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Control of the Glycolytic Flux in *Saccharomyces cerevisiae* Grown at Low Temperature

A MULTI-LEVEL ANALYSIS IN ANAEROBIC CHEMOSTAT CULTURES[*] [S]

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Growth temperature has a profound impact on the kinetic properties of enzymes in microbial metabolic networks. Activities of glycolytic enzymes in *Saccharomyces cerevisiae* were up to 7.5-fold lower when assayed at 12 °C than at 30 °C. Nevertheless, the *in vivo* glycolytic flux in chemostat cultures (dilution rate: 0.03 h^{-1}) grown at these two temperatures was essentially the same. To investigate how yeast maintained a constant glycolytic flux despite the kinetic challenge imposed by a lower growth temperature, a systems approach was applied that involved metabolic flux analysis, transcript analysis, enzyme activity assays, and metabolite analysis. Expression of hexose-transporter genes was affected by the growth temperature, as indicated by differential transcription of five *HXT* genes and changed zero trans-influx kinetics of [^{14}C]glucose transport. No such significant changes in gene expression were observed for any of the glycolytic enzymes. Fermentative capacity (assayed off-line at 30 °C), which was 2-fold higher in cells grown at 12 °C, was therefore probably controlled predominantly by glucose transport. Massive differences in the intracellular concentrations of nucleotides (resulting in an increased adenylate energy charge at low temperature) and glycolytic intermediates indicated a dominant role of metabolic control as opposed to gene expression in the adaptation of glycolytic enzyme activity to different temperatures. In evolutionary terms, this predominant reliance on metabolic control of a central pathway, which represents a significant fraction of the cellular protein of the organism, may be advantageous to limit the need for protein synthesis and degradation during adaptation to diurnal temperature cycles.

Changing ambient temperature, for example, as a result of diurnal temperature cycling, is among the most common environmental changes that microorganisms have to contend with in nature. Temperature effects on microbial physiology are also relevant for the industrial exploitation of microorganisms. For example, the temperature in industrial processes for production of alcoholic beverages with the yeast *Saccharomyces cerevisiae* (commonly 8–20 °C) is much lower than the optimum temperature range for growth (25–30 °C) (1).

Much of the current knowledge on adaptation of the model eukaryote *S. cerevisiae* to suboptimal temperatures has been derived from studies on cold shock. These studies have identified cold-induced changes in membrane lipid composition (2), transport functions, translational efficiency, protein folding, and nucleic acid structure (for a review, see Ref. 3). Transcriptome analysis of cold adaptation in *S. cerevisiae* has revealed several types of responses that depend on the temperature range applied, namely "cold shock" (between 20 and 10 °C) (4, 5) and "near freezing" (<10 °C) (6) conditions. Responses to low temperature are also affected by the exposure period to low temperature (early phase and late phase responses (4, 6)). The early response in cold shock experiments encompasses up-regulation of low-temperature marker genes such as *NSR1* (7), *TIR1*, *TIR2* (8), and *TIP1* (9). The late phase is characterized by up-regulation of genes involved in protein folding, trehalose synthesis, and stress responses (*e.g. HSP12* and *HSP26* (10)), which suggests involvement of *MSN2* and *MSN4* in the regulatory circuit for cold adaptation (4, 10). Recent reports have implicated the HOG pathway in the transduction of the low temperature signal, resulting in the production of glycerol and trehalose required for resistance to freezing but not for growth at 12 °C (11).

Temperature also has a drastic effect on the catalytic properties of enzymes. The temperature dependence of enzyme kinetics on the catalytic rate constant, k_{cat}, of a reaction is partially governed by the Arrhenius equation (Equation 1).

$$k_{cat} = Ae^{\left(\frac{-E_a}{RT}\right)} \quad \text{(Eq. 1)}$$

This equation dictates that increasing the temperature or decreasing the activation energy (for instance, through the use of a catalyst) will result in an increase of k_{cat} (12). Each enzyme has unique catalytic properties, including temperature optimum and specific binding to allosteric effectors that contribute to regulation of its *in vivo* activity (these effects are not taken into consideration by the Equation 1).

In natural environments, both specific growth rate and the

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1.

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fermentation rate are key parameters in determining evolutionary fitness of microorganisms. To optimize rates at lower temperatures, microorganisms can, in principle, resort to different strategies. First, the synthesis of rate controlling enzymes may be increased. This "vertical" regulation may be effected at the level of transcription, mRNA degradation, protein synthesis, and degradation and/or post-translational modification. Alternatively or additionally, temperature-compensatory shifts of *in vivo* enzyme activity may be accomplished by "metabolic" regulation altering intracellular and extracellular concentrations of metabolites and effectors. The impact of metabolic control may also result from the temperature-dependent expression of (iso)enzymes with different kinetic properties (13).

The aim of the present study is to assess the contribution of vertical and metabolic regulation in the physiological adaptation of *S. cerevisiae* to lower temperature. In batch cultures, the tight coupling of temperature and specific growth rate makes it exceedingly difficult to dissect effects of these two parameters. For example, it is well documented that, even at a fixed growth temperature, different specific growth rates result in different gene expression patterns (14) as well as different intracellular metabolite concentrations (15). In the present study, we circumvent this problem by comparing growth of *S. cerevisiae* at 12 and 30 °C in glucose-limited chemostat cultures. Because, in chemostat cultures, the specific growth rate is equal to the dilution rate, this cultivation technique enables investigation of the impact of temperature at a fixed specific growth rate. Our study is focused on glycolysis and alcoholic fermentation, a pathway that plays a central role in anaerobic growth and represents a substantial fraction of the cellular protein of the yeast (16). To dissect the regulation of *in vivo* glycolytic flux, data from physiological studies are integrated with *in silico* metabolic flux analysis, transcriptome analysis, enzyme-activity assays, and intracellular metabolite analyses.

EXPERIMENTAL PROCEDURES

Strain and Growth Conditions—The *S. cerevisiae* prototrophic haploid reference strain CEN.PK113-7D (MAT***a***) (17) was grown at a dilution rate (D) of 0.03 h^{-1} at both 12 or 30 °C in 2-liter chemostats (Applikon, Schiedam, The Netherlands) with a working volume of 1.0 liter as described in Ref. 18. A temperature probe connected to a cryostat controlled cultures grown at 12 °C. Cultures were grown in a defined synthetic medium that was limited by glucose with all other growth requirements in excess as described (18). The dilution rate was set at 0.03 h^{-1} with pH measured online and kept constant at 5.0 by automatic addition of 2 M KOH using an Applikon ADI 1030 Biocontroller. The stirrer speed was set to 600 rpm. Anaerobic growth and steady-state conditions were maintained as described (18). Biomass dry weight, metabolite, dissolved oxygen, and gas profiles were constant at least three volume changes prior to sampling.

To measure the specific maximal growth rate at 12 and 30 °C, anaerobic batch cultivations were performed in 2-liter chemostats (Applikon, Schiedam, The Netherlands) with a working volume of 1.0 liter. Precultures were grown in mineral medium with 2% glucose until stationary phase in shaker flask at 200 rpm at 30 °C. Fermentors were inoculated with precultures at an A_{660} of 0.1. Cultures were grown in a predefined synthetic medium for anaerobic growth (18) with 2% glucose. pH, temperature, and stirrer speed were maintained as previously described for chemostat anaerobic cultures.

Analytical Methods—Culture supernatants were obtained with the method described in Ref. 19. For the purpose of glucose determination and carbon recovery, culture supernatants and media were analyzed by high performance liquid chromatography on an AMINEX HPX-87H ion exchange column using 5 mM H_2SO_4 as the mobile phase. Ethanol evaporation from cultures was determined as described in Ref. 20. Residual ammonium concentrations were determined using cuvette tests from DRLANGE (Dusseldorf, Germany). Culture dry weights were determined as described in Ref. 21, whereas whole cell protein determination was carried out as described in Ref. 22.

Trehalose and Glycogen—Trehalose and glycogen concentration measurements were according to Parrou and François (23). Trehalose was determined in triplicate measurements for each chemostat. Glycogen was determined in duplicate for each chemostat. Glucose was determined using the UV method based on Roche kit 0716251.

Fermentative Capacity Assay—Fermentative capacity assays were as previously described in Ref. 24. The fermentative capacity can be defined as the specific maximal production rate of ethanol per g of biomass (mmol/g/h) under anaerobic and excess glucose conditions.

Microarray Analysis—Sampling of cells from chemostats, probe preparation, and hybridization to Affymetrix Genechip® microarrays were performed as described previously in Ref. 25. RNA quality was determined using the Agilent 2100 Bioanalyzer. The results for each growth condition were derived from three independently cultured replicates. Data acquisition and analysis as well as statistical analysis were performed as described previously in Ref. 18. The microarray data have been deposited at Genome Expression Omnibus data base under series number GSE6190.

In Vitro Enzyme Assays—Each *in vitro* enzyme assays for the glycolytic pathway was performed as previously described (24). All enzyme assays were performed at 12 and 30 °C (with exception of enolase that has not been measured). Protein determinations of cell extracts were as previously described (26).

Metabolic Flux Distribution—Intracellular metabolic fluxes were calculated through metabolic flux balancing using a compartmented stoichiometric model derived from the model developed (27). The setup for the model was performed using the dedicated software (SPAD it, Nijmegen, The Netherlands). For both 12 and 30 °C chemostats, the specific rates of growth, substrate consumption, carbon dioxide, and ethanol production during steady-state cultivation were calculated from the measured concentrations and flow rates from three independent chemostats. Assumptions and the theory of the model were previously described (28).

Zero Trans-influx Uptake Assays with Labeled [^{14}C]Glucose—For both cultures from different temperatures, cells were harvested from duplicate fermentations by centrifugation at 4 °C (5 min at 5000 rpm) and zero trans-influx uptake of labeled [^{14}C]glucose was determined at 30 °C according to Ref. 29. The

TABLE 1

Physiological and biochemical characteristics of glucose-limited anaerobic chemostats grown at 12 and 30 °C

Values represent the mean ± S.D. of data from three independent steady-state chemostat cultures.

Culture temp (°C)	Physiological data							Biochemical data		
	$Y_{Glc\text{-}X}$	q_{Glu}	$q_{ethanol}$	q_{CO_2}	Carbon recovery	Residual glucose	Fermentative capacity	Trehalose	Glycogen	Whole cell proteins
	$g_{glucose} \cdot g_{dry\ wt}^{-1}$	$mmol \cdot g_{dry\ wt}^{-1} \cdot h^{-1}$			%	g/liter	$mmol \cdot g_{dry\ wt}^{-1} \cdot h^{-1}$	$g_{eq} \cdot glucose \cdot g_{dry\ wt}^{-1}$		$g_{proteins} \cdot g_{dry\ wt}^{-1}$
12	0.07 ± 0.01	−2.5 ± 0.2	3.8 ± 0.3	4.4 ± 0.3	100 ± 3	0.5 ± 0.2	19.3 ± 1.6	<0.005	0.06 ± 0.0	0.40 ± 0.0
30	0.07 ± 0.00	−2.3 ± 0.0	3.5 ± 0.0	3.8 ± 0.2	95 ± 1	0.05	11.4 ± 0.6	0.02 ± 0.0	0.04 ± 0.0	0.43 ± 0.0

TABLE 2

***In silico* glycolytic fluxes**

Reaction number	Enzymes	*In silico* fluxes analysis (mmol g^{-1} h^{-1})		12 versus 30 °C
		12 °C	30 °C	
1	Hexokinase	2.4 ± 0.1	2.1 ± 0.1	1.1
2	Phosphoglucose isomerase	2.3 ± 0.1	2.0 ± 0.1	1.1
3	Phosphofructokinase	2.3 ± 0.1	2.0 ± 0.1	1.1
4	Fructose 1,6 bisphosphate aldolase	2.3 ± 0.1	2.0 ± 0.1	1.1
5	Triose-phosphate isomerase	1.9 ± 0.1	1.7 ± 0.1	1.1
6	Glyceraldehyde-3-phosphate dehydrogenase	4.3 ± 0.2	3.8 ± 0.1	1.1
7	3-Phosphoglycerate kinase	4.3 ± 0.2	3.8 ± 0.1	1.1
8	3-Phosphoglycerate mutase	4.3 ± 0.2	3.8 ± 0.1	1.1
9	Enolase	4.3 ± 0.2	3.8 ± 0.1	1.1
10	Pyruvate kinase	4.3 ± 0.2	3.8 ± 0.1	1.1
11	Pyruvate decarboxylase	4.1 ± 0.2	3.6 ± 0.1	1.1
12	Alcohol dehydrogenase	4.1 ± 0.2	3.6 ± 0.1	1.1

parameters of sugar transport were derived according to single component Michaelis-Menten kinetics.

Intracellular Metabolite Determination—The procedure and equipment for rapid sampling of intracellular metabolites were based on Ref. 19 using the cold methanol quenching method. Two independent chemostats for each culture temperature were run and metabolite measurements were done in triplicate for each chemostat. Glycolytic intracellular metabolites were analyzed by ESI-LC-MS/MS according to Ref. 30 and the quantification was performed following the IDMS concept (31). Nucleotide concentration in the cell extract was analyzed by an ion pairing ESI-LC-MS/MS method and quantified following the IDMS concept. The adenylate charge (AC)[2] was calculated as shown in Equation 2.

$$AC = \left(\frac{[ATP] + \frac{1}{2}[ADP]}{[ATP] + [ADP] + [AMP]} \right) \quad \text{(Eq. 2)}$$

RESULTS

Physiological and Biochemical Analysis of Chemostat Cultures Grown at Different Temperatures—The chemostat cultures were performed at 0.03 h^{-1} at a dilution rate compatible with the temperature of 12 °C as the maximal specific growth rate of the *S. cerevisiae* CEN.PK113-7D strain was 0.035 ± 0.002 h^{-1} at 12 °C (*versus* 0.34 ± 0.01 h^{-1} at 30 °C). Biomass yields, as well as specific rates of glucose consumption, ethanol, and carbon dioxide production in anaerobic, glucose-limited chemostat cultures of *S. cerevisiae* differed by less than 15% at 12 and 30 °C (Table 1), indicating that growth energetics were not drastically affected by temperature. The only physiological characteristic that clearly distinguished the cultures was a 10-fold higher residual glucose concentration at 12 °C compared with 30 °C (Table 1).

Whereas trehalose accumulation is a commonly observed phenomenon in studies on transient cold stress (10), the trehalose concentration during steady-state growth at 12 °C was much lower than at 30 °C. This is consistent with previous studies indicating that trehalose is essential for freezing tolerance, but not for growth at temperatures above 10 °C (4, 11). The cellular glycogen content was 1.5-fold higher at 12 than at 30 °C (Table 1).

In vivo fluxes in glycolysis were calculated via flux balancing using a stoichiometric model (Table 2) (28). To calculate *in vivo* fluxes, the model was fed with quantitative data on the biomass composition of *S. cerevisiae* (32) and with the substrate consumption and product formation rates measured in the carbon-limited chemostat cultures (Table 1). Consistent with similar culture kinetics (Table 1), glycolytic flux was not substantially different in cultures grown at 12 and 30 °C (Table 2). Conversely, off-line measurements of the fermentative capacity in glucose-rich medium at 30 °C yielded values that were 70% higher for cells grown at 12 °C than for cells grown at 30 °C (Table 1 and supplemental materials Table S1).

The increased fermentative capacity of cells grown at 12 °C suggested that an up-regulation of key rate-controlling enzymes involved in fermentative glucose metabolism and/or glucose transport is part of the mechanism by which *S. cerevisiae* maintained a constant glycolytic flux in the chemostat cultures grown at 12 and 30 °C. We subsequently analyzed the

[2] The abbreviations used are: AC, adenylate charge; HXT, hexose transporter; FBA, fructose 1,6-bisphosphate aldolase; PGI, phosphoglucose isomerase; PGK, phosphoglycerate kinase; PFK, phosphofructokinase; TPI, triose phosphate isomerase; PGM, phosphoglycerate mutase; PYK, pyruvate kinase; PDC, pyruvate decarboxylase; TDH, glyceraldehyde-3-phosphate dehydrogenase; ADH, alcohol dehydrogenase; PEP, phosphoenolpyruvate; HXK, hexokinase.

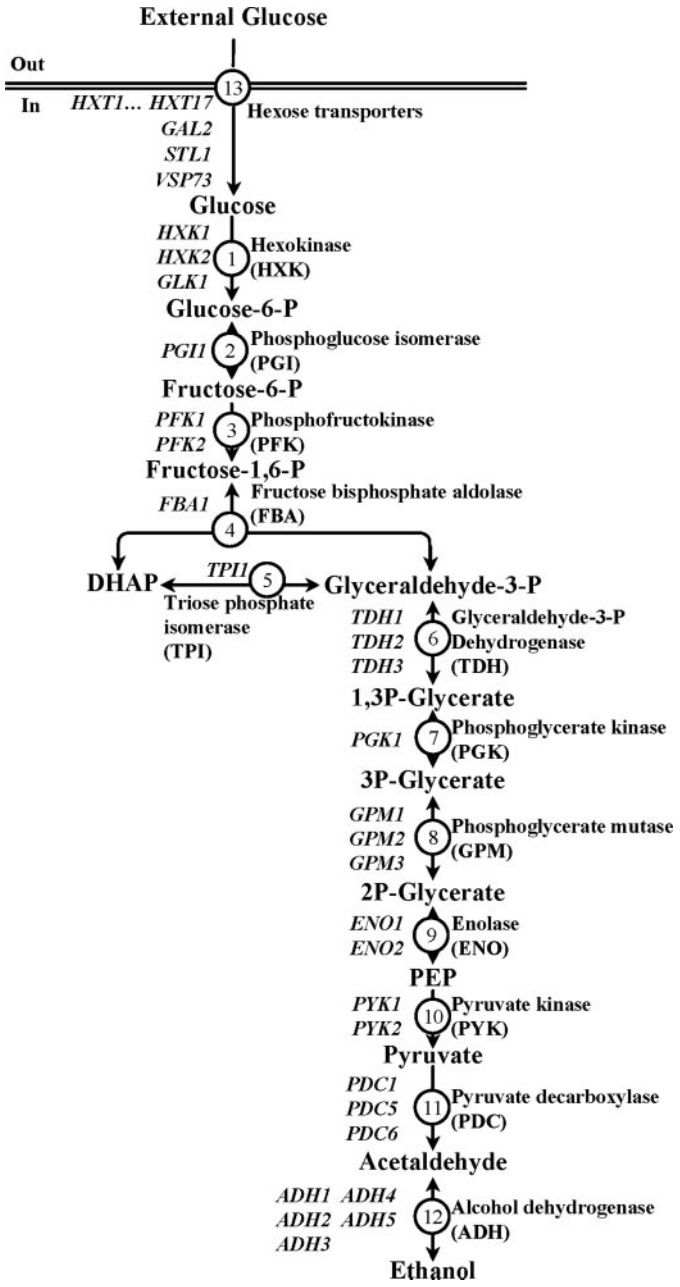

FIGURE 1. **Central carbon metabolism in *S. cerevisiae* from external glucose to the production of ethanol.**

contribution of different levels of cellular control to the regulation of glycolytic flux in more detail.

Expression Analysis of the Fermentation Pathway Genes in Low Temperature Chemostat Cultivations—DNA microarrays were used for a genome-wide comparison of transcript levels in anaerobic, glucose-limited chemostat cultures grown at 12 and 30 °C. Here, we focus on transcripts that encode key enzymes involved in glucose transport, glycolysis, and alcoholic fermentation (Fig. 1).

The most striking differences were observed in the expression of several hexose-transporter (*HXT*) genes. Out of the seven *HXT* genes that were significantly expressed in at least one condition, five were differentially expressed at 12 and 30 °C (Table 3). *HXT2* (+2.6-fold), *HXT3* (+3.7), and especially *HXT4* (+33.5) were expressed at higher levels at 12 °C, whereas *HXT5* (−40.4) and *HXT16* (−10.0) were expressed at a lower level at 12 °C. Interestingly, these changes did not seem to follow a simple logic that could be deduced from previous classification of the encoded hexose transporters as low (*HXT3*), high (*HXT2*), or moderate affinity carriers (*HXT4*) (33) were coordinately changed. Transcription of high affinity transporter genes, *HXT6* and *-7* was not significantly affected by the culture temperature.

Only four genes encoding enzymes of the glycolytic pathway were differentially expressed at 12 °C compared with 30 °C (Table 2). Three were down-regulated (*GPM2*, *PYK2*, and *PDC6*), whereas *PDC5* was up-regulated. Despite their significant change in expression, all four genes encode minor isoforms of the corresponding enzyme activities and were transcribed at low level compared with the genes encoding the main isoforms (*GPM1*, *PYK1*, and *PDC1*) (Table 3).

Glucose Uptake Assay and Glycolytic Enzyme Activities in Low Temperature Chemostat Cultivations—To investigate whether the different transcript levels of the five *HXT* genes were reflected in glucose transport kinetics, zero trans-influx uptake assays with [^{14}C]glucose were performed at 30 °C. Cells grown at 12 and 30 °C exhibited high-affinity transport with substrate-saturation constants (K_m) of 1.1 and 0.8 mM, respectively (Fig. 2). The maximum transport capacity (V_{max}) was 60% higher at low temperature (Fig. 2), probably as a consequence of the increased transcript levels of several *HXT* genes (Table 3).

In anaerobic carbon-limited chemostat cultures, substrate level phosphorylation via glycolysis is the main metabolic route for ATP supply. One mechanism to compensate for the decreased turnover numbers of glycolytic enzymes at low temperature might be an increased abundance (expression) of glycolytic enzymes. Hence, *in vitro* assays of the glycolytic and fermentative enzymes were carried out with cell extracts from chemostats cultures at 12 and 30 °C (Table 3). To gain insight in the effect of the temperature on enzymes, the assays were performed at both 12 and 30 °C.

Irrespective of the culture temperature, activities measured in cell extracts at 12 °C were strongly reduced (2.1–7.5-fold) when compared with *in vitro* measurements at 30 °C (Table 4). Fructose-1,6-biphosphate aldolase was most significantly affected by temperature with a 7-fold reduction for both growth temperatures. Hexokinase (HXK), phosphoglucose isomerase (PGI), glyceraldehyde-3-phosphate dehydrogenase (TDH), and 3-phosphoglycerate kinase (PGK) showed a 2–3-fold lower activity when measured at 12 °C. Meanwhile phosphofructokinase (PFK), triose-phosphate isomerase (TPI), phosphoglycerate mutase (PGM), pyruvate kinase (PYK), and pyruvate decarboxylase (PDC) activities were 3–7-fold lower (Table 4). These observations show that the glycolytic enzymes have different temperature/activity relationships. To our surprise, the alcohol dehydrogenase activity did not show any difference in activity when assayed at 12 and 30 °C and this is irrespective of the cultivation temperature. Addition of Zn^{2+}, Cu^{2+}, or the concentration of the cell extract by filtration on membrane with a cut-off of 10 kDa did not yield any significant differences higher than 20% between 12 and 30 °C *in vitro* ADH activity.

TABLE 3

Hexose transporters and glycolytic pathway transcript levels in *S. cerevisiae* grown in glucose-limited anaerobic chemostat cultivations at 12 and 30 °C

Values represent the mean ± S.D. of data from three independent steady-state chemostat cultivations. Numbers between brackets correspond to the glycolytic pathway displayed in Fig. 1.

Activity	Gene	Expression levels, culture temperature		FC[a]
		12 °C	30 °C	
Glucose transport (13)	*HXT1*	17 ± 8	12 ± 0	1.4
	HXT2	**557 ± 160**	**214 ± 29**	**2.6**
	HXT3	**840 ± 240**	**230 ± 27**	**3.7**
	HXT4	**1020 ± 290**	**31 ± 2**	**33.5**
	HXT5	**12 ± 0**	**485 ± 100**	**−40.4**
	HXT6	3320 ± 510	3020 ± 480	1.1
	HXT7	2590 ± 310	2090 ± 360	1.2
	HXT8	19 ± 8	13 ± 1	1.4
	HXT9	23 ± 2	12 ± 0	1.9
	HXT10	12 ± 0	19 ± 6	−1.6
	HXT11[b]	NA[c]	NA	
	HXT12	43 ± 22	45 ± 10	1.0
	HXT13[b]	NA	NA	
	HXT14	12 ± 0	22 ± 3	−1.8
	HXT15[b]	NA	NA	
	HXT16	**34 ± 8**	**337 ± 96**	**−10.0**
	HXT17[b]	NA	NA	
	STL1	25 ± 6	28 ± 4	−1.1
	GAL2	15 ± 3	12 ± 0	1.3
	VSP73	82 ± 31	52 ± 2	1.6
HXK (1)	*HXK1*	2050 ± 470	2960 ± 230	−1.4
	HXK2	1660 ± 18	979 ± 130	1.7
	GLK1	1280 ± 37	1700 ± 170	−1.3
PGI (2)	*PGI1*	3120 ± 180	2840 ± 280	1.1
PFK (3)	*PFK1*	1270 ± 140	1100 ± 94	1.2
	PFK2	1350 ± 40	1230 ± 91	1.1
FBA (4)	*FBA1*	3480 ± 840	2700 ± 410	1.3
TPI (5)	*TPI1*	4180 ± 540	3700 ± 380	1.1
TDH (6)	*TDH1*	2520 ± 110	1810 ± 290	1.4
	TDH2	3670 ± 160	2750 ± 380	1.3
	TDH3	4190 ± 860	5410 ± 1210	−1.3
PGK (7)	*PGK1*	4310 ± 240	3750 ± 350	1.1
GPM (8)	*GPM1*	3350 ± 100	3050 ± 230	1.1
	GPM2	**91 ± 24**	**196 ± 35**	**−2.1**
	GPM3	127 ± 2	87 ± 8	1.5
ENO (9)	*ENO1*	3420 ± 370	2880 ± 290	1.2
	ENO2	3490 ± 760	2840 ± 420	1.2
PYK (10)	*PYK1*	2820 ± 210	2040 ± 157	1.4
	PYK2	**47 ± 24**	**133 ± 34**	**−2.8**
PDC (11)	*PDC1*	2190 ± 45	1410 ± 190	1.6
	PDC5	**130 ± 12**	**56 ± 8**	**2.3**
	PDC6	**15 ± 2**	**66 ± 13**	**−4.4**
ADH (12)	*ADH1*	4740 ± 180	3980 ± 590	1.2
	ADH2	40 ± 7	57 ± 17	−1.4
	ADH3	749 ± 55	871 ± 110	−1.2
	ADH4	237 ± 80	252 ± 35	−1.1
	ADH5	937 ± 180	830 ± 130	1.1

[a] Fold change of transcription intensities of 12 °C over 30 °C.
[b] Open reading frame not available on Affymetrix Genechip® YG-S98.
[c] N/A, not applicable.

A comparison of the *in vitro* enzymatic activities done at the same temperature revealed only minor differences between yeast cultures grown at 12 and 30 °C. Only the pyruvate decarboxylase activity was significantly higher (p value <0.01) after growth at 12 °C than after growth at 30 °C (Table 4). Surprisingly, four activities were significantly lower at the low cultivation temperature (HXK, PGI, PFK, and PGK) (Table 4), irrespective of the assay temperature. The capacity of the glycolytic and fermentative enzymes was thus generally kept constant or even decreased at low temperature. The observations are in good agreement with the transcript levels (Table 3).

The glycolytic enzyme activities of yeast cultures grown at 12 °C and assayed at 12 °C were much lower than those of cultures grown at 30 °C and assayed at 30 °C (Table 4). A comparison of estimated *in vivo* fluxes (Table 2) with the *in vitro* enzyme activities (Table 4) showed that for all reactions, except PFK, the enzyme capacity exceeded the *in vivo* fluxes (2–1000-fold, Fig. 3). The degree of *in vivo* saturation of PFK was well above 100% (Fig. 3), indicating that this enzyme activity measured *in vitro* could not account for the estimated *in vivo* fluxes. Because mRNA and enzyme activity measurements did not provide an indication for regulation of the *in vivo* activity of glycolytic enzymes at the level of enzyme synthesis (vertical control), we subsequently analyzed the intracellular concentration of metabolites of central carbon metabolism.

Intracellular Metabolite Concentrations at 12 and 30 °C—The activities of glycolytic and fermentative enzymes are affected by their substrate and product concentrations and moreover regulated by allosteric effectors such as adenosine nucleotides (*e.g.* ATP, ADP, and AMP), glycolytic intermediates (*e.g.* fructose 1,6-biphosphate and PEP), and pyridine nucleotide cofactors. The intracellular concentrations of most

of the measured compounds were significantly and markedly different in the glucose-limited chemostat grown at 12 and 30 °C. The concentrations of the intermediates of both upper and lower glycolysis were concertedly increased by 1.5–5.7-fold (Glc-6-P, Fru-6-P, FBP, Glc-3-P, 2-PG/3-PG, PEP, and PYR) (Table 5).

Adenine nucleotides act as allosteric effectors on several glycolytic enzymes (PFK (34) and PYK (35)) but are also involved as substrates and products of glycolytic reactions (HXK, PFK, PGK, and PYK). The intracellular ATP concentration was 2-fold higher at 12 °C, while ADP and AMP levels were much lower (2.3- and 6.9-fold respectively), resulting in a higher adenylate sum (ΣAXP) and AC at 12 than at 30 °C (Table 5).

The concentration of trehalose 6-phosphate dropped 5.7-fold at 12 °C, concomitantly with the concentration of intracellular trehalose (Table 5). Besides its role in trehalose biosynthesis, trehalose 6-phosphate is a potent inhibitor of the hexokinase activity (36). Its lower concentration may participate in controlling the glucose phosphorylation and consequently the glycolytic flux (37). The increase in UTP and glucose 1-phosphate concentrations was consistent with the coordinated increased concentration of glycogen at 12 °C (Tables 1 and 5) (38).

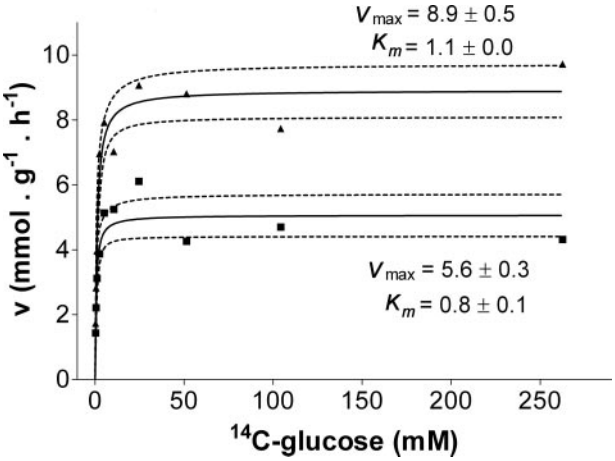

FIGURE 2. **Zero trans-influx uptake kinetics of labeled [^{14}C]glucose of strain CEN. PK113-7D measured at 30 °C with chemostat cultures grown at 12 (▲) and 30 °C (■).** Data and mean ± S.D. result from single component Michaelis-Menten fits the averaged triplicates of two independent chemostat cultures, with K_m in mM and V_{max} in mmol·$g_{dry\ weight}^{-1}$ h^{-1}. The *dotted lines* indicate the 95% confidence interval.

DISCUSSION

This study investigates how *S. cerevisiae* deals with the strong temperature dependence of key enzymes in glycolysis and alcoholic fermentation when it is grown at a suboptimal temperature of 12 °C. The activity assays of glycolytic enzymes, performed at 12 and 30 °C (Table 4), indicate that this temperature dependence is very strong. It should be realized that these assays were performed at a single concentration of substrates and effectors. Consequently, effects of temperature on the affinity for substrates and effectors may further increase the kinetic challenges posed by a low cultivation temperature (39, 40).

Glucose transport across the plasma membrane was shown to be regulated at different levels. The higher residual glucose concentration at low temperature (Table 1) is likely to contribute to a higher degree of substrate saturation of hexose transporters and thereby compensate for a reduced capacity of transporters at 12 °C. An additional level of regulation was observed by an analysis of mRNA levels for the *HXT* genes. Whereas levels of the dominant *HXT* transcripts *HXT6* and *HXT7* (which encode transporters that, at 30 °C, catalyze high affinity glucose transport (33)) were not affected by temperature, other *HXT* genes, which exhibit lower affinities and as such would be less sensitive to an increased intracellular glucose concentration, showed clear transcriptional regulation. Because no kinetic data are available for the individual Hxt transporters at low temperature, it is not possible to interpret the significance of these changed transport levels. In addition to adjusting the overall capacity and/or affinity of hexose transport, this altered expression of *HXT* genes may represent an adjustment to temperature-dependent changes in membrane composition and/or fluidity (41). Kinetic analysis of glucose transport by cells pre-grown at 12 and 30 °C in chemostat cultures showed a clear increase in capacity of trans-

TABLE 4

***In vitro* enzyme assays measurement in cell-free extract of *S. cerevisiae* grown in glucose-limited anaerobic chemostat cultivations at 12 and 30 °C**

Assay conditions were carried out at both 12 and 30 °C. Mean ± S.D. of data represented are from duplicate measurements from three independent chemostat cultures. Fold change (FC) of glycolytic enzymes with enzyme assays measured at 12 and 30 °C. *p* values shown are from standard Student *t* test. *In vitro* enzyme activity of enolase was not measured. Numbers by the last column correspond to the glycolytic pathway displayed in Fig. 1. Shading indicates $p < 0.01$.

	Enzyme activities (μmol·$mg_{protein}^{-1}$·min^{-1})				A vs B		C vs D		B vs D		A vs C		
Culture T	**12 °C**	**12 °C**	**30 °C**	**30 °C**	**12 12**		**30 30**		**12 30**		**12 30**		
Assay T	*12 °C*	*30 °C*	*12 °C*	*30 °C*	*12 30*		*12 30*		*30 30*		*12 12*		
Enzyme	*A*	*B*	*C*	*D*	FC	p-value	FC	p-value	FC	p-value	FC	p-value	
HXK	0.7 ± 0.0	2.1 ± 0.0	2.0 ± 0.0	5.7 ± 0.1	-2.9	*1.5E-05*	-2.8	*1.6E-04*	-2.7	*1.6E-04*	-2.7	*3.5E-05*	1
PGI	1.7 ± 0.1	3.9 ± 0.3	2.2 ± 0.1	5.3 ± 0.1	-2.3	*1.4E-03*	-2.4	*1.1E-05*	-1.4	*3.5E-03*	-1.3	*5.1E-03*	2
PFK	0.1 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.5 ± 0.0	-4.3	*2.3E-03*	-3.7	*3.5E-04*	-1.4	*2.1E-03*	-1.7	*8.1E-03*	3
FBA	0.3 ± 0.0	2.4 ± 0.2	0.3 ± 0.0	1.9 ± 0.0	-7.3	*1.6E-03*	-7.4	*1.4E-05*	1.2	*2.9E-02*	1.3	*9.5E-03*	4
TPI	37.6 ± 5.7	170.0 ± 16.0	29.3 ± 3.8	116.0 ± 3.5	-4.5	*1.1E-03*	-4.0	*4.5E-06*	1.5	*1.2E-02*	1.3	*5.6E-02*	5
TDH	4.2 ± 0.4	9.4 ± 2.0	3.6 ± 0.2	10.1 ± 1.8	-2.2	*1.9E-02*	-2.8	*1.2E-02*	-1.1	*3.5E-01*	1.2	*6.7E-02*	6
PGK	6.3 ± 0.4	13.2 ± 0.8	9.9 ± 0.4	20.9 ± 1.8	-2.1	*3.3E-04*	-2.1	*3.6E-03*	-1.6	*4.7E-03*	-1.6	*2.5E-04*	7
GPM	2.9 ± 0.8	14.5 ± 1.8	3.9 ± 0.3	15.5 ± 1.4	-5.0	*1.6E-03*	-4.0	*1.7E-03*	-1.1	*2.4E-01*	-1.3	*7.2E-02*	8
PYK	1.9 ± 0.3	9.1 ± 1.4	0.9 ± 0.1	7.0 ± 0.8	-4.9	*4.6E-03*	-7.5	*3.0E-03*	1.3	*5.2E-02*	2.0	*1.8E-02*	10
PDC	0.4 ± 0.0	1.5 ± 0.1	0.3 ± 0.0	0.8 ± 0.1	-3.8	*2.2E-03*	-2.4	*3.7E-03*	1.9	*2.1E-03*	1.2	*9.5E-03*	11
ADH	2.3 ± 0.1	2.2 ± 0.7	2.3 ± 0.1	2.4 ± 0.2	1.1	*4.0E-01*	1.0	*2.5E-01*	1.1	*3.2E-01*	1.0	*4.1E-01*	12

port. A plausible explanation for this increased capacity can be found in the differential expression of transcription of *HXT2*, *-3*, *-4*, *-5*, and *-16*. The fact that the maximum specific rates of glucose consumption (measured at 30 °C) for cells pre-grown at 12 and 30 °C closely corresponded to the fermentative capacity of the same cultures (Table 1, Fig. 2, and supplemental materials Table S1) would support this hypothesis. However, the possibility cannot be excluded that glucose-transport activity was increased as a result of changes outside the hexose transporters, such as membrane composition. The change in glucose transport capacity, together with the minor changes in the levels of glycolytic enzymes (see below), strongly suggests that glucose transport also controls the fermentative capacity in these slowly growing chemostat cultures as well as at 30 °C.

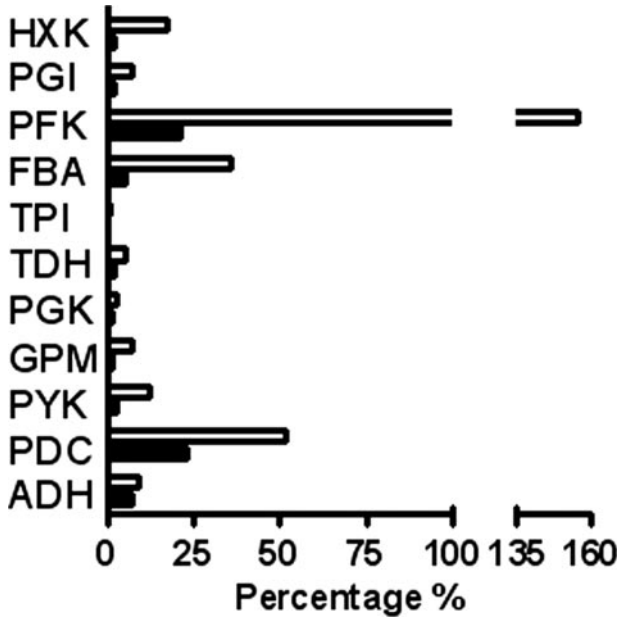

FIGURE 3. **Degree of *in vivo* saturation of glycolytic enzymes, calculated from estimated *in vivo* fluxes and enzyme-capacity estimates from enzyme assays in cell extracts.** □, 12 °C; ■, 30 °C. *In vitro* enzyme activities were expressed in grams per g dry weight per min by assuming a soluble protein content of 33% in dry biomass.

In contrast to sugar transport, there was virtually no evidence that regulation of glycolysis and alcoholic fermentation at the enzyme synthesis level (vertical regulation) contributed to the maintenance of *in vivo* glycolytic flux at low temperature. Indeed, the few glycolytic enzymes that did show a clear change at transcript levels and/or enzyme activity level appear to show a lower level at low temperatures, thus augmenting the effect of temperature on enzyme activity rather than compensating for it. The absence of a clear up-regulation of the synthesis of glycolytic enzymes at low temperatures is perhaps less surprising when it is taken into account that, in fermenting yeast cultures grown at 30 °C, glycolytic enzymes already make up a significant fraction of the total cell protein (up to 21%, estimation extrapolated from Ref. 16). It would take very significant increases in the concentrations of these glycolytic enzymes resulting in a significantly increased energetic demand to counteract the effects of the reduction in temperature.

The minor role of vertical control in the regulation of glycolytic flux at low temperature was in marked contrast to drastic differences in the intracellular concentrations of glycolytic intermediates and effectors. Several of the observed changes may contribute to a higher degree of saturation of the glycolytic capacity in the low temperature cultures, thus compensating for the reduction of enzyme activity at low temperature. The lower intracellular concentration of trehalose 6-phosphate may have a similar effect, as this compound is a well documented inhibitor of the *S. cerevisiae* hexokinases (42) that prevents "glucose-accelerated death" (36, 43). The lower intracellular trehalose 6-phosphate concentration, an intermediate in trehalose biosynthesis, is also con-

TABLE 5
Intracellular metabolite concentrations

Values represent the mean ± S.D. of data from two independent steady-state chemostat cultivations measured in triplicates. 2PG/3PG, 2-phosphoglycerate/3-phosphoglycerate; PYR, pyruvate.

	Metabolite concentration, $\mu mol \cdot g_{dry\ wt}^{-1}$ Culture temperature		Student's *t* test, *p* value	FC[a]
	12 °C	30 °C		
Glycolysis				
Glc-6-P	21.6 ± 0.6	9.6 ± 0.2	*1.1E-07*	2.3
Fru-6-P	2.5 ± 0.4	1.1 ± 0.1	*1.8E-04*	2.3
Fru-1,6-P	69.8 ± 5.5	30.3 ± 1.5	*1.5E-06*	2.3
Glc-3-P	3.9 ± 0.1	1.0 ± 0.1	*1.5E-10*	3.8
2PG/3PG	1.1 ± 0.1	0.4 ± 0.0	*1.9E-06*	2.5
PEP	0.6 ± 0.1	0.1 ± 0.0	*2.9E-06*	5.7
PYR	3.0 ± 0.1	2.1 ± 0.2	*6.8E-06*	1.5
Reserve carbohydrate				
Trehalose 6-P	0.1 ± 0.0	0.4 ± 0.0	2.8E-09	−5.7
Glc-1-P	2.6 ± 0.1	1.2 ± 0.0	*3.7E-09*	2.2
Nucleotides[b]				
ATP	51.0 ± 1.1	26.7 ± 1.5	*1.1E-10*	−1.9
ADP	4.6 ± 0.7	10.4 ± 2.5	*4.6E-04*	−2.3
AMP	0.9 ± 0.2	6.5 ± 1.3	*1.0E-04*	−6.9
AC[c]	0.94	0.73		1.3
UTP	10.3 ± 0.5	4.1 ± 0.1	*3.7E-05*	2.5
UDP	0.5 ± 0.1	1.4 ± 0.6	*9.4E-03*	−2.7

[a] Fold change of metabolite concentrations of 12 over 30 °C.
[b] UMP, CXP, and GXP were also measured, however, the concentrations were too low to be accurately quantified.
[c] Adenylate charge of the cell (see Equation 2).

sistent with the lower trehalose concentrations in the cultures grown at 12 °C.

Extreme changes were observed in the intracellular concentrations of adenine nucleotides. Whereas the ATP concentration was higher at low temperature, the ADP and AMP concentrations were much lower, thus leading to an AC (see "Experimental Procedures" Equation 2) of 0.94 in the chemostat cultures grown at 12 °C (Table 5). This change seems counterintuitive in a situation where the *in vivo* activity of glycolytic enzymes has to be boosted to compensate for low temperature-induced reduction of their turnover numbers. An increased AC is generally correlated with a decrease of the activity of enzymes and/or pathways involved in ATP production (44) and in *S. cerevisiae*, intracellular ATP concentration is negatively correlated with glycolytic flux (45). This unexpected relation between growth temperature and AC may be related to strong changes of the kinetic and/or allosteric properties of enzymes at low temperature and to the changes of other relevant metabolites. For example, inhibition of PYK by the increased intracellular ATP concentration at 12 °C may be compensated by the strongly increased intracellular concentration of its activator Fru-1,6-P (34, 46) (Table 5). Similarly, a negative effect of increased ATP and decreased AMP on PFK activity (34) may be compensated for by changes of the positive allosteric regulator Fru-2,6-P (15), which could not be accurately measured in our experimental set-up.

An important factor in the interpretation of the intracellular metabolite data is that the kinetics of glycolytic enzymes and, in particular, the impact of low molecular weight effectors, has been extensively studied at 30 °C, but not at 12 °C. For example, if the kinetics of allosteric regulation are strongly temperature-dependent, this may affect the impact of the AC on glycolytic flux by removing or reducing bottlenecks at the lower temperature. Indeed, several studies show that the affinity for allosteric effectors is reduced at low temperature (*i.e.* inhibition and activation constants are increased) (39, 40, 47). The apparent discrepancy between *in vivo* and *in vitro* PFK activities at 12 °C represents a case in point (Fig. 3). In previous studies at 30 °C, the maximum capacity of PFK estimated in cell extracts was close to the *in vivo* flux through this enzyme (48). The difference between these parameters in cultures grown at 12 °C may reflect suboptimal conditions in the enzyme assays due to temperature-dependent changes in the complex allosteric regulation of this enzyme (49, 50). A deeper understanding of the *in vivo* kinetics of glycolysis at low temperature, involving the application of kinetic modeling, will require quantitative data on the impact of temperature on the kinetics and allosteric regulation of the entire glycolytic pathway in *S. cerevisiae*.

In addition to providing ATP for anaerobic growth, glycolysis also is a key cellular supply line for biosynthesis. The temperature-dependent changes of the intracellular concentrations of glycolytic intermediates are therefore also likely to affect biosynthetic pathways. Clearly the relative demands placed on anaerobic glycolysis in terms of energetics and biosynthetic supplies are not the same at 12 and 30 °C. In addition to vital biomass components, the products derived from these biosynthetic pathways include important flavor compounds (51, 52). Therefore, a further analysis of the mechanisms by which the entire yeast metabolic network adapts to temperature is highly relevant for yeast-based industrial fermentations that are performed at low temperature. Further insights will be gained from a similar study to this one using an industrial yeast strain, which is adapted to the low temperature conditions as opposed to a lab strain, which is probably adapted to the higher temperature conditions.

The present study demonstrates that, at low specific growth rate, the *in vivo* glycolytic flux at low temperature is maintained by a combination of vertical and metabolic regulation. Glucose transport represents the clearest case of vertical (transcriptional) regulation through the synthesis of additional hexose transporters. However, the increased extracellular glucose concentration at low temperature is likely to be just as decisive in maintaining a constant cellular glucose import rate. Vertical regulation did not appear to contribute to compensating for suboptimal kinetics of glycolytic enzymes at low temperature. Instead, metabolic control, reflected by massive changes of intracellular concentrations of glycolytic metabolites and effectors, was dominant.

From an evolutionary perspective, a dominant role of metabolic regulation seems logical for a pathway that represents a substantial fraction of the cellular protein, in an organism that is subjected to circadian temperature cycles. In nature, *S. cerevisiae* is likely to encounter relatively exposed, and sugar-rich niches such as fruits and nectar. Moreover, growth is probably slow due to the low availability of nitrogen. In such environments, a preliminary vertical regulation of glycolytic activity would require wasteful cycles of massive synthesis (as temperature decreases in the evening) and degradation (in the morning) of glycolytic enzymes. This interpretation suggests that the high fermentative capacity of wild-type *S. cerevisiae* strains (assayed at 30 °C) may, at least in part, reflect an evolutionary adaptation to fluctuating temperatures in its natural environment.

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