The effect of altered expression of transcriptional regulators of catabolism on the transcription profile and physiology of Saccharomyces cerevisiae

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Chapter 3

The effect of *hxk2* deletion and *HAP4* overexpression on fermentative capacity in *S. cerevisiae*

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Abstract

To describe the fermentative potential of a yeast cell, the fermentative capacity (FC) has been defined as the specific rate of ethanol and CO_2 production under anaerobic conditions. We compared the effect of growth rate on FC of glucose-limited grown *Saccharomyces cerevisiae* strains with altered expression of two major glycolytic regulators, Hap4p and Hxk2p, with their parent strain. Whereas overproduction of Hap4p behaved similar to the wild type strain, deletion of *hxk2* resulted in a very different FC profile. Most importantly, with maltose as carbon and energy source, the latter strain expressed an FC twofold that of the wild type. Further analysis at the level of gene expression showed large changes in *ADH2* transcripts and to a lesser extent in hexose transporters and genes involved in the glyoxylate cycle. With respect to primary glucose metabolism, a shift in the type of hexose transport to one with high affinity was induced. In accordance with the phenotype of the mutant strain, the maltose transporter was constitutively expressed under glucose-limited conditions and synthesis increased in the presence of maltose.

Introduction

The yeast *Saccharomyces cerevisiae* has been used for centuries to make several foods and beverages, such as bread, beer and wine. Although this organism is one of the best studied organisms today, many aspects of the mechanisms of fermentation are not well understood yet. The presence of oxygen, the availability of glucose and many other physicochemical parameters of the environment play a role in the net distribution of the carbon fluxes that conserve energy, *i.e.* fermentation and respiration. In industrial application, control of this distribution is essential and a fermentative flux may be desired when CO_2 and/or ethanol are the preferred end products: this is the case *e.g. d*uring the raising of dough where the yeast should ferment sugars to CO_2 with minimal increase in biomass. To quantify the potential to produce CO_2 and ethanol, the rate at which this process occurs is defined as the fermentative capacity (FC) of the particular yeast strain used. Maximalization of the FC is important, for example because in the late industrial production phase *S. cerevisiae* tends to grow very slowly due to oxygen- and heat-transfer problems in the fermentors. Slower growing cells express a lower FC (Van Hoek, et al., 1998) and attempts to increase their performance by overexpression of the lower part of glycolysis have been only partly successful (Smits, et al., 2000). Another problem during production of yeast is the tendency of this organism to ferment even in the presence of excess oxygen. This so-called Crabtree-effect (Van Urk, et al., 1989a) affects dramatically the biomass yield as a consequence of the low energy efficiency of fermentation. Altering the metabolic network in key places could shift metabolism to a greater respiratory output, thus increasing the biomass yield. An important role in controlling the catabolic flux distribution over respiration and fermentation has been ascribed to the global glycolytic regulators Hap4p and Hxk2p (Blom, et al., 2000; Diderich, et al., 2001b; van Maris, et al., 2001).. In this study we set out to investigate the effects on FC of a hexokinase II deletion strain (*hxk2Δ*) and the haeme-activated protein 4 overexpression strain (*HAP4*↑). Both strains show an increased respiratory output under glucose excess conditions (Blom et al, 2000) suggesting that their ability to ferment under anaerobic conditions could be impaired. We studied the effects when cells were grown on glucose, which is baker's yeast's preferred carbon source as well as on maltose, which is the most abundant sugar in bread dough. The observed differences between the *hxk2Δ* and the wild type strain with respect to FC and physiological behaviour are complemented by an analysis at the level of the transcriptome, of the glycolytic proteome including the sugar transport proteome.

Materials & Methods

Strains and growth conditions

Strains used were CEN.PK113-7D (MATa URA3 LEU2 HIS3 TRP1 SUC2 MAL MEL GAL) provided by Dr. P. Koetter (Frankfurt, Germany), a HAP4 overexpression strain (previously described (Blom, et al., 2000), a hxk2Δ:KanMX4 mutant (previously described (Diderich, et al., 2001a)). These strains were grown in laboratory fermenters (L.H. Engineering, Maidenhead, UK) under aerobic glucose-limited conditions (Van

Hoek, et al., 1998). A defined mineral medium described by Verduyn et al. (Verduyn, et al., 1992) was used. The concentration of glucose in the feed was 5 $g \cdot l^{-1}$. The working volumes used varied between 0.65 and 0.8 l. pH was kept at 5.0 ± 0.1 by automatic addition of 1 M NaOH. BDH Laboratory Supplies Silicone anti-foaming agent was used in a 50 µl/l concentration to prevent foam development. The cultures were sparged with air at a flow rate of approximately 1 volume of air per vessel volume per minute. The cultures were stirred with double impellers (LH Engineering) at an equal rpm as the culture volume. The cultures were grown in continuous mode by steady addition of medium while the overflow medium was disposed as waste. After 6-7 volume throughputs, a stead-state was considered to be reached as biomass and CO_2 and O_2 did not vary more than 5% after this time. Oxygen consumption and carbon dioxide production were determined by passing the effluent gas through an paramagnetic oxygen analyzer type 1100 (Servomex, Crowborough, UK) and an IR gas analyzer type 4100 (Servomex, Crowborough, UK), respectively.

Sample extraction and analysis

Protein concentrations were determined according to the method of Lowry et al. using fatty-acid free bovine serum albumin as a standard. Samples taken for metabolite analysis were immediately treated with 35% cold (4°C) perchloric acid and stored at -20°C. Upon analysis samples were neutralized with 7M KOH and filtered through a 0.45 µm filter. Dry weight was determined by putting 2x 10 ml of culture in pre-weighed tubes, the culture was then spun down at 5000 rpm (Sorvall, SS-34 rotor) at 4°C, then washed with demineralized water, spun down again and dried overnight at 100°C. The difference in weight was used to determine the dry-weight of the culture.
Metabolites were determined by means of high performance liquid chromatography (LKB, Bromma, Sweden) with a Rezex organic acid analysis column with an 8-µm particle size, 8% cross-linking and a hydrogen ionic form (Phenomenex, Torrance, CA, USA) at a temperature of 45°C and with 7.2 mM H_2SO_4 as eluent. Detection was done with an RI1530 refractive index detector (Jasco, Tokyo, Japan). Peak integration and data processing were done with BORWIN (Le Fontanil, France) chromatography software.

Fermentative capacity assays

The method used was essentially as described by Van Hoek et al (1998). Samples containing 100 mg of dry-weight of biomass from a steady-state culture were harvested by centrifugation at 5000 rpm (Sorvall, SS-34 rotor) for 10 min.,washed once with defined mineral medium (Verduyn, et al., 1992) containing no carbon source and resuspended in 10 ml of a 5x concentrated medium again lacking a carbon source. These cell suspensions were the introduced into a thermostatted (30 °C) vessel, the volume was adjusted to 45 ml with demineralized water. After 10 min. of incubation 5 ml of a 20% glucose (w/v) or maltose solution was added. Samples were taken at appropriate time intervals. The headspace was continuously flushed with water-saturated N_2-gas at a flow rate of approximately 50 $ml \cdot min^{-1}$. Metabolite concentrations were determined using the HPLC. Fermentative capacity was calculated from the increase in ethanol concentration

over 30 min. for glucose and over 60 min. for maltose. Increase in biomass was negligible during the assay.

Preparation of cell extracts

Culture samples were harvested by centrifugation, washed twice with 10 mM potassium-phosphate buffer, pH 7.5, containing 2 mM of EDTA, concentrated 4-fold and stored at -20°C. Before use, the samples were thawed, washed and resuspended in 100 mM potassium-phosphate buffer, pH 7.5, containing 2 mM $MgCl_2$ and 1 mM DTT. Extracts were prepared by sonication with 0.7 mm diameter glass beads at 0°C in a MSE sonicator (Abcoude, The Netherlands) (150 W output, 7 µm peak-to-peak amplitude) for 3 min. with 0.5 min. intervals of cooling Unbroken cells were removed by centrifugation at 4 °C for 20 min at 10000 rpm (Sorvall SS-34). The supernatant was used as the cell extract.

Preparation of total RNA and labeled cRNA

Samples for RNA isolation were collected from steady-state chemostats, flash-frozen in liquid nitrogen and stored at –80 ^{o}C. Total RNA was extracted using the method described in Zakrzewska et al. (Zakrzewska, et al., 2005). The concentration and quality of RNA were determined by measuring absorbance at 260, 280, and 230 nm on a Nanodrop spectrophotometer. The purity and integrity of the RNA samples were further validated with RNA LabChip on a 2100 Bioanalyzer from Agilent Technologies.
Total RNA was labeled according to the manufacturer's protocol (Affymetrix). Total RNA (20 µg) was used for first strand cDNA synthesis. This was followed by synthesis of second strand cDNA. cDNA was purified using the GeneChip Sample CleanUp Module from Qiagen. The cDNA was used for synthesis of biotin-labeled cRNA, which was performed with the ENZO BioArray HighYield RNA Transcript Labeling Kit from Affymetrix. The synthesized cRNA was purified with the GeneChip Sample CleanUp Module from Qiagen. The concentration and quality of labeled cRNA were tested using a spectrophotometer. Subsequently, the cRNA fragmentation reaction was carried out according to the manufacturer's protocol. The degree of fragmentation was confirmed with RNA LabChip. The samples were stored at –20 ^{o}C prior to hybridization.

Hybridization and scanning of the DNA microarrays

The biotin-labeled cRNA samples were hybridized to the Affymetrix GeneChip® Yeast Genome S98 Array according to Affymetrix protocols (http://www.affymetrix.com). This chip contains 25-mer oligonucleotide probes for approximately 6,400 *S. cerevisiae* ORFs. Each ORF is represented by approximately 16 probes, covering different parts of its sequence. Every probe is neighboured by a probe that is identical, except for one nucleotide in the middle of its sequence. This probe is called the 'mismatch' probe (MM), as opposed to the 'perfect match' probe (PM). The arrays were scanned with the GeneArray Scanner System on standard settings at 3 mm resolution. The data were extracted from the scanned images with MAS 5.0 (Microarray Suite 5.0).

Data processing

DChip was used to normalize the raw data. dChip is a software package implementing model-based expression analysis of oligonucleotide arrays and several high-level analysis procedures ((Li, 2001)). The model-based approach allows probe-level analysis on multiple arrays. The arrays were normalized by adjusting the overall brightness of the arrays to a similar level. Background subtraction was performed prior to calculating the expression values. Expression values were calculated using the perfect match model only, as this is unaffected by adverse effects of mismatch probes. A more detailed description of the used procedure is given in (Zakrzewska, et al., 2005).

Enzyme determinations

Enzyme assays were performed in a COBAS BIO automatic analyzer (Roche Diagnostics, Mannheim, Germany) at 30 ^{o}C, measuring at 340 nm (E340nm of reduced pyridine-dinucleotide cofactors = 6.3 mM-1) with freshly prepared cell extracts. All enzyme activities are expressed as $\mu mol \cdot min^{-1} \cdot (mg\ protein)^{-1}$. When necessary, samples were diluted with demi-water. All assays were performed with three concentrations of cell extract to confirm that reaction rates were proportional to the amount of cell extract added. All enzymes were assayed according to van Hoek et al. (Van Hoek, et al., 1998)

Zero trans-influx assays

Zero trans-influx rates of sugars were determined in a 5 sec assay according to Walsh et al. (Walsh, et al., 1994) at 30 °C in the same medium that was used for growth (pH=5.0). Maltose experiments were performed in the same way using ^{14}C labelled maltose. For the maltose experiments, the transport time was increased from 5 seconds to 15 seconds. Kinetic parameters of sugar transport were derived from least square fitting of the data to one- or two- component Michaelis-Menten models using Sigmaplot software.

Results and Discussion

Table 1: Physiology of the wild-type, *hxk2Δ* and *HAP4*↑ strains.

D (h^{-1})	$q_{glucose}$ (mmol g^{-1} h^{-1})	$q_{ethanol}$ (mmol g^{-1} h^{-1})	qO_2 (mmol g^{-1} h^{-1})	qCO_2 (mmol g^{-1} h^{-1})	$Y_{glucose}$ (g/g)	C%
WT						
0.1	1.1	0	2.6	2.8	0.51	106
0.31	3.4	0	8.3	8.8	0.52	106
0.32	4.4	7.1	7.1	9.5	0.41	102
hxk2Δ						
0.1	1.1	0	2.2	2.5	0.52	100
0.31	3.2	0	6.2	6.2	0.54	97
0.32	3.4	0	8.5	8.9	0.53	107
HAP4 ↑						
0.1	1.1	0	3.0	3.0	0.52	110
0.31	3.1	0	7.6	7.8	0.57	108
0.32	4.3	2.7	7.0	9.7	0.42	110

Strains were grown in glucose-limited aerobic chemostats at various dilution rates. Fluxes were determined for glucose, ethanol, CO_2 and O_2. The biomass yield on glucose and the carbon balance are also shown. Shown are the means of two independent experiments

The CEN.PK113-7D (wild-type), *hxk2Δ* and *HAP4*↑ strains were grown in glucose-limited aerobic chemostats at three different specific growth rates, 0.1 h^{-1} (slow growth), 0.31 h^{-1} (fastest growth rate with fully respiratory catabolism) and slightly higher (0.32 h^{-1}) where steady state catabolism starts to become fermentative for the wild type *i.e.* just above its critical specific growth rate. For all data presented here, carbon balances all fitted within the 90-110% range. At low growth rates all strains are growing fully respiratory and thus do not form ethanol. Fluxes and yields are equal under these conditions, indicating their energetic demands are the same. This appears to be true up to a specific growth rate of 0.31 h^{-1}. The critical dilution rate (D_c) for the wild type and *HAP4*↑ turned out to be 0.32 h^{-1}: both strains catabolize glucose at this growth rate respire-fermentatively with a concommitant drop in $Y_{glucose}$ of 20%. The *hxk2Δ* strain however continued to grow fully respiratory and as a consequence maintained its biomass yield value on glucose (see Table 1). The higher respiratory capacity in the *hxk2Δ* strain has been observed under other growth conditions as well and has been subject to a number of studies (Diderich, et al., 2001a; Petit, et al., 2000; Raghevendran, et al., 2004) From the steady state chemostat cultures described above, samples for fermentative capacity analysis were taken.

From figure 1 it can be seen that the fermentative capacity on glucose ($FC_{glucose}$) for both the wild-type and the *HAP4*↑ strain hardly changed with changing growth rates whereas the $FC_{glucose}$ of the *hxk2Δ* strain drops dramatically with increasing growth rates, resulting in a 75 percent reduction of capacity at the highest growth rate tested.

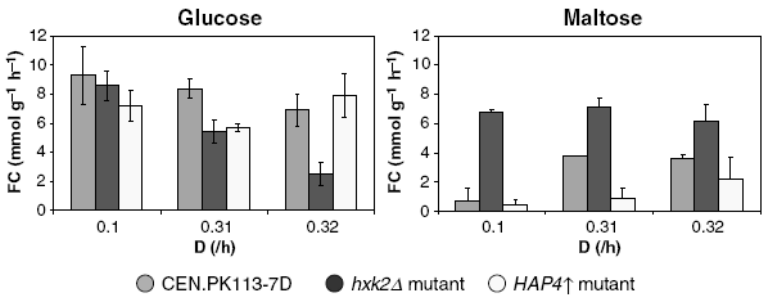

Figure 1: Fermentative capacity (FC) of the wild-type, hxk2Δ and HAP4↑ strains. Left: $FC_{glucose}$ of the wild-type (blue*) hxk2Δ* (red) and *HAP4*↑ (yellow) strains. Right: $FC_{maltose}$ of the wild-type (blue) *hxk2Δ* (red) and *HAP4*↑ (yellow) strains. The results of at least two independent experiments are shown, and the SD is shown in error bars.

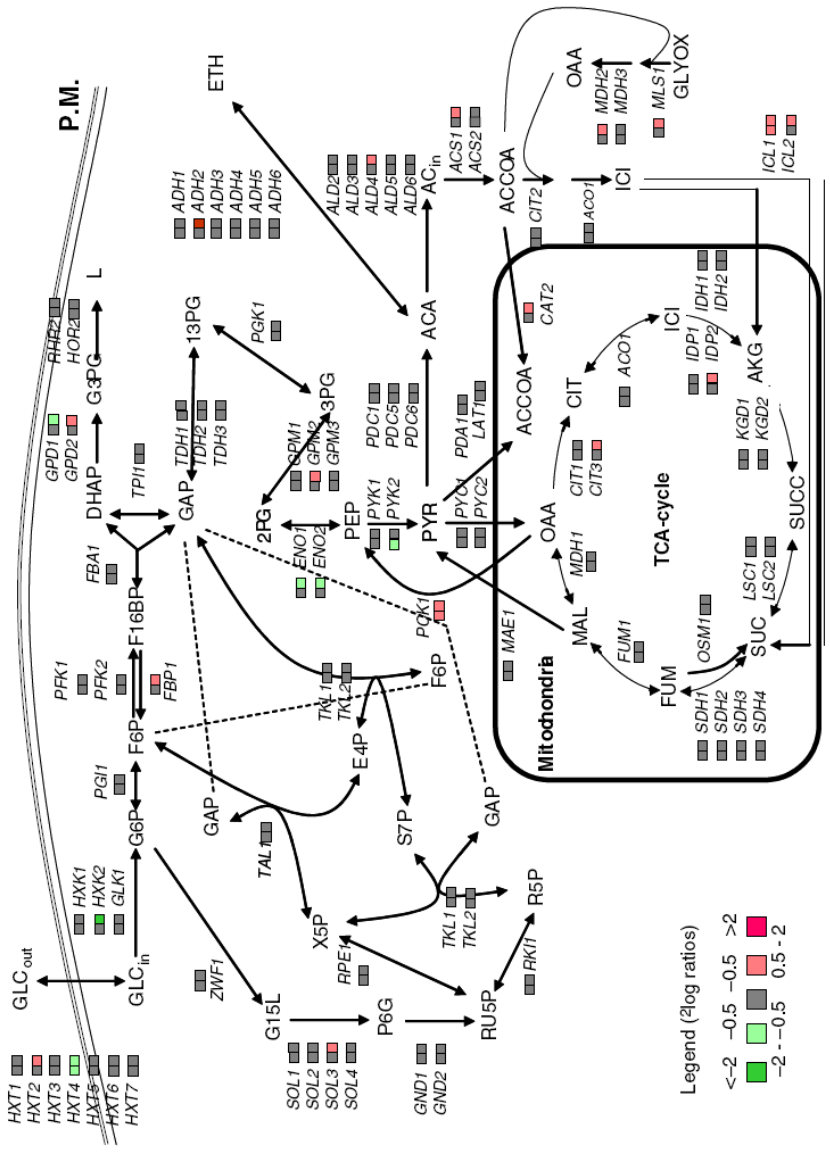

Figure 2: Gene expression ^{2}log-ratio's in the central carbon metabolism of yeast. Transcriptome data from the mutants and the wild-type, taken during steady-state growth at D=0.32 h^{-1}, were normalized and expression ratios were calculated relative to the wild-type. Shown here are the expression ratio's of most of the genes involved in the depicted pathways. The values are expressed as ^{2}log-ratio's and are relative to the wild-type. The left squares show the *HAP4* ↑/WT ratio and the right squares show the *hxk2Δ*/WT ratio. The given values are the mean of two independent experiments.

Even more remarkable is the observation that deletion of *hxk2* resulted in a strikingly different behaviour when maltose was used as the carbon and energy source. Nor the wild type nor the *HAP4*↑ strain expressed a significant fermentative capacity on maltose ($FC_{maltose}$) upon transfer from glucose-limited growth conditions to the FC assay with maltose whereas the *hxk2Δ* strain was able to catabolize this sugar immediately at a rate that was some 70% of the $FC_{glucose}$ of the wild type. In addition, the $FC_{maltose}$ did not change with growth rate. In contrast, the behavior on maltose of the *HAP4*↑ strain was opposite to that of the *hxk2Δ* strain (figure 1) as that it expressed a lower $FC_{maltose}$ than the wild-type.

Further comparative studies were done with the wild-type and *hxk2Δ* strains, at a dilution rate of 0.32 h^{-1}, because here the largest physiological differences were observed. Transcriptome profiles were analyzed as well as the activities of putatively important enzymes such as those of glycolysis and the sugar transporters. The analyses were carried out for three points in time, namely before $FC_{maltose}$ incubation, after 30 min. and after 60 min. of $FC_{maltose}$ incubation. To express the differences in $FC_{glucose}$ we refer to the samples prior to incubation on maltose.

In figure 2, a comparison of all transcripts in the central carbon metabolism of yeast is shown. In accordance with the similar behaviour of the wild type and the *HAP4* ↑ strain under the conditions tested, the transcriptome profiles of these strains do not show major differences with the exception of down regulation of the *HXT4* transcript and some upregulation for *ICL1* and *PCK1* transcripts in the *HAP4* ↑ strain.

Also in accordance with major physiological changes due to deletion of hxk2, the *hxk2Δ* strain shows several more distinct differences at the expression level. As expected *HXK2* transcript is hardly present in the mutant. Further, the expression of *ADH2* has more than doubled as has been observed earlier for batch cultures with excess glucose (Schuurmans, et al., 2008). Also several genes involved in the glyoxylate cycle, the TCA cycle and *FBP1* (needed for gluconeogenesis) are increased. Although these results point towards gluconeogenic growth in the *hxk2Δ* strain, from alcohol as substrate, this cannot explain the difference in $FC_{glucose}$, as gluconeogenesis in yeast requires oxygen.

Further information at the proteome level of glycolysis is presented in Figure 3 where glycolytic enzyme activities are presented. These show the V_{max} of each of the glycolytic enzymes and thus represent the maximum sustainable flux per enzyme, (and not the total *in situ* flux, as shown in table 1). Assuming cells contain approximately 40% protein, the values can be recalculated to allow direct comparison to the measured fluxes of glucose and ethanol. Thus, triose phosphate isomerase (TPI) activities are much higher than any of the other glycolytic enzymes, in the range of 25-45 µmol/min./mg DW opposed to a 0.1-3 µmol/min./mg DW for all the other enzymes for both yeast strains. Relative low activities are found for phosphofructokinase (PFK), aldolase (ALD) and alcohol dehydrogenase (ADH) but according to the calculation above, the sustainable flux through these enzymes still greatly exceeds the *in situ* flux, *e.g* for PFK (wild-type prior to FC incubation) the V_{max} is 0.22 $\mu mol \bullet min^{-1} \bullet mg^{-1}$ DW, which corresponds to 13.2 $mmol \bullet h^{-1} \bullet g^{-1}$ DW, whereas the *in situ* flux is 4.4 $mmol \bullet h^{-1} \bullet g^{-1}$ DW. HXK activity is much lower in the *hxk2Δ* strain, therefore it can be concluded that the lack of one of its three hexokinase iso-enzymes is not compensated by enhanced expression of either or both of the two other. Again, the maximum activity greatly exceeds the *in vivo* flux, (7.8 $mmol \bullet h^{-1} \bullet g^{-1}$ DW versus 3.4 $mmol \bullet h^{-1} \bullet g^{-1}$ DW respectively). We also observed changes

in enzyme activity during the $FC_{maltose}$. ,wild-type enzyme activity appears to increase after 30 minutes, but in the end is lower in all cases than the starting activity, whereas enzyme activity in the hxk2Δ strain increases in 30 minutes, but does not drop like in the wild-type after an hour.

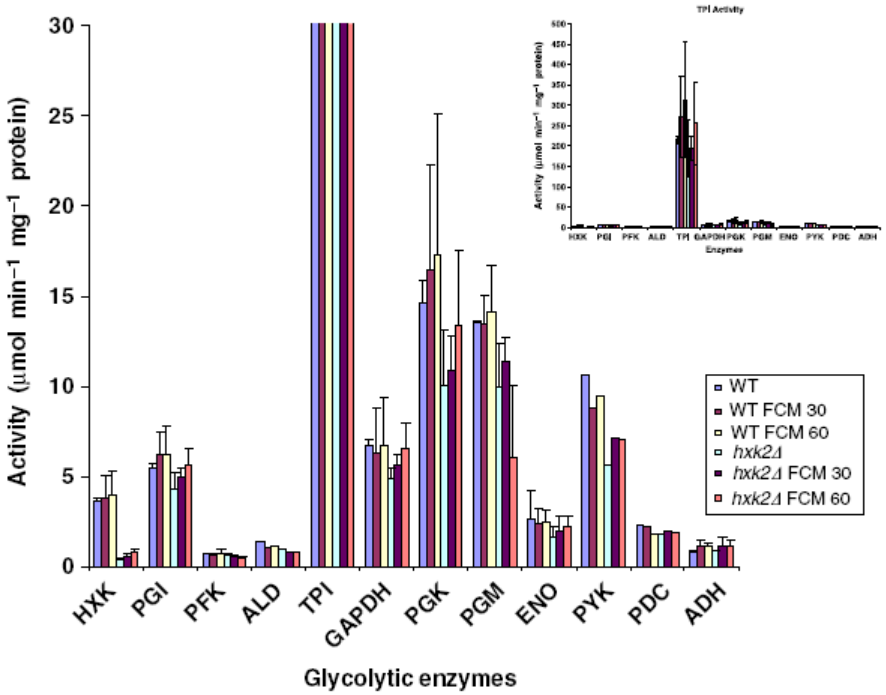

Figure 3: Glycolytic enzyme activities in the wild-type and hxk2Δ strain, before, during and after FC incubation on maltose. Shown are activities of the wild-type strain prior to (blue), during (red) and after (yellow) incubation with maltose. Activities of the *hxk2Δ* strain are shown in cyan (prior to maltose incubation), purple (during maltose incubation) and orange (after maltose incubation).

An adaptation to maltose occurs during the incubation, but the observed changes in $FC_{maltose}$ in time can not be explained by changes in glycolytic enzyme activities. Likewise the poor performance of the *hxk2Δ* strain on glucose is not entirely clear, although the reduction in hexokinase activity in this strain can account for at least a 40% reduction in $FC_{glucose}$. This still leaves 35 % of the reduction in $FC_{glucose}$ unexplained.
To further investigate the changes in $FC_{glucose}$, glucose transport kinetics were studied by zero trans-influx transport assays. These results are presented in figure 4. The wild-type strain, when sampled from glucose-limited chemostat cultures ($D = 0.32\ h^{-1}$) shows a one-component transport system, similar as was found in earlier studies ((Diderich, et al., 1999). The V_{max} found in this study is a bit lower, however our experiments were performed in unbuffered medium at pH 5.0, whereas the earlier experiments were done in phosphate buffer at pH 6.5.

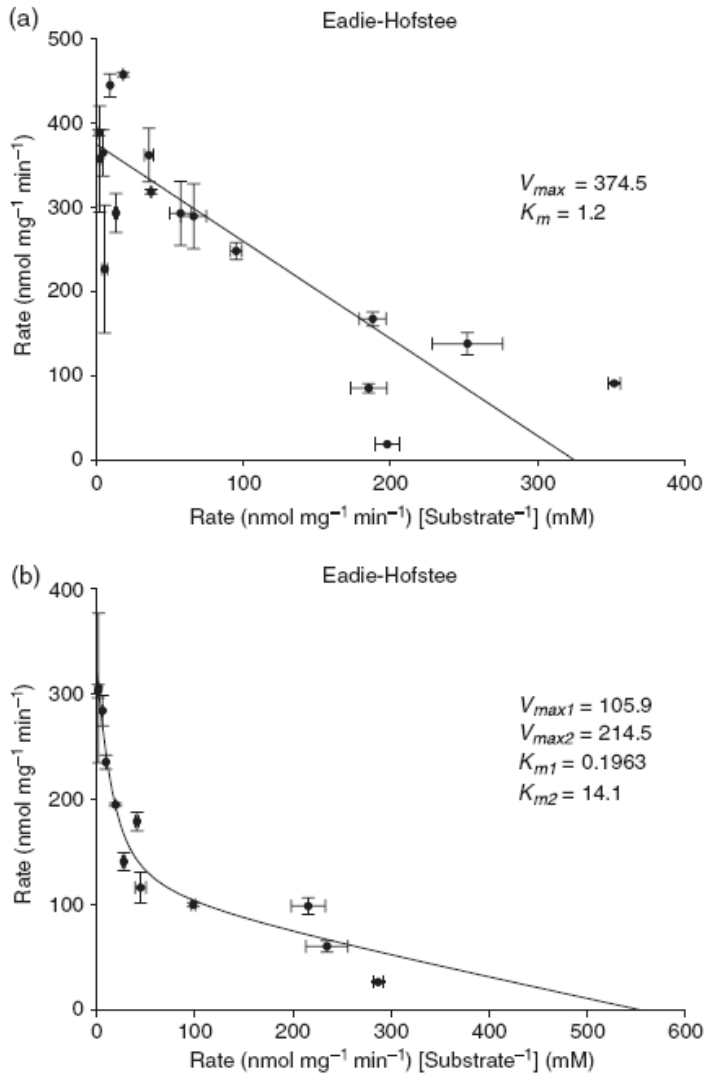

Figure 4: Eadie-Hofstee plots of the glucose transport kinetics of the wild-type (a) and *hxk2Δ* strain (b). Samples were obtained from glucose-limited aerobic cultures at a dilution rate of 0.32 h^{-1}. Velocities are shown in nmol•min.$^{-1}$•mg prot.$^{-1}$, the affinity constants are shown in mM. SE (standard error) *hxk2Δ* V_{max1} 35.9, V_{max2} 32.5, K_{m1} 0.19, K_{m2} 8.2. SE Wild Type V_{max} 14.0, K_m 0.24

The overall transport is characterized as a high-affinity system ($K_{m,\ glucose}$=1.2 mM), which is most likely solely attributable to Hxt6p/Hxt7p. In contrast, the *hxk2Δ* strain shows a two-component system, with a $K_{m,\ glucose}$ of 14.1 mM, suggesting significant contribution of the Hxt2-transporter and a very high-affinity component with a K_m of

only 0.2 mM. This very high-affinity component has been observed before (Diderich, Walsh, unpublished data), but it is unknown which hexose transporter manifests this very high-affinity. Fermentative capacity on glucose is measured in an excess glucose environment and since the total V_{max} for glucose transport of both strains does not vary greatly, the different values for the $FC_{glucose}$.cannot be explained by a change in transport capacity.
It was tested whether the capacity to ferment maltose related to the presence of a maltose transport system. Indeed, it was found (fig. 5) that maltose transporters are functional in the *hxk2Δ* strain during growth on glucose whereas this was not the case for the wildtype. The latter strain expressed transport activity only after an induction period in the presence of maltose and reached after approximately one hour an activity that equalled the activity of the *hxk2Δ* strain from the start of maltose incubation. These differences can account for the exceptional performance of the *hxk2Δ* strain compared to the wild-type, as it can start fermenting maltose immediately, plus more maltose transporters are induced, just like in the wild-type, by the presence of maltose.

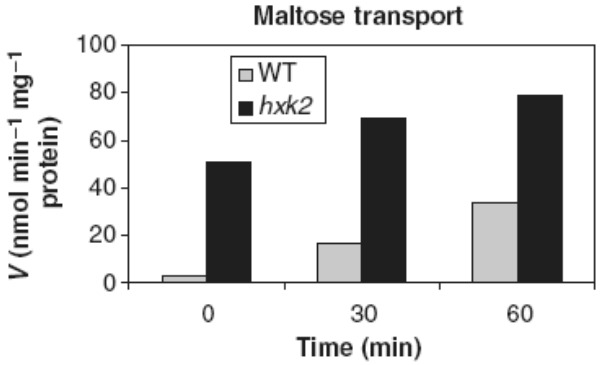

Figure 5: Rate of maltose transport by the wild-type and the hxk2Δ strains.
Samples were obtained prior, during and after FCM. The cells originated from glucose-limited aerobic cultures at a dilution rate of 0.32 h^{-1}. Velocities are shown in nmol•min.$^{-1}$•mg protein.$^{-}$1. Shown are the means of at least two independent experiments

Previously it has been shown that both the *hxk2Δ* and *HAP4*↑ strains in batch cultures express a more efficient glucose catabolism due to an increase in respiratory capacity and altered regulation of carbon source utilization, resulting in higher biomass yield values (Schuurmans, et al., 2008). Increased yield values may have an economic advantage in the production process but since under aerobiosis these mutants rely less on their fermentative branch of glycolysis, the question then arises as to the capacity of these strains to ferment sugars when transferred to anaerobic conditions, (as for example occurs when yeast is applied in dough). We therefore set out to quantify the trade-off between better growth performance and fermentative capacity. The results of this study illustrate the complexity of the regulatory network of yeast glycolysis: loss of hxkp results in a decreased capacity to ferment glucose but maltose can be fermented immediately, a property that is not observed in wild type cells. Overproduction of Hap4p on the other hand has no effect on the capacity to ferment glucose but does not endow the cells to

cope directly with maltose. The question then arises whether these physiological responses can be explained at the level of the transcriptome in general or at the level of specific enzyme activities. Although a lower hexokinase activity in the *hxk2Δ* strain was observed, mostly due to the deletion of one of the hexokinase iso-enzymes the altenative phosphorylating enzymes, *GLK1* and *HXK1,* still can account for enough activity to sustain a higher glucose influx than observed *in vivo*. This is true for all the other enzyme activities in the glycolytic path. Interestingly TPI activity was much higher than the other glycoltyic enzymes. This has been observed by others (Rossell, et al., 2007), and could possibly be seen as a mechanism to warrant fast conversion between di-hidroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP) in order to maintain redox neutrality. When the *hxk2Δ* strain was transferred to fermentative conditions with maltose glycolytic enzyme activity was induced, where in the wild-type activity was decreased after an hour. It has been shown (Gancedo, 1998) that the *hxk2Δ* strain lacks carbon catabolite repression. This is in accordance with our observations that both maltose transporter and maltase activity may be already present during growth on glucose, whereas in the wild-type it is still actively repressed. Thus, it maybe that in contrast to the wild type cells, the *hxk2Δ* strain produces a low level of glucose upon transfer to a maltose containing environment by the activity of maltase which subsequently diffuses out of the cells through the glucose transporters (as was reported earlier (Jansen, et al., 2002). These low levels may then result in upregulation of glycolysis.

Cells lacking hxk2p and hence being partially de-repressed express a more oxidative glucose catabolism. Indeed, transfer to an anaerobic environment with glucose results in much lower fermentative capacity than observed for the wild type. However, from the results with maltose, where high ethanol production rates are found, it must be concluded that the capacity of the pathway from glucose-6-phosphate to ethanol is not diminished by the absence of hxk2. This implies that the cause of the shift to a more oxidative physiology resides in the early steps of glucose metabolism: transport and/or the initial phosphorylation. Although a change in expression of glucose transporters does occur, it remains to be elucidated whether the catabolic shift is evoked directly by transcriptional changes or indirectly by the accompanying changes in glycolytic intermediates (and therefore by alterations in the steady state kinetics of glycolysis). Hierarchical regulation analysis (Rossell, et al, 2006) could be the appropriate tool to solve this question.

Whereas we have shown previously (Schuurmans, et al., 2008) that hxk2 and HAP4 act synergistically with respect to their control on the flux distribution over oxidative and fermentative glucose catabolism, no significant role could be seen for HAP4 with respect to adaptation to fermentative maltose conditions. We conclude therefore that the impact of hxk2p on the physiology of *S. cerevisiae* with sugar metabolism is more profound and more related to catabolite repression and the utilization or exclusion of alternative carbon and energy sources than that of HAP4 which seems to be limited to the regulation of the two catabolic modes only.

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