nash *et al.* 1988; Wittenburg *et al.* 1990). It thus became necessary to establish that the phenotype we observed was caused by the overexpression of pyruvate kinase and not some other locus. We disrupted the pyruvate kinase coding region of plasmid pPK by Tn5 mutagenesis and transformed RRY78 with the new plasmid pPK::Tn5 (see Figs 4 and 5). These transformants present levels of pyruvate kinase activity indistinguishable from yeast transformed with the vector alone (data not shown). We then compared the sporulating ability of pPK::Tn5 transformants and pPK transformants under conditions identical to those described above (see Table 2). pPK::Tn5 transformants demonstrate final yields of asci similar to that of other transformants and controls in the previous experiments. We concluded that in the RRY78 background, overexpression of pyruvate kinase inhibits sporulation.

4. Discussion

Current models of metabolic regulation stress interdependence in biochemical networks. Flux is held to be an emergent property of the system rather than a function of activity at highly regulated steps. Based on steady-state analysis of linear pathways, one prominent theory (Kacser & Burns, 1973, 1979, 1981; Kacser, 1985) predicts that large increases in the activity of one enzyme will have negligible impact on flux compared to large decreases. These findings have recently been extended and offered as a general theory for the evolution of selective neutrality (Hartl *et al.* 1985; Dykhuizen *et al.* 1987; Dean, 1989). According to this theory, wild-type alleles occupy the plateau of a saturating, hyperbolic function which describes the relationship between fitness and variation in the activity of one enzyme. Unless selection alters the shape of this function, the alleles remain equivalent, regardless of the differences in catalytic parameters which may distinguish them *in vitro*.

In the present work we have examined the effects of thermal stress and nitrogen starvation on yeast overexpressing glycolytic enzymes. By so doing, we have relaxed the assumption of steady-state and tested a major prediction of the theory in a branched pathway. Both environmental challenges are known to elicit complex responses which result in major reorganizations of metabolism that remove the organism from steady-state. Significantly, both heat shock and sporulation are known to alter the pool sizes of storage carbohydrates. Previous work (Rosenzweig, 1991) had established that overexpression of certain glycolytic enzymes can markedly perturb these pools, consequently, we expected that potential fitness differences, measured either as survivorship under thermal stress or ascospore yield under nutrient limitation, would be realized under these challenges.

Few differences could be detected in the response of cells to heat shock either in exponential or late stationary phase. Only exponential phase pPGI transformants showed enhanced thermotolerance. This result might not have been unexpected insofar as exponential phase pPGI transformants demonstrate abnormally high glycogen levels (Rosenzweig, 1991). Moreover, despite the fact that it is already one of the most abundant cytosolic proteins, greater increases in activity are obtained for phosphoglucose isomerase than for any other glycolytic enzyme. We might have expected pPGI transformants to become thermotolerant by an aggregative mechanism similar to that postulated for certain low-molecular-weight HSP homologues of mammalian α-crystalline (Ingolia & Craig, 1982). However, cells transformed with a plasmid bearing a Tn5 disrupted copy of *PGI* also show increased thermotolerance. Either an additional protein encoded by both pPGI and pPGI::Tn5 plasmids or a truncated, catalytically inactive form of PGI is responsible for this phenotype. The mechanism by which thermotolerance is conferred remains unclear. Since pPGI::Tn5 restores normal patterns of carbohydrate accumulation (Rosenzweig, 1991), we can eliminate any mechanism which invokes differences in glycogen metabolism. These results are consistent with those of Farkas *et al.* (1991) who found no difference in the heat shock phenotype between wild-type yeast and a glycogen-deficient strain that was defective at both glycogen synthase loci.

Overexpression of 3 out of 4 glycolytic enzymes had no effect on cellular response to nitrogen starvation in acetate media. The kinetics of ascospore formation and final yield of asci were indistinguishable among the vector-only control, pHKB, pPGI, and pPFK transformants. However, pPK transformants sporulated poorly. This apparent defect was corrected by disruption of the plasmid borne *PYK* gene. Previous work has established (Rosenzweig, 1992) that pPK transformants display a number of unusual phenotypes with respect to growth. Compared to isogenic strains harbouring only YEp13, haploid and diploid pPK transformants from two genetic backgrounds grow more slowly under non-limiting conditions, and attain higher stationary phase densities. In addition, glucose grown cultures of diploid pPK transformants

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display reduced levels of both major storage carbohydrates in late stationary phase (Rosenzweig, 1991). For each of these characters, pPK::Tn5 transformants demonstrate the control phenotype.

Taken together these data suggest possible regulatory role(s) for pyruvate kinase, in addition to its catalytic function. This suggestion is strengthened by the observation that a lesion at the *PYK1* locus was first isolated as a cell division cycle mutation, *cdc19* (Sprague, 1977; Pringle & Hartwell, 1982). Since cells overexpressing pyruvate kinase grow more slowly, and continue to divide under conditions when other cells arrest, it is not surprising that they respond aberrantly to conditions which normally induce a starvation response. However, the role which *PYK1* may play in either signal transduction or cell cycle regulation remains unclear. In this context, we note that another glycolytic enzyme, hexokinase B, has long been implicated as a regulator of yeast catabolite repression. Recently this protein has been demonstrated to have protein kinase activity (Herrero *et al.* 1989).

In summary, the integrated responses of yeast to heat shock and nitrogen starvation appear to be insensitive to overexpression of hexokinase, phosphofructokinase, and phosphoglucose isomerase. Given our results, it is difficult to imagine selective regimes involving these agents which would favour increases in enzyme activity over wild type. For these enzymes, our study confirms the Saturation Model. However, high activity of a multifunctional protein may have effects on fitness not predicted by analyses that incorporate only its catalytic parameters for one reaction. In the present case, we can easily imagine conditions which would place selective value on alleles that repress sporulation. For example, in environments where the survivorship of quiescent, vegetative cells equalled that of ascospores, there might be a selective disadvantage to incurring the cost of meiosis. The difficulty commonly encountered in attempts to sporulate wild strains suggests that such conditions may be prevalent.

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