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

Schuurmans, J.M.

Citation for published version (APA):
The effect of altered expression of transcriptional regulators of catabolism on the transcription profile and physiology of *Saccharomyces cerevisiae*

J. Merijn Schuurmans

Uitnodiging
tot het bijwonen van de openbare verdediging van het proefschrift van
Jasper Merijn Schuurmans
op donderdag 13 november 2008 om 14.00 uur in de aula van de Universiteit van Amsterdam,
Agnietenkapel
Amsterdam
Oudezijds Voorburgwal 231

Paramenten:
Milou Schuurmans &
Martijn Bekker
The effect of altered expression of transcriptional regulators of catabolism on the transcription profile and physiology of *Saccharomyces cerevisiae*. 

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof.dr. D.C. van den Boom
ten overstaan van een door het college van promoties ingestelde commissie,
in het openbaar te verdedigen in de Agnietenkapel
op donderdag 13 november 2008, te 14.00 uur

door

Jasper Merijn Schuurmans

geboren te Amsterdam
Promotiecommissie

Promotor: Prof. dr. K.J. Hellingwerf
Co- promotor: Prof. dr. M.J. Teixeira de Mattos

Overige Leden: Prof. dr. J.M. Thevelein
Prof. dr. J.T. Pronk
Prof. dr. S. Brul
Dr. B.M. Bakker
Dr. M.C. Walsh

Faculteit der Natuurwetenschappen, Wiskunde en Informatica

The research described in this thesis was supported by the Dutch Technology Foundation STW, applied science division of NWO and the Technology Program of the Ministry of Economic Affairs. The research was carried out at the Swammerdam Institute for Life Sciences (SILS), Department of Molecular Microbial Physiology of the University of Amsterdam.

Heineken Technical Services, Unilever Vlaardingen and DSM Bakery Ingredients are acknowledged for their financial contributions to the research reported in this thesis.
Chapter 1

Introduction
General introduction

Mankind has been using yeast for several millennia. Historians have traced the roots of the first alcoholic fermentations back to African, Egyptian and Sumerian tribes, some 6000 years ago (Jackson, 1977). In the old world the art of making alcohol-containing beverages and bread spread from Egypt to Greece and Rome, but in the Far East it had been developed even earlier. However, it took several thousand years before the agent causing alcohol production was discovered and described. The first yeast cells to be observed were identified by Antonie van Leeuwenhoek in 1680 using his self-built microscope but at that time yeast was not seen as a living organism and not thought to be responsible for the fermentation process (Schierbeek, 1963). It took another 100 years, before the Frenchman Lavoisier first described the fermentation process (1789) in the form of a chemical equation: “le mout de raisin = acide carbonique + alkool” (Barnett, 1998).

After this, the concept of yeast being a living organism developed, and between 1855 and 1875, the French chemist Louis Pasteur unequivocally showed yeast to be the organism solely responsible for the fermentation of malt sugars into alcohol and carbon dioxide. At last, fermentation was no longer a “magical” phenomenon and yeast became a popular organism to study (Barnett, 2000). Around 1881 Emile Hansen isolated the first yeast for use in “single cell” cultures from a production fermentation of the Carlsberg brewery in Denmark. It was called *Saccharomyces carlsbergensis*, which is a yeast very closely related to *Saccharomyces cerevisiae* (Barnett, 2000). Eduard Buchner, a German physiologist and chemist demonstrated in 1897 for the first time a cell-free fermentation by use of extracts from yeast cells. These experiments, together with studies in muscle, stood at the basis of the discovery of glycolysis (also known as the Emben-Meyerhof-Parnas pathway, named after its discoverers (Barnett, 2000)), a series of chemical reactions responsible for the conversion of glucose to pyruvate. The end product of glycolysis serves as the substrate that is finally converted to ethanol and carbon dioxide in the fermentation proper.

Hexose Transport

Before anything else, the very first obligate step in sugar catabolism is transport of the sugar molecules, and here we will limit ourselves to glucose, over the plasma membrane. *Saccharomyces cerevisiae* transports glucose, but also other hexoses such as fructose and mannose, over the membrane by means of facilitated diffusion. This transport step exerts a high degree of control on the glycolytic flux and has been considered for long the sole rate-determining step for sugar metabolism (Oehlen, et al., 1994). Not only is this transport step crucial for energy- and carbon metabolism; the availability and transport process of glucose are also important factors for a large set of extensive and complex regulatory mechanisms, which trigger large alterations in the transcript profile of the cell in response to the presence of glucose. This regulatory program is known as glucose repression (or induction) and is described in the next paragraph. As in nature *S. cerevisiae* typically grows on decomposing fruits, it needs to be able to adapt to very large changes in sugar concentration, from amounts as high as 1.5 M glucose, down to micromolar
concentrations (Barnett, 1976). Whereas facilitated diffusion is most suitable as the mechanism for uptake of extracellular glucose into the yeast cell when the extracellular glucose concentration is high and fairly constant, it is far from ideal for low ambient glucose concentration. One may wonder why this particular yeast species does not have energy driven (e.g. proton symport) systems available for the transport and intracellular accumulation of this sugar as this would be beneficial to the cell at low sugar conditions (van Urk, et al., 1989). Interestingly, baker’s yeast does possess sugar symporters for disaccharide sugars such as maltose (Lagunas, 1993). To accommodate optimal transport against steep concentration gradients, \textit{S. cerevisiae} has developed a large diversity of hexose transport proteins (Hxtp), each with their own specific kinetic properties and environment-dependent (transcriptional) regulation (Kruckeberg, 1996). The expression of these hexose transporters is tightly regulated by the availability of the corresponding substrate, to allow for optimal transport rates at each sugar concentration.

In \textit{S. cerevisiae} a large family of hexose transporters has been identified on the basis of sequence analyses and similarities to sugar transporters of other organisms. This family (the HXT family) consists of 20 hexose transporter (-like) proteins (Hxt1p - Hxt17p, Gal2p, Snf3p and Rgt2p). A dendrogram of sequence similarity of this family of transporters is shown in figure 1 (taken from A. Kruckeberg (Kruckeberg, 1996)). This figure suggests that the large diversity in hexose transporters of \textit{S. cerevisiae} may have arisen from several duplication events throughout evolution. The HXT family in turn belongs to a large transporter super-family, called the major facilitator super-family (MFS). The MFS consists of a large variety of transporters for a large range of metabolites in eukaryotes and prokaryotes (Marger and Saier, 1993).

Sequence alignment of the transporters from the MFS family showed particular conservation throughout that part of the sequence that encodes 12 putative transmembrane helices (Kruckeberg, 1996). The N- and C-terminal regions of the different proteins differ considerably in length and sequence; nevertheless they all are predicted to be located on the cytosolic side of the cytoplasmic membrane.

Glucose transport kinetics of whole cells is complicated by the presence of so many transporters. In wild-type cells under de-repressing conditions a glucose-repressible high-affinity component with a $K_m$ of about 0.7 mM and of approximately 2 mM under glucose-repressed conditions, as well as a low-affinity component with a $K_m$ of approximately 25 mM (under de-repressed conditions) and of approximately 45 mM (under repressed conditions) has been observed (Bisson and Fraenkel, 1984; Coons, et al., 1995). In view of the complexity of glucose transport, it is noteworthy that the uptake rate of glucose is fairly constant during batch cultivation with a starting concentration of glucose of 100 mM. Yet, the affinity for glucose increases as glucose concentrations decrease (Walsh, et al., 1994). Modulation of the expression level of various transporters allows for these changes and is brought about in various ways, \textit{e.g.} by changing the expression levels of \textit{HXT} transcripts, encoding transporters with different affinities (Reifenberger, et al., 1997), by removal of the transporter proteins from the cytoplasmic membrane and subsequent inactivation of these hexose transporters (Bisson and Fraenkel, 1984; Horak and Wolf, 1997), by modulating the affinity of a specific transporter (Reifenberger, et al., 1997). Mutant analysis showed that deletion (or mutation) of any single Hxt protein does not lead to a significant phenotype, with the exception of \textit{GAL2} and \textit{SNF3} (Boles and Hollenberg, 1997).
Figure 1: Dendrogram of the sequence similarity among the yeast hexose transporter proteins. The dendrogram shows the clustering relationship of the sequences based on their mutual similarity; the length of the horizontal branches is inversely proportional to the sequence similarity of the sequences at each branching point. The sequences were clustered by the GCG PILEUP program, and the dendrogram was plotted by the GCG FIGURE program (Genetics Computer Group 1994). The chromosomal location of each gene is shown next to the name of the corresponding transporter protein. (taken from (Kruckeberg, 1996))

These latter two proteins do not allow the cells to grow on galactose nor on low concentrations of glucose. Growth on glucose is fully abolished, even when this sugar is present at high concentrations, only when HXT1 to HXT7 are all deleted (Reifenberger, et al., 1995). In such a “hxt null” strain no significant rate of glucose transport can be detected. Reintroduction of either HXT1, -2, -3, -4, -6 or -7 restores growth on glucose, although not at all glucose concentrations, for every transporter (Reifenberger, et al., 1995). These results show that these seven Hxt proteins are the major proteins used for transport of glucose, and it further illustrates that non-facilitated diffusion through the membrane does not contribute significantly to glucose uptake (Gamo, et al., 1995). These ‘single-HXT strains’ allowed the biochemical characterization of the respective transporters (i.e. with respect to $K_m$ and $V_{max}$) (Reifenberger, et al., 1997; Reifenberger, et al., 1995). Further details about the members of the Hxt family are described in detail below, with the exception of Snf3p and Rgt2p, which are discussed in the next section.
under glucose signaling. Table 1 summarizes some key features of the various hexose transporters. Further details about each transporter follow below.

Reintroduction of HXT1 into the hxt null strain restores growth on high concentrations of glucose (or fructose, mannose), but not on low concentrations of glucose, which is consistent with its low-affinity. HXT1 expression is highly induced at high levels ( >200 mM) of glucose, but not by galactose. Expression is also induced during chemostat growth at very high dilution rates ( $\mu > 0.36$), as revealed by Northern blot analysis (Diderich, et al., 1999). Additionally, HXT1 expression is induced by hyperosmotic stress (addition of 0.1 M NaCl or sorbitol) (Hirayama, et al., 1995). This induction is controlled by the Hog1p pathway. Activation of this pathway leads to the formation of glycerol inside the cell, in order to protect the cells against the osmotic stress. Taken together, these properties make Hxt1p an excellent glucose transporter when yeasts are growing on freshly decomposing fruits, where glucose (or fructose) concentrations can be as high as 1.5 M.

As seen in figure 1, the closest relative of Hxt2p is Hxt10p. The genes encoding these two proteins are 72% identical, but only in the coding regions. Hxt2p has been identified as a medium- to high- affinity transporter, with shifting affinities for glucose, depending on the extracellular glucose concentration. At a glucose concentration of 100 mM, an apparent $K_m$ value of approximately 10 mM was observed. In contrast, a strain that only contained Hxt2p grown at low glucose concentrations expressed biphasic uptake kinetics, with a high affinity component ($K_m= 1.5$ mM) and a low affinity component ($K_m= 60$ mM) (Reifenberger, et al., 1997). Interestingly, reintroduction of HXT2 in the hxt null strain restored growth on very low glucose concentrations, whereas that is not the case for HXT4, a transporter with a similar affinity for glucose. During chemostat growth HXT2 is induced at low to medium growth rates, peaking between a $\mu$ of 0.15 and 0.20 h$^{-1}$ (Diderich, et al., 1999). In this study no induction on batch growth was observed. Strangely this has to do with growth also, as there is no detectable HXT2 when cells are grown from 100 mM glucose to glucose exhaustion, but there is a large amount of HXT2 expressed when cells are shifted to a new medium with low glucose concentrations. The complexity of the regulation of HXT2 expression goes even further as its expression is both activated and repressed by glucose (Ozcan and Johnston, 1995; Wendell and Bisson, 1994). At high glucose levels, HXT2 is repressed by the Mig1p repressor protein (Nehlin and Ronne, 1990). More generally speaking, Mig1p is involved in the glucose-dependent expression of a number of genes and is discussed below. At low levels of glucose, Mig1p is released from the promoter region. This process requires Snf1p (Celenza and Carlson, 1986) via the Snf1p kinase that regulates the activity of Mig1p (see below). Adding to the complexity once more, this release itself is not sufficient, as HXT2 expression in the absence of glucose is also repressed by the Rgt1p repressor. Only on low concentrations of glucose, neither of the two pathways is active and this therefore results in HXT2 expression. Interestingly, the Snf1p-Mig1p pathway seems to control most of its expression, as in a sof1Δ mutant, there is continuous repression by Mig1p regardless of the glucose concentrations (Ozcan and Johnston, 1995), whereas in a rgt1Δ mutant expression is constitutive in the absence of glucose, but still effectively repressed at high levels of glucose (Boles and Hollenberg, 1997). Unlike Htx2p, Hxt10p despite its high similarity doesn’t seem to be able to transport glucose at all (or very little) (Reifenberger, et al., 1995) and will not be discussed here.
Hxt3p is most closely related to Hxt1p with 86.4% identity. Reintroduction of Hxt3p in the \textit{hxt} null mutant restores growth at high glucose concentrations exclusively, consistent with its low-affinity. During glucose-limited chemostat growth, \textit{HXT3} is only expressed at or near the \( \mu_{\text{max}} \) of \textit{S. cerevisiae}, and in batch cultures only in the very first phase of growth (Diderich, et al., 1999). Expression of \textit{HXT3} is regulated by the Snf3p-Rgt2p pathway and, surprisingly, is independent of the Snf1p pathway.

Hxt4p is most closely related to Hxt6p and Hxt7p with 83.4% identity to both. Reintroduction of \textit{HXT4} in the \textit{hxt} null mutant restored growth on intermediate and high glucose concentrations, but not on 5mM (low) glucose. Under glucose-limited chemostat conditions, however \textit{HXT4} is expressed at high growth rates (0.36 h\(^{-1}\)) and in batch culture only in the first phase of growth on glucose, which doesn’t seem to fit with the affinities reported (Diderich, et al., 1999). Expression of \textit{HXT4} is sufficient to suppress the growth defects of a \textit{snf3A} mutant. Regulation of \textit{HXT4} expression appears to be similar to that of \textit{HXT2}, as it is induced under low glucose conditions and repressed at high glucose concentrations. Although the promoter sequence of \textit{HXT4} contains a Mig1p and a Rgt1p binding site, it seems that \textit{HXT4} is repressed solely by Mig1p, although this could be strain dependent (Ozcan and Johnston, 1996). Unlike \textit{HXT2}, expression of \textit{HXT4} in the \textit{hxt} null mutant restores a near wild-type growth rate on ~250 mM glucose, whereas upon expression of \textit{HXT2} in the \textit{hxt} null mutant, growth on high glucose concentrations was strongly inhibited.

A rather enigmatic putative glucose transporter is Hxt5p. Interestingly, it is repressed by high-, intermediate-, as well as very low glucose concentrations. Only in the absence of glucose (i.e. growth on an ethanol/glycerol mixture), \textit{HXT5} will become expressed, be it not at a very high level when \textit{S. cerevisiae} is grown in batch cultures. The only condition reported to result in high expression of \textit{HXT5} is sporulation, as determined by a GFP-tagged Hxt5p. Reintroduction of \textit{HXT5} in the \textit{hxt} null mutant does not restore growth on glucose, and these cells can only be grown on a non-fermentable carbon source or sugars requiring different transporters (like galactose or maltose). Upon growth on maltose and galactose, however, \textit{HXT3} is also repressed. Affinities for glucose were determined by growing the corresponding strain on an ethanol/glycerol mixture, until sufficient \textit{HXT5} was expressed, after which glucose uptake was measured. An apparent \( K_m \) value for glucose of 11 mM was determined (Diderich, et al., 2001b). In glucose-limited chemostat cultures \textit{HXT5} is expressed at a dilution rate of 0.1 h\(^{-1}\), but not at 0.05 h\(^{-1}\). The expression at 0.1 h\(^{-1}\) is rather inconsistent with the findings that \textit{HXT5} is repressed by glucose (Diderich, et al., 1999). Deletion of \textit{HXT5} does not result in a clear phenotype, although a slightly longer lag phase was observed after transfer from glucose exhausted medium to fresh medium, and the \textit{hxt5A} strain resumed with pseudo-hyphal growth instead of the normal budding that the wild-type displays (Diderich, et al., 2001b).

Hxt6p and Hxt7p are the most closely related hexose transporters in yeast, with only two amino acid differences. These two residues are positioned outside the 12 membrane spanning domains and do not show up in any of the other hexose transporters, making these two proteins virtually identical in their membrane intrinsic domains. Interestingly, strains with a \textit{HXT6/7} chimaeric gene have been reported. This chimaera can also arise spontaneously by intrachromosomal rearrangement between highly related genes (Boles and Hollenberg, 1997). Reintroduction of one or both of these genes restores growth on low concentrations of glucose (5mM) (Reifenberger, et al., 1997). During normal growth...
HXT7, however, is far more abundant than HXT6 and in glucose-limited chemostat cultures HXT7 is expressed at all growth rates, whereas HXT6 was only found at intermediate growth rates (0.15-0.28 h⁻¹) ([Diderich, et al., 1999]). Both genes are strongly repressed by glucose concentrations exceeding 25 mM, whereas Hxt6p in addition seems to be degraded post-transcriptionally by high glucose ([Boles and Hollenberg, 1997]). HXT6 and HXT7 are strongly expressed when there is no glucose available in the environment (for example during growth on substrates like maltose, ethanol and galactose, etc.), most likely to immediately be able to resume glucose consumption when this preferred substrate becomes available. Both genes are regulated by Snf3p; however, only HXT6 requires Snf3p for maximal expression during growth on low glucose concentrations.

Hxt8p does not contribute significantly to glucose transport. Only overproduction of Hxt8p can partially restore growth on glucose in the hxt null strain, which qualifies it as a functional hexose transporter that is not used to a significant extent under the growth conditions reported in literature.

With respect to Hxt9p, Hxt11p and Hxt12p the functionality seems somewhat like that of Hxt8p. These are three hexose transporters that again do not contribute to the transport of glucose. Interestingly, these transporters are not only very similar in their coding region; the similarities extend into the entire promoter sequence as well. It has been found that these three transporters are not regulated by glucose at all but seem to be involved in pleiotropic drug resistances ([Nourani, et al., 1997]). This is a phenotype closely related to the mammalian multidrug resistance phenotype ([Balzi and Goffeau, 1995]). This phenotype causes the rapid expression of plasma membrane-bound non-proton ATPases, which confer resistance to various drugs by acting as efflux pumps. Expression of genes, involved in this response in yeast, is under control of Pdr1p and Pdr3p, and indeed HXT9 and HXT11 are under control of these very same proteins. Hxt9p, Hxt11p and Hxt12p, however, cause sensitivity to various drugs. A Pdr3p mutation, which dramatically increases HXT9 and HXT11 expression, can still not restore growth on glucose in the hxt null mutant, which is a further indication that these genes are most likely not functional hexose transporters, but rather multi-drug efflux pumps ([Nourani, et al., 1997]).

Very little if anything is known about Hxt13p, Hxt14p, Hxt15, Hxt16p and Hxt17p. HXT13, HXT15, HXT16 and HXT17 are closely related, whereas HXT14 is the most distantly related member of the hxt family (see figure 1). Deletion of either one or more of these proteins does not show a noticeable phenotype, and no significant expression of any of these was detected. The only exception to this is HXT13, which was found to be regulated by the Hap2p regulator, although no function has been assigned ([Boles and Hollenberg, 1997]).

Gal2p, although a member of the hxt family, predominantly transports galactose. Of the 17 Hxts, it is most closely related to Hxt6p and Hxt7p. Expression of GAL2 is strongly induced by galactose and repressed by glucose. Induction is controlled by the Gal11p-Gal80p-Gal4p pathway, which is initiated by binding of intracellular galactose to the Gal1p galactokinase. This, however, requires galactose to be present inside the cell, for GAL2 expression to start. It has been shown that the glucose transporters can transport small amounts of galactose, thus transferring the initial signal intracellularly. Similarly, the Gal2p can transport glucose. GAL2 is repressed by the Snf1p pathway and Gal2p is
furthermore inactivated by endocytosis into the vacuole and subsequent proteolysis when glucose is present (Horak and Wolf, 1997).

Table 1: Properties of the major Hexose Transporters of *S. cerevisiae*

<table>
<thead>
<tr>
<th>Transporter</th>
<th>ORF Name</th>
<th>Protein Length</th>
<th>Affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>HXT1</td>
<td>YHR094C</td>
<td>570 a.a.</td>
<td>Low ($K_m$ ~ 100mM)</td>
</tr>
<tr>
<td>HXT2</td>
<td>YMR011W</td>
<td>541 a.a.</td>
<td>Moderate to High ($K_m$ ~ 1.5 mM, $K_m^2$ ~ 60 mM)</td>
</tr>
<tr>
<td>HXT3</td>
<td>YDR345C</td>
<td>567 a.a.</td>
<td>Low ($K_m$ ~ 60 mM)</td>
</tr>
<tr>
<td>HXT4</td>
<td>YHR092C</td>
<td>576 a.a.</td>
<td>Moderate ($K_m$ ~ 9 mM)</td>
</tr>
<tr>
<td>HXT5</td>
<td>YHR096C</td>
<td>592 a.a.</td>
<td>Moderate ($K_m$ ~ 11 mM)</td>
</tr>
<tr>
<td>HXT6</td>
<td>YDR343C</td>
<td>570 a.a.</td>
<td>High ($K_m$ ~ 1-2 mM)</td>
</tr>
<tr>
<td>HXT7</td>
<td>YDR342C</td>
<td>570 a.a.</td>
<td>High ($K_m$ ~ 1-2 mM)</td>
</tr>
<tr>
<td>GAL2</td>
<td>YLR081W</td>
<td>574 a.a.</td>
<td>High ($K_m$ ~ 2 mM)</td>
</tr>
</tbody>
</table>

Glucose Signaling (repression and induction)

*Saccharomyces cerevisiae* can use a large variety of compounds as carbon and energy source, ranging from polysaccharides, monosaccharides such as glucose, fructose, mannose and galactose, linear alcohols such as ethanol, to organic acids like acetate and lactate. However, glucose is by far the preferred carbon and energy source for *Saccharomyces cerevisiae*, as reflected by its intricate and complex mechanisms to detect, adapt and finally metabolize this sugar. In this paragraph the main sensors and regulators that play a role in recognizing and responding to different amounts of glucose will be discussed. These are the Ras-cAMP-, the Snf1-, the Snf3- and Rgt2-pathway. Although these are discussed here as separate pathways, they most likely mutually communicate, which leads to additional, emerging properties of the entire regulatory network and creates a dynamic system of regulation and responses to glucose, and as such one might even consider them to all be part of one large system.

The Ras-cAMP Pathway

One of the major mechanisms of glucose signaling is the Ras-cAMP Pathway (see also figure 2). Ras proteins are monomeric GTPases that can act as molecular switches, depending on whether GDP (inactive) or GTP (active) is bound. These proteins are always accompanied by GAPs (GTPase Activating Proteins), which stimulate the hydrolysis of GTP to GDP, thus rendering the GTPase in an inactive state and GEFs (Guanine Exchange Factors), which stimulate the dissociation of GDP and the binding of GTP, rendering the GTPase active. The two Ras proteins in *S. cerevisiae* are encoded by...
RAS1 and RAS2. Deletion of either of these has no effect on growth of glucose, however, deletion of both causes a growth arrest in the G1 phase of the cell cycle (Tatchell, et al., 1984; Toda, et al., 1985). Several important effectors mediate the activity of the Ras proteins in S. cerevisiae. CDC25, an essential gene in yeast (which also causes G1 cell cycle arrest, upon deletion (Hartwell, et al., 1973)), is a GEF for the Ras proteins and activates them (Cameron, et al., 1988). When Ras is activated, it initiates the production of cAMP in the cell by the adenylate cyclase (CYR1) (Matsumoto, et al., 1982). On the other hand IRA1 and IRA2 inactivate Ras proteins by stimulating GTP hydrolysis, thereby lowering cAMP levels (Tanaka, et al., 1991; Tanaka, et al., 1989). Additionally, cAMP levels are modulated by the cAMP phosphodiesterases (PDE1 and PDE2) (Nikawa, et al., 1987).

When glucose is added to cells growing on a non-fermentable carbon source (or when they are in the stationary phase), a signal is generated by one or more sensors. These signals converge on membrane-bound Ras. This subsequently triggers a rapid, but transient increase in cAMP levels within 1-2 minutes and returns to normal within 20 minutes (Thevelein, et al., 1989).

Protein Kinase A (PKA) is a heterotetrameric protein consisting of 2 regulatory subunits and 2 catalytic subunits (Toda, et al., 1987). The cAMP spike that has been triggered by the Ras proteins, leads to the dissociation of the 2 regulatory subunits (encoded by BCY1) from the 2 catalytic subunits (encoded by the genes TPK1, TPK2 and TPK3). This results in an active PKA, allowing for the start of a phosphorylation cascade. Active PKA is thought to phosphorylate many regulatory proteins in the nucleus and thereby initiate a wide-spread change in the transcriptional network needed for adaptation to the availability of glucose (Griffioen and Thevelein, 2002). Interestingly, the Ras-cAMP pathway is associated with many more functions and as such appears to be a hub for regulation of transcription in response to many factors, such as thermotolerance (Zhu, et al., 2000), stress resistance, glycogen accumulation (Smith, et al., 1998) and aging (Lin, et al., 2002). Another G-protein has been found to contribute to the adenylate cyclase activity and subsequently the activation of PKA. This protein, consisting of an α, β and presumably a γ subunit (if analogous to other G-proteins), can also, like the monomeric Ras, bind to guanine nucleotides. The α subunit Gpa2p and its GAP Rgs2p have been found to act in the glucose signaling cascade (Versele, et al., 1999). Beta subunits (Gpb1p and Gpb2p) have been identified by interaction with Gpa2p, however so far no γ subunit has been found (Harashima and Heitman, 2002). The Gpbp1p and Gpb2p proteins are not β-subunits in the classical sense, instead they are proteins with a different structure, also identified as the Kelch-repeat proteins Krh1p and Krh2p (Batlle, et al., 2003). These proteins facilitate the binding of Bcy1p to the Tpk subunits, and are capable of bypassing the cAMP dependent regulation of PKA (Peeters, et al., 2006). Also the Krh1p and Krh2p can bind to the Irl1p and Ira2p proteins, inhibiting Ras inactivation and thus stimulating cAMP production. Deletion of RAS2 and GPA2 leads to a severe synthetic growth defect which can be restored by removal of PDE1 and PDE2. It has been proposed therefore that the essential missing part is the cAMP synthesis needed to trigger PKA activity (Xue, et al., 1998). Indeed expressing activated Ras and/or activated Gpa2 shows the full response even in absence of glucose (Wang, et al., 2004). Gpa2p presumably is activated by Gpr1p, a membrane-bound g-protein coupled receptor (Xue, et al., 1998). Deletion of GPRI causes the same effects as deletion of GPA2 and in combination with RAS2.
deletion causes a severe synthetic growth defect. This indicates that Gpr1p is the only receptor coupled to Gpa2p to activate the cAMP response. As expression of either Ras or Gpa2 in their activated form causes the same effect as addition of glucose, this raises the question as to how they are activated by glucose. Gpr1p is a membrane bound protein and therefore could directly interact with glucose and as such activate Gpa2 to activate the cAMP signaling cascade.

Figure 2: Cytoplasmic events during PKA Signaling. Glucose regulation is mediated through the intracellular signaling molecule cAMP. Cyr1p (adenylate cyclase) responds to both Gpa2p and Ras1/2p. In GTP-bound form Ras1/2p and Gpa2p bind independently to Cyr1p and stimulate production of cAMP. RasGEFs (Cdc25p and Sdc25p) and RasGAPs (Ira1p and Ira2p) activate and deactivate Ras, respectively. The signal generated by glucose transport and/or phosphorylation stimulates Ras1/2p activation in a still unknown way. The receptor protein Gpr1p acts upstream of Gpa2p in glucose signaling and is a member of the G protein-coupled receptor (GPCR) superfamily. Gpa2p has a high similarity to the mammalian Gs subunits of heterotrimeric G proteins, and based on this similarity the putative Gs subunits Gpb1p and Gpb2p were detected via their interaction with Gpa2p. However, a corresponding G subunit has yet to be identified (shown as a question mark). Rgs2p is a GAP for Gpa2p; a GEF for Gpa2p has not yet been identified. Phosphodiesterases (Pde1p and Pde2p) antagonize glucose signaling via conversion of cAMP to AMP. cAMP activates the PKA tetramer by binding to the regulatory subunit Bcy1p, triggering a conversion and releasing the catalytic subunits of PKA, namely Tpk1p, Tpk2p and Tpk3p. The catalytic domain is then ready to phosphorylate corresponding target proteins (adapted from (Rolland, et al., 2002)).

On the other hand, Gpr1p does not activate Ras signaling. Ras signaling appears to be activated by phosphorylated intracellular glucose as a glucose-phosphorylation deletion mutant (hck1Δhxk2Δglk1Δ mutant) does not cause an increase of Ras2-GTP protein, the activated form of Ras2p. It has been suggested that phosphorylated glucose inhibits the RasGAP proteins Ira1p and Ira2p (Colombo, et al., 2004).
Chapter 1 General introduction

Clearly, many more experimental data is needed before we can unravel the precise mode of action by glucose on the Ras and Gpa2 proteins or their regulatory GEFs and GAPs. A schematic overview of mode of action is shown in figure 2.

The glucose sensors Snf3p and Rgt2p

Another way of sensing glucose by \textit{S. cerevisiae} is by means of the 2 glucose receptors Snf3p and Rgt2p. As outlined above, \textit{SNF3} and \textit{RGT2} are considered as members of the hexose transporter family despite the fact that they respond to the availability of glucose rather than transporting it and thereby mainly regulate the expression of the main 7 hexose transporters in yeast (\textit{HXT1-7}) (Ozcan, et al., 1996a). One piece of evidence that they are sensors and not transporter comes from the observation that expression of either of these proteins in the \textit{hxt null} mutant (\textit{hxt1-7/g39}) does not restore growth on glucose. Additionally two dominant mutations in these proteins (\textit{RGT2-1} and \textit{SNF3-1}) cause constitutive expression of \textit{HXT1} to \textit{HXT4}, even in the absence of glucose, which normally is not associated with their expression (Ozcan, et al., 1996a). Both sensors are required for the glucose induction or glucose repression of the various hexose transporters (see figure 3). Snf3p acts as a high-affinity sensor, sensing low levels of glucose. It is required for the induction of \textit{HXT2} and \textit{HXT4}, but not for the induction of \textit{HXT1}, a low affinity glucose transporter that is expressed under high glucose conditions. This is also supported by the observation that \textit{SNF3} is repressed at high glucose concentrations and cannot exert its function under those conditions. Rgt2p acts as the low affinity sensor, sensing high levels of glucose. It is required for the maximal induction of \textit{HXT1}, but has no function in inducing \textit{HXT2} and \textit{HXT4} at low glucose concentrations (Ozcan and Johnston, 1999).

These 2 sensors consist of two functional domains, a predicted 12 trans-membrane spanning domains and a long C-terminal intracellular tail, which is not present in other hexose transporters (Marshall-Carlson, et al., 1990). The trans-membrane domains are similar to the functional hexose transporters and almost certainly enable the sensors to bind glucose. However, the divergence from the functional hexose transporters might cause the inability to transport glucose (Ozcan and Johnston, 1999).

Upon glucose binding on the outside, the C-terminal tail generates a signal (of which the precise nature is unknown) that presumably consists of a conformational change in the C-terminal tail, in order to interact with the next signaling proteins in the signaling pathway. Interestingly swapping the long C-terminal tail onto the Hxt1p or Hxt2p protein restores the induction of hexose transporters in a \textit{snf3Argt2A} mutant (Ozcan, et al., 1998). After glucose binding the signal is transduced to Grr1p, possibly via Mth1p/Std1p. Grr1p is an inhibitor of a repressor Rgt1p (mentioned below). Mutants in Grr1p were originally identified as strains resistant to 2-deoxyglucose (a non metabolizable form of glucose) (Bailey and Woodword, 1984). These mutants were defective in various aspects of glucose repression such as the repression of maltase, invertease and mitochondrial cytochrome c oxidase. Grr1p appears to have more functions then repressing the genes involved in glucose metabolism as this mutation causes pleiotropic effects, such as elongated cell morphology and increased sensitivity to stress factors, i.e. osmotic stress and nitrogen starvation (Bailey and Woodword, 1984; Flick and Johnston, 1991).
Figure 3: Three different modes of modulation of the expression of individual \textit{HXT} genes by different levels of glucose.

Mode 1: At high glucose concentrations, signals are generated by glucose transport itself and by the low-affinity glucose sensor Rgt2p. The signal from the transporters is mediated by the Reg1p/Glc7p phosphatase complex and an unknown activator, which induces expression of \textit{HXT1}. Additionally, the signal generated by Rgt2p activates Grr1p, which inactivates the Rgt1p repressor allowing expression of \textit{Hxt1}. Mode 2: At both low and high levels of glucose signals are generated by both Snf3p and Rgt2p, which activate Grr1p, which in turn inactivates Rgt1p, allowing expression of \textit{HXT3}. No other ways of regulation of \textit{HXT3} expression by Snf3 and Rgt2p has been found so far. Mode 3: At intermediate to low levels of glucose, a signal generated via the Snf1p pathway, generated by Hxk2p is also required to allow expression of \textit{HXT2} and \textit{HXT4} (adapted from Ozcan and Johnston, 1999).

A \textit{grr1}\Delta strain is unable to induce \textit{HXT} genes in response to glucose and is impaired in glucose uptake (Ozcan, et al., 1994; Vallier, et al., 1994). This is due to the inability to inactivate the transcriptional regulator Rgt1p. Overexpression of \textit{RGT1} in a \textit{grr1}\Δ mutant restores the expression of \textit{HXT} genes, restoring their lowered transport capability (Marshall-Carlson, et al., 1991; Ozcan and Johnston, 1995). However, it does not restore the altered morphology of these mutants, which shows that Grr1p acts upon other proteins as well.

Rgt1p is a transcriptional regulator of \textit{HXT} expression. It is a bi-functional protein that can act as a repressor of transcription in the absence of glucose and as an activator under high level glucose conditions (Ozcan, et al., 1996b). At low concentrations of glucose it acts neither as a repressor nor as an activator. During repression Rgt1p is associated with Snf6p/Tup1p, a repressor complex that associates with many other repressors, including
Mig1p. At low concentrations of glucose it dissociates from this complex losing its functionality. High concentrations of glucose convert Rgt1p into an activator, in which function it is independent of Ssn6p/Tup1p (Ozcan, et al., 1996b). Figure 3 summarizes the regulation by the two glucose sensors upon the various hexose transporters.

HXT3 is activated by both low and high levels of glucose, conditions that result in Grr1p activation and hence Rgt1p inactivation. It has been reported that the presence of glucose may leading to a 10-fold increase in expression (Ozcan and Johnston, 1995). HXT2 and HXT4 are two moderate affinity transporters that are induced only under low levels of glucose. Snf3p activates Grr1p, relieving repression by Rgt1p. An additional signal, possibly intracellular glucose or glucose-6-phosphate, deactivates Reg1p, a regulatory protein involved in Snf1p kinase activity. In its activated form, Snf1p kinase phosphorylates Mig1p rendering it inactive. These two converging signals then allow for full induction of HXT2 and HXT4 (Ozcan and Johnston, 1999). Also at high glucose concentrations relief of repression by Rgt1p via Rgt2p signalling to Grr1p occurs. Rgt1p is then converted into an activator of transcription instead, furthermore the high amounts of glucose coming into the cell lead to the activation of a regulator, which is yet to be identified. What is known that this activation requires Hxk2p and Reg1p.

General glucose repression pathway (Snf1p pathway)

As mentioned before, intracellular glucose or phosphorylated glucose appears to be an important factor in the initiation of glucose signaling. Either of these compounds is implied in Ras2 activation and also seems to play a role in hexose transporter regulation. In de-repressed cells intracellular glucose can reach detectable levels, which can reduce transport activity by 50%. This elevation of intracellular glucose and/or the reduction of the transport activity may trigger the glucose response (Teusink, et al., 1998).

Yeast has 3 hexokinases, of which only HXK2 is abundantly expressed in cells growing on glucose. This particular hexokinase also appears to be one of the bases of the glucose repression induction machinery (see figure 4) (Moreno and Herrero, 2002). In glucose grown cells approximately 14% of Hxk2p is nuclear as has been shown by various techniques (Herrero, et al., 1998). It implies that Hxk2p might have a direct regulatory function on at the level of DNA. Indeed, it has been shown that Mig1p (a repressor of transcription) can bind to Hxk2p, and Mig1p even appears to mediate the nuclear localization of Hxk2p to the nucleus (Ahuatzi, et al., 2004).

Additionally, interactions with Med8p have been described. Med8p is a mediator protein and can bind directly to DNA to either positively or negatively act on glucose regulatory elements in the promoter regions (Chaves, et al., 1999).

Whether the action is positive or negative is most likely determined by the proteins it is associated with. The mode of action of Hxk2p, in this way shows many similarities with Gal1p, the galactokinase, which phosphorylates galactoses and activates the transcription factor Gal4p by binding to Gal80p a repressor protein of Gal4p (Zenke, et al., 1996). Here too, the exact signaling role of Hxk2p still hasn’t been elucidated and contradictory reports have been made. The deletion of a small N-terminal fragment (residue 7-16) was reported to abolish nuclear localization of Hxk2p and restored repression of invertase.
Figure 4: Simplified view of the glucose signaling pathway in *S. cerevisiae*. Extracellular glucose is transported into the cell by the various hexose transporters. Intracellular glucose is phosphorylated by hexokinase. Upon phosphorylation by Hxk2p, a signal is created which presumably indirectly affects Snf1p kinase, as direct interactions between Hxk2p and Snf1p or Reg1p/Glc7p have never been shown. An active Snf1p, at low glucose concentrations, inactivates the Mig1p and Mig2p repressor proteins, thereby relieving repression of many genes needed for respiration and TCA cycle activity and when glucose is depleted also genes needed for gluconeogenesis. Hap4p is an activator of respiratory- and TCA cycle genes and is also controlled by activity of the Mig1p and Mig2p repressors. A direct interaction between Snf1p and Hap4p and/or Cat8p hasn’t been shown, but a still unknown factor might facilitate this activation. The catalytic activity of this truncated Hxk2p was reported not to be affected (Rodriguez, et al., 2001). In contrast, deletion of residues 1-16, resulted in the opposite result, abolished glucose repression and correct localization (Mayordomo and Sanz, 2001). Hxk2p is considered in transferring a signal to the Glc7p phosphatase and the Snf1 kinase. No direct interactions have been shown between Hxk2p and either of these proteins however, which once more illustrates the need to find out exactly how Hxk2p signaling works. Not only would the top end of the Snf1p pathway then be unraveled, it might give insight as to how Hxk2p signaling is linked to the PKA and Snf3-Rgt2 pathways.

*SNF1* was identified in two screens, one for regulatory factors in the glucose response, designated *C47I* at that time, and in a screen for mutants that could ferment glucose, but not sucrose, and designated *SNF1* (*Sucrose Non-Fermenting*) (Carlson, et al., 1981; Ciriaey, 1977; Entian and Zimmermann, 1982). Deletion (or inactivation) of *SNF1* leads to various defects in the utilization of galactose, maltose, sucrose and non-fermentable carbon sources, and as such it was recognized as a very important regulator of the glucose
repression response. SNF1 was shown to encode a serine/threonine protein kinase (Celenza and Carlson, 1986). Additionally, in the same screen SNF3 (discussed above) and SNF4 were found (discussed above). The phenotypes of snf1Δ and snf4Δ are nearly identical. A hyperactive allele of SNF1, can however partially restore the defect of the snf4Δ strain (Celenza, et al., 1989). Further studies showed that Snf4p co-immuno precipitates with Snf1p and is required for maximal Snf1p activity. This led to the conclusion that Snf4p is an activator subunit of the Snf1p complex (Estruch, et al., 1992). More proteins were identified that act together with Snf1p. Three proteins, namely Sip1p, Sip2p and Gal83p strengthen Snf4p and Snf1p association and appear to form a bridge between the two proteins, as each of the three proteins have a Snf1p binding internal region and a Snf4p binding C-terminal region (Jiang and Carlson, 1997). In another search for mutants showing consecutive expression of SUC2, many proteins were found, of which most have been mentioned already: Snf1p, Ira1p, Ras2p and Hxk2p (Neigeborn and Carlson, 1987). In addition, another gene was identified, later named GLC7. This gene encodes the yeast homolog of the mammalian protein phosphatase 1 (PPI) (Feng, et al., 1991). Glc7p appears not only to have a function in glucose repression but also to play a crucial role in other cellular processes as well, including cell cycle progression through G2/M phase (Bloecher and Tatchell, 2000), actin organization (Chang, et al., 2002), sporulation (Ramaswamy, et al., 1998) and glucose induced degradation of fructose-1,6-bisphosphatase (Cui, et al., 2004). For a specific function PPI needs a subunit, as PPI itself shows hardly any substrate specificity. Most of these subunits have been identified. Reg1p is needed for glucose repression (Tu, et al., 1996), Gac1p and Pig1p are needed for storage carbohydrate metabolism (Cheng, et al., 1997; Stuart, et al., 1994). Reg2p and Sds22p are used in the cell cycle progression (Frederick and Tatchell, 1996; Peggie, et al., 2002), Scd5p for actin reorganization (Chang, et al., 2002)and Gip1p for sporulation (Tu, et al., 1996).

Reg1p (first identified as the gene HEX2) regulates glucose repression by Glc7p as is shown in various ways. Both originally identified alleles of glc7 and reg1 are involved in transcription of the gene SUC2, encoding invertase (Neigeborn and Carlson, 1987). Combinations of these alleles showed no synergy in release of glucose repression but overexpression of REG1 suppresses the repression defect (constitutive expression) caused by the GLC7-T152K allele (Tu and Carlson, 1995). This creates a balance between 2 complexes, one that phosphorylates the target proteins (discussed below) involved in repression and one that de-phosphorylates them. Under high glucose conditions Snf1p is in an auto-inhibited form as the regulatory domain is bound to the catalytic domain (Jiang and Carlson, 1996; Sanz, et al., 2000). When glucose concentrations become lower, Snf4p will compete for the regulatory domain and its binding will release the catalytic domain allowing it to phosphorylate its target proteins (Jiang and Carlson, 1996). When glucose is present again, PPI then presumably de-phosphorylates Snf1p, resulting in the release of Snf4p and conversion back into the auto-inhibited state. Interestingly, this de-phosphorylation was reported to occur not in the nucleus but in the cytoplasm, and it is postulated that Snf1p shuttles in and out of the nucleus to exert its functions (Dombek, et al., 1999). This also may explain the role of the two 14-3-3 like proteins, Bmh1p and Bmh2p in glucose regulated gene expression (Ichinura, et al., 2004) as they are known to respond to PKA signaling and might act as mediators for Reg1p-Glc7p function on Snf1p (Dombek, et al., 2004).
Several downstream repressors have been identified that are phosphorylated by Snf1p. The first identified was Mig1p (Multicopy Inhibitor of GAL expression) (Nehlin and Ronne, 1990). Deletion of Mig1p abolishes repression of numerous genes known to be glucose repressed (Klein, et al., 1998). Deletion of Mig1p in a snf1Δ mutant suppresses many snf1Δ mutant specific defects and therefore Mig1p is presumed to function directly downstream of Snf1p, as Snf1p also interacts with Mig1p (Vallier and Carlson, 1994). Upon phosphorylation of Mig1p by Snf1p, the repressor can no longer bind to DNA and loses its repressor function allowing gene expression of glucose repressed genes (Treitel, et al., 1998).

Mig2p, Mig3p, Nrg1 and Nrg2p are 4 additional Snf1p regulated repressors. Mig2p appears to have exactly the same function as Mig1p although in a less pronounced way as there are much less Mig2p molecules present than Mig1p (Klein, et al., 1999). The function of mig3p is unclear so far. The protein is very similar to Mig1p and can bind to Mig1p binding sites, but physiologically relevant target genes of Mig3p have not been identified. Mig3p was found in a screen for multicopy suppressors of Rad53-GFP toxicity and as such could be involved in the DNA damage response, but no clear evidence has been found so far (Dubacq, et al., 2004).

Nrg1p and Nrg2p are, like Mig1p, zinc-finger proteins. Nrg1p was identified in a screen for factors mediating repression of glucose-regulated genes (Park, et al., 1999) whereas Nrg2p was identified as a binding partner of Snf1p in a 2-hybrid screen (Vyas, et al., 2001). Both these proteins show glucose-dependent repression of a heterologous reporter gene and they are both able to interact with Snf1p although neither of them appears to be phosphorylated by Snf1p (unlike the Mig proteins) (Berkey, et al., 2004; Vyas, et al., 2001). They are implied to have functions in various processes such as glucose repression, filamentous invasive growth and biofilm formation but this vague notion is all that can be said about these proteins since their regulatory relationship with Snf1p nor their functioning still needs to be elucidated (Vyas, et al., 2001).

The Hap2/3/4/5p complex is a global regulator complex activating genes involved in the TCA cycle and oxidative phosphorylation (de Winde and Grivell, 1993; Forsburg and Guarente, 1989). The Hap4p regulator of this complex is under control of the Snf1p pathway and plays an important role in induction of genes needed for respiration. The Hap2p, Hap3p and Hap5p members of this complex are continuously expressed and as such do not appear to control the amount of expression of genes needed for respiration. In contrast, the Hap4p regulator is responsive to glucose via the Snf1p pathway, by means of Mig1p and Mig2p (Gancedo, 1998). When glucose is abundant, the HAP4 gene is repressed by Mig1p and Mig2p, as well as the genes involved in the TCA-cycle and oxidative phosphorylation (shown in figure 4). When glucose concentrations become lower, Snf1p phosphorylates Mig1p, allowing expression of HAP4 and respiratory genes. Hap4p subsequently binds to the Hap2/3/5p complex to form a transcriptional activator, which further activates genes containing a CCAATCA like promoter element (Dang, et al., 1994). This promoter element is present in many of the genes (e.g. the QCR genes) encoding for enzymes that catalyze respiration. Overexpression of HAP4 generally results in an increase in respiration by activating a whole set of genes (Blom, et al., 2000; van Maris, et al., 2001). Not only does Snf1p interact with repressors of transcription, it also regulates (at least) three transcriptional activators (Cat8p, Sip4p and Adr1p) needed for proper utilization of
non-fermentable carbon sources. Cat8p and Sip4p both bind specifically to a set of genes containing the carbon source responsive elements (CSRE) (Vincent and Carlson, 1998). Although both proteins are closely related structurally and can both be phosphorylated by Snf1p, Cat8p seems to be the most important one as a cat8Δ mutant causes defective growth on non-fermentable carbon sources, whereas deletion of Sip4p shows no detectable phenotype (Hedges, et al., 1995; Lesage, et al., 1996). Cat8p activates the expression of a wide array of genes needed for utilization of non-fermentable carbon sources, such as genes required for ethanol utilization (ACS1 and ACR1) (Bojunga, et al., 1998), genes needed for gluconeogenesis (Hedges, et al., 1995), the glyoxylate cycle (Haurie, et al., 2001), lactate utilization and isocitrate metabolism (Bojunga and Entian, 1999). Interestingly it also regulates the activity of Sip4p, which could explain why cat8Δ shows a phenotype and sip4Δ does not (Vincent and Carlson, 1998). Under glucose repressing conditions, the CAT8 promoter is bound by Mig1p and as such it needs two subsequent phosphorylations by Snf1p to be activated, the first being the phosphorylation of Mig1p, to relieve its binding of the promoter sequence of CAT8 and the second being phosphorylation by Snf1p on Cat8p itself to become active (Randez-Gil, et al., 1997). Upon addition of glucose, Cat8p is de-phosphorylated by PPI and becomes inactive again. As a consequence Mig1p will bind to the promoter and expression will also stop. A third factor is Adr1p which works together with Cat8p for maximal expression of a small group of genes (e.g. ADH2, ACS1 and ALD6). Although proper activation of Adr1p requires Snf1p, its regulatory mechanism is virtually unknown (Young, et al., 2003).

Yeast performance in industrial applications

As S. cerevisiae is used worldwide for many applications, such as the making of bread, the brewing of beer and the production of fine chemicals and specific proteins, decades of research have been devoted to understand and optimize the performance of yeast, both during these processes and in the production phase of the yeast in large scale fermentors. One of the most important quality parameters is the “raising power” or fermentative capacity. Fermentative capacity is defined as the ability to make ethanol and CO₂ under anaerobic conditions, which occurs during the raising process of bread dough. Research by P. van Hoek showed that the fermentative capacity of an industrial yeast is far from optimal at low growth rates (see figure 5 (Van Hoek, et al., 1998)). Due to the nature of the production process in fed-batch cultivations, the growth rates of yeast is very low at the end of the production process, expressing only one-third of the capacity found at high growth rates.. Many attempts have been made to increase the fermentative capacity. One of these attempts focused on increasing the expression of genes involved in glycolysis but generally speaking to no avail or with only limited success. With further insight in metabolic control analysis and hierarchical regulation, it could be understood that aiming for increased cellular capacity by enhancing one catalytic step in a multi-step reaction sequence may not be a proper approach; shifts in control of other steps will result in only moderate improvement of the overall fluxes (Smits, et al., 2000).
In this thesis we took another approach, instead of attempting to increase the fermentative capacity we have looked at strains with increased respiration capacity, allowing for higher yields on the used carbon source (glucose) during the production process (which is aerobic), while maintaining the fermentative capacity of the wild-type at various growth rates. Rather than focusing on individual enzymes in glycolysis, we have sought to achieve increased cell production by studying the effects of general glycolytic regulators. Several strains have been investigated, a hxs2Δ strain, a HAP4Δ strain, a mig1Δmig2Δ strain and the hxs2ΔHAP4Δ strain. Although one might intuitively assume that strains with an increased respiration might be impaired in fermentation, it is shown that this is not always the case.

Outline of this Thesis

This thesis describes the effects of various alterations in the expression of global catabolic regulators on the catabolism of \textit{S. cerevisiae}. As \textit{S. cerevisiae} is an organism that is often used in food and beverage applications, this organism has been studied extensively. However, the balance between respiration (the preferable mode of metabolism during yeast production processes) and fermentation (which is preferable during beer and bread production) cannot yet be tuned sufficiently to make these industrial processes proceed optimally. Attempts along these lines to increase the performance of yeast by over-expression of single enzymes were only moderately successful (Smits, et al., 2000). By changing the expression level of global regulators, a whole set of genes, often encoding all the enzymes required for an entire metabolic
pathway, can be affected. This could change the performance of that particular module as a whole, and thereby the overall metabolism of the organism as such.

Chapter 2 describes the effects of alteration of the expression of two key regulators in central carbon metabolism, HXK2 and HAP4. Both mutants, when either HXK2 is deleted or HAP4 is overproduced show an increase in respiration at high glucose concentrations, as compared to the wild-type. A transcriptome analysis of these mutant strains indeed revealed many increases in the transcript level of enzymes primarily involved in the TCA-cycle and in oxidative phosphorylation. These findings are consistent with the observed alterations in metabolic fluxes. Additionally, significant effects on transcription of genes involved in metal ion homeostasis were observed. It is not clear however, whether these are primary or secondary effects.

In Chapter 3 the results are presented of growth experiments of the strains described in Chapter 2 in a chemostat. As these strains show increased respiration, their ability to ferment under anaerobic conditions was tested in an off-line assay. Increased respiration might imply decreased fermentation; however, at low growth rates no effects were observed in the rate of fermentation of these strains. At the critical dilution rate, the growth rate at which the wild-type strain switches to respiro-fermentative metabolism, the performance of the \textit{hxk2}\textgreek{G39} strain was largely reduced, whereas the \textit{HAP4}\textgreek{G} strain showed the same capacity as the wild-type. In contrast, the \textit{hxk2}\textgreek{G39} strain performed well on maltose (which is the most abundant sugar in bread dough) under all conditions. To investigate the molecular mechanisms responsible for these changes, transcript profiles, enzyme activities and the sugar-transport capacity of these strains were measured. When the cells were grown on glucose, only the activity of hexokinase was found to be significantly affected, even though this activity should still allow a wild-type flux. With maltose as the catabolic substrate, however, there was a very clear difference between transport capacity of the \textit{hxk2}\textgreek{G39} and wild-type strains.

In chapter 4, a detailed analysis is presented of transcript profiling experiments performed with the Hap4 over-expression strain, under a large range of physiological conditions. This analysis shows a set of specific transcription factors of which their targets genes have been changed in expression in response to the different conditions and some which are specific for the \textit{HAP4}\textgreek{G} strain. As expected we found the target genes of \textit{HAP4} to be significantly upregulated in the \textit{HAP4}\textgreek{G} strain under conditions, where glucose repression is present (high glucose). Additionally we found the target genes for Mig1p to be changed when comparing conditions with glucose repression to conditions in which the cells are de-repressed. For genes involved in iron and zinc homeostasis, changes were found as well, specific for the \textit{HAP4}\textgreek{G} strain as well as condition dependent. Especially genes under control of Zap1p (zinc activator protein) were changed significantly. \textit{HAP4}\textgreek{G} cells under batch conditions, downregulate genes need for zinc homeostasis, although the cause of this event is largely unknown. Target genes of Cat8p were found to be upregulated in de-repressed conditions. The target genes of transcription factors Msn2-4p were significantly present under nutrient-limited conditions, combined with low-growth rates.
Chapter 5 describes a theoretical study on the relationship between the Monod constant \( K_s \) and the affinity constant \( K_m \) of the transporter. It is shown that it is very well possible that the former has a lower value than the latter i.e. the cell as a whole may have a better affinity for a limiting substrate than the transporter of that substrate. The two constants turn out to be related by the control of the transporter on the specific growth rate. It is shown further that the unexpected result is confirmed by the experimental data for *Saccharomyces cerevisiae* grown in glucose-limited chemostats.

Chapter 6 summarizes the most important findings reported in this thesis and discusses the implications of these findings and possible future research.
Chapter 2

On the role of $HXK2$ deletion and $HAP4$ overproduction on the respiro-fermentative flux distribution and gene expression in $S.\ ceres\! i\! s\! a\! e$

J. Merijn Schuurmans§, André Boorsma§, Romeo Lascaris§, Klaas J. Hellingwerf§ and M. Joost Teixeira de Mattos§

§Department of Molecular Microbial Physiology, Swammerdam Institute for Life Sciences, University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV Amsterdam, The Netherlands

Keywords: $Saccharomyces\ ceres\! i\! s\! a\! e$, Hap4p, Hsk2p, Glucose repression, Yield, fermento-respirative metabolism, T-profiler

Published in modified form in FEMS Yeast Research (2008) 8: 26-34 as “Physiological and transcriptional characterization of $Saccharomyces\ ceres\! i\! s\! a\! e$ strains with modified expression of catabolic regulators”
Abstract

We present a comparative physiological and transcriptional study on wildtype *Saccharomyces cerevisiae* and mutants with altered levels of catabolic regulators: *hxk2Δ* lacking hexokinase2, *HAP4†* overproducing Hap4p and *hxk2ΔHAP4†*. Relative to the wildtype, *HAP4†* expressed the same growth rate with some increased yield on glucose, *hxk2Δ* grew 28% slower but with a dramatically improved yield. *Hxk2ΔHAP4†* grew 14% slower but virtually fully oxidative. Higher yield correlated with increased respiration.

For both *hxk2Δ* strains glucose-repression was relieved, (upregulation of high-affinity sugartransporters, invertase and oxidative phosphorylation). T-profiler analysis showed that genes under control of the hap2/3/4/5 binding motif were significantly altered in expression in all strains. *HAP4†* overexpression, directly or in *hxk2* knockouts, led to repression of the genes containing the Zap1p-motif including *ZAP1* itself, indicating altered zinc metabolism. Whereas *HAP4†* overexpression results in a shift towards oxidative metabolism only, deletion of *HXK2* results in a strain that besides being oxidative, almost completely lacks the ability to sense glucose. Since the double mutant has an energetic efficiency close to the maximum even with excess glucose and is derepressed to a larger extent and over a broader range, we conclude that the functioning of the two regulators is in general synergistic.
Chapter 2  
Physiological and transcriptional characterization

Introduction

Saccharomyces cerevisiae is a unicellular fungus, which in its natural habitat encounters rapidly environmental changes. In its original habitat, either ripening or decomposing fruits, especially the concentration of various sugars changes dramatically. It is therefore not surprising that to effectively cope with these changes, S. cerevisiae has developed a very complex regulatory network to control their uptake and utilization as carbon and energy source. S. cerevisiae favors glucose (or fructose) for the latter purpose above other sources, and the presence of these sugars will have the effect that genes encoding enzymes needed to utilize other sugars, such as maltose, saccharose and galactose, will remain repressed. Additionally, the concentration of glucose per se is important, as at high concentrations (>30 mM), the organism also represses genes encoding enzymes needed for respiration (Gancedo, 1998). Thus at high glucose concentrations, S. cerevisiae partly ferments glucose to ethanol even in the presence of an excess oxygen, the so-called Crabtree effect (De Deken, 1966).

In some industrial processes fermentation is undesirable since it significantly reduces the biomass yield on glucose, and ethanol at high concentrations can become a growth inhibitor. Hence the catabolic regulatory network, also known as the glucose repression pathway, has been studied quite extensively. Many of the proteins involved in this regulation network have been identified and characterized (Gancedo, 1998). Signaling pathways can be divided into three parts, namely the G-coupled protein Gpr1p (Kraakman, et al., 1999), the Snf3p and Rgt2p glucose sensors (Ozcan, et al., 1996a) and the glucose transport systems plus hexokinase II (Teusink, et al., 1998). Gpr1p senses glucose and can activate the Ras-cAMP pathway (Rolland, et al., 2002). Since this pathway also controls many genes involved in nitrogen source utilization, Gpr1p links glucose availability to the nitrogen status of the cell. Snf3p and Rgt2p are involved in the control of expression of various hexose transporter (HXT) genes (Ozcan, et al., 1998), thereby directly exerting control on the glucose influx rate, as each individual hexose transporter has a different affinity for glucose transport (Diderich, et al., 1999). With respect to glucose transport itself and hexokinase II (Elbing, et al., 2004; Moreno and Herrero, 2002), the exact signal received and transmitted is not exactly known. It should be realized that the above three systems are intricately linked and interact with each other at various levels. For instance, changes in HXT expression mediated by Snf3p or Rgt2p may change the glucose uptake rate, which again will affect the glycolytic flux and Hxk2p activity and this subsequently may affect the expression of HXT genes again.

Hexokinase II is not only the primary glucose phosphorylating enzyme, it also has a regulatory function in glucose repression (Moreno and Herrero, 2002). It has been shown that in the presence of high glucose concentrations (>30 mM), hexokinase II migrates to the nucleus to exert its regulatory function, together with Mig1p (Ahuatzi, et al., 2004). It has also been shown that a strain lacking the HXK2 gene is largely shifted towards respiratory metabolism (Diderich, et al., 2001a) and shows co-consumption of various sugars and even ethanol (Raamsdonk, et al., 2001). The genes needed for utilization of these alternative sugars are normally repressed most predominantly by Mig1p and to a lesser extent by Mig2p. These latter proteins also repress genes involved in respiration. A mig1Δmig2Δ strain indeed shows an increase in respiration (Klein, et al., 1999), although not as profound as might have been expected due to the absence of the main repressor
Chapter 2  Physiological and transcriptional characterization

genes. For a fully functional respiratory chain and TCA-cycle, genes in the corresponding processes also need to be properly induced and activated. The transcriptional activator Hap4p mediates this process. It has been shown indeed that overproduction of this protein results in a considerably higher respiration rate and an increased biomass yield for *S. cerevisiae* grown on glucose (Blom, et al., 2000; van Maris, et al., 2001).

Clearly, the regulatory mechanisms that result in the net glycolytic, fermentative and respiratory activity of the yeast cell are complex and the contributions of the various components of the network to achieve the final output are still largely unknown. Each of the regulators has its own characteristics, often studied in isolation (Gancedo, 1998; Santangelo, 2006). The question arises to what extent the various regulatory proteins communicate with each other within the regulatory network. It may be that each of them affects gene expression and hence the physiology of the cell for some specific adaptive response or they may act in a concerted manner to provide the cell with a means to cope with a variety of conditions. In this study we quantitatively analyze the separate contribution of two of the key regulators (Hap4p and Hxk2p) to the metabolic activity of baker’s yeast as well their synergistic functioning by a comparative study of the wild type strain, and single and double mutants.

Materials & Methods

**Strains and cultivation**

Strains used were CEN.PK113-7D (MATa URA3 LEU2 HIS3 TRP1 SUC2 MAL MEL GAL), a HAP4 overexpression strain (van Maris, et al., 2001), a hsk2 Δ:KanMX4 mutant (Diderich, et al., 2001a), and a hsk2 Δ:KanMX4 HAP4 overexpression strain (Lascaris, et al., 2004). All mutants are derived from the CEN.PK113 background. The strains were precultured overnight at 30°C in 0.67% YNB 2% glucose 0.1 M phtalic acid, pH=5.0 using KOH. Cells were inoculated to an OD600 of 0.2 in the same medium in batch fermentors with a 0.5 liter working volume, aerated with 1 vvm and stirred at 500 rpm. Growth was followed in time both by measuring OD600 and biomass. All results are typical results representing a series of experiments.

**Metabolite and flux analysis**

Extracellular metabolites were measured every hour by HPLC. 1 ml of culture was mixed with 100 μl 35% (v/v) cold PCA. After 10 min., the PCA was precipitated by adding 55 μl 7 M KOH. After centrifugation, the supernatant was filtered and analyzed for glucose, ethanol, glycerol, acetate, succinate, phosphate and pyruvate by HPLC (Phenomenex type Rezex Organic Acid column; eluent, 7.2 mM H2SO4 at 45º C). From the data on changes in dry weight and on metabolites the specific flux of compound x during a given time span could be calculated, using the following formula:

\[
q_x = \frac{[x](\Delta t)}{\text{average}DW} \]  

expressed in mmol·g dry weight\(^{-1}\)·h\(^{-1}\). 

Biomass yield on glucose (\(Y_{\text{glc}, \text{g dry weight,g glucose}^{-1}}\)) was calculated from 

\[
Y = 1000.(\mu - q_{\text{glc}}^{-1})/180 
\]

and is presented in percentages.
The $Y_{\text{ATP}}$ value was calculated assuming that net 1 mole ATP per mole ethanol produced was synthesized and that 2.4 mole of ATP per mole of $O_2$ reduced (Verduyn, et al., 1991) was synthesized. The following relations were used:

$$q_{\text{ATP}} = q_{\text{ethanol}} + 2.4 \cdot q_{O_2} \text{ (mmol•g dry weight}^{-1} \text{•h}^{-1})$$

and

$$Y_{\text{ATP}} = \mu \cdot q_{\text{ATP}} \text{ (g•mol}^{-1})$$

**Preparation of total RNA and labeled cRNA**

Samples for RNA isolation were collected from a batch fermentor’s culture at early-mid log phase, flash-frozen in liquid nitrogen and stored at $-80^\circ$C. Total RNA was extracted using the method of Llinas, in the same way as was done by Zakrzewska and co-workers (Zakrzewska, et al., 2005). The concentration and quality of RNA was 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). Twenty μg of total RNA 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 was tested using a Nanodrop spectrophotometer. Subsequently, the cRNA fragmentation reaction was carried out according to the manufacturer’s protocol. The degree of fragmentation was confirmed with RNA LabChip on a 2100 Bioanalyzer from Agilent Technologies. The samples were stored at $-20^\circ$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 6400 $S.\text{cerevisiae}$ 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 substraction 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 by Zakrzewska (Zakrzewska, et al., 2005).

**T-profiler analysis of DNA microarray data**

To assess the contribution of the expression of genes from specific gene classes to the total gene expression, T-profiler was used (Boorsma, et al.). This algorithm uses an unpaired t test to classify a difference between the mean of a set of a specific class of genes and the mean of the remaining genes of the total gene expression profile. We have looked at classes of genes containing a similar promoter element. The elements used are within 600 basepairs of the start codon and are based on literature and computational analysis results. GO: Ontology (http://www.yeastgenome.org) has been used to classify genes into specific categories. Further analysis was done to see whether specific GO categories are overrepresented within our arrays. The experiments are separately analyzed and no arbitrary cutoffs were applied prior to T-profiler analysis. T-profiler and additional information can be found on http://www.t-profiler.org.

**Results**

**Physiological characterization**

In order to characterize the effects of mutations in the glycolytic regulatory network on the physiological and transcriptional level, wild type and mutant cells were grown in batch fermentors and growth was followed from the early to mid-log phase. During the growth phase samples were withdrawn and quickly quenched (see material and methods) to determine metabolite concentrations. Off-gas was analyzed for CO₂ and O₂ just prior to sampling for micro-arrays. From these data the physiological parameters as given in Table 1 were calculated. At an OD₆₀₀ of approximately 1, the cells were harvested for micro-array analysis. The WT strain was found to grow on the medium used (see methods and materials) at a maximal specific growth rate (μ) of 0.36 h⁻¹, with a respiratory quotient (RQ, defined as the q_CO₂/q_O₂ ratio and indicative for the fermentative catabolism over the oxidative catabolism ratio with a value of approx. 1 representing fully oxidative metabolism) of 3.4, showing both respiration and fermentation. A biomass yield on glucose of 18 % was calculated. The data obtained for the HAP4Δ strain are in accordance with a previous report (Blom, et al., 2000): it showed a similar growth rate as the wild-type, with a slight reduction in ethanol production, glucose consumption and RQ, resulting in a biomass yield value on glucose that is 30% higher than the value found for the wild type. The absence of the other major global glycolytic regulator, HXK2, resulted on the one hand in a growth rate that was significantly lower (0.26 h⁻¹) but on the other hand in a much higher catabolic efficiency, i.e. catabolism was shifted towards respiration as can be deduced from the lower RQ ratio. Indeed, under the conditions tested this strain showed nearly full respiration, concomitant with a doubling of the biomass yield on glucose. Virtually maximal energetic efficiency, i.e. a completely oxidative catabolism resulting in the highest energy conservation rate (Bruinenberg,
1986), with little decrease in growth rate capacity, was obtained with the hsk2ΔHAP4 strain. This strain had a growth rate of 0.31 h⁻¹ and an insignificant ethanol flux and hence an even higher biomass yield of 47%, which is comparable to values found for fully respiring wild type cultures, e.g. as found in glucose-limited chemostat cultures (Bruinenberg, 1986). For all strains, a $Y_{\text{ATP}}$ value of 11.5 ± 1.0 g•mol⁻¹ was calculated assuming a P/O ratio of 1.2 (Verduyn, et al., 1991). Carbon balances were consistently found to be 110±/- 1% and therefore suggest a technical error. Even if this is due to a systematic overestimation of the gas flow analysis, none of the calculations can be affected to any significant extent.

Table 1: Physiological characteristics of the wild type and the mutants as determined in aerobic batch cultures.

<table>
<thead>
<tr>
<th>Strain</th>
<th>$\mu$ (h⁻¹)</th>
<th>$Q_{\text{ext}}$ (mmol g·dw⁻¹ h⁻¹)</th>
<th>$Q_{\text{C2}}$ (mmol g·dw⁻¹ h⁻¹)</th>
<th>$Y_{\text{ATP}}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt</td>
<td>0.26</td>
<td>17.00</td>
<td>7.16</td>
<td>3.4</td>
</tr>
<tr>
<td>hsk2</td>
<td>0.26</td>
<td>2.40</td>
<td>8.43</td>
<td>1.3</td>
</tr>
<tr>
<td>hap4</td>
<td>0.35</td>
<td>11.30</td>
<td>6.04</td>
<td>2.9</td>
</tr>
<tr>
<td>hsk2hap4</td>
<td>0.31</td>
<td>0.84</td>
<td>9.95</td>
<td>1.1</td>
</tr>
</tbody>
</table>

The mutants and the wild-type were grown in aerobic cultures in batch-fermentors as described in Methods and Materials. Fluxes and the maximal specific growth rate were determined during early log growth. The data present typical results as observed for various time spans for one growth experiment and for separate growth experiments. No other products were detectable. The given values are the mean of two independent experiments.

**Transcriptome analysis**

Transcript profiles were made of samples taken from the mid-log phase at an optical density between 0.8 and 1.0. A comparative analysis of these profiles of the mutants and the wild type strain was carried out. With respect to the central carbon metabolism, (sugar transport, glycolysis, the pentose phosphate pathway, the tricarboxylic acid cycle and the glyoxylate cycle, shown in figure 1) the following interesting points can be seen. In strains lacking HXX2, an upregulation of expression of the high affinity glucose transporters HXT6 and HXT7, and a concomitant decrease in expression of the low affinity transporter HXT1 is seen. Expression of HXT4, a glucose transporter with a rather high affinity increased as well, whereas HXT3 (medium to low affinity) mRNA levels remained unchanged. Apparently, the modification of the regulatory network results in a shift from low-affinity glucose transport to high affinity glucose transport. In addition HXT5 is upregulated in the hsk2 strains, whereas under these conditions HXT5 is completely shut off in the wild-type.

Quite in contrast to the significant changes in the glucose transport machinery, it is seen that the expression of most glycolytic genes is hardly changed by the modification of the level of the general regulators. The only exception seems to be the expression levels of the sugar phosphorylating enzymes HXX1 and (to a minor extent) GLK1, both being strongly upregulated when HXX2 is absent, but not when only HAP4 is overproduced.
Figure 1: Expression of individual genes within glycolysis/gluconeogenesis, the PP pathway, the glyoxylate cycle and the TCA cycle. Transcriptome data from the early log phase of the mutants and the wild-type were normalized and expression ratios were calculated relative to the Wild-type. Shown here are the expression ratio’s of most of genes involved in the depicted pathways. The values are expressed as log2-ratio’s and are relative to the Wild-type. The first squares show the HAP4/g313/WT ratio, the middle squares shows the hxk2/g39/WT ratio and the right squares show the hxk2ΔHAP4/g313/WT ratio. The given values are the mean of two independent experiments.
Further noteworthy changes in the upper part of glycolysis include upregulation of PFK1 and PFK2 on the one hand and downregulation of ENO1, both in the hxk2A/HAP4 χ strain. More dramatic effects of altered levels of HAP4p and/or hxk2p are observed for the lower part of glycolysis; here, of all the alterations, the change in expression of ADH2 in the hxk2A/HAP4 χ strain stands out most. It should be noted that ADH2 is the alcohol dehydrogenase that in the wild type only is expressed after glucose depletion and is associated with gluconeogenic growth. As exemplified by ADH2 and also FBP1 there is an upregulation of genes associated with gluconeogenic growth in strains lacking hxk2p but not in the strain that only overproduces HAP4p. PDC5, ADH4 and ADH5 are also downregulated in the hxk2A strains. ALD4 is strongly upregulated in the latter cells, presumably to facilitate strains resshuttling acetate into the TCA-cycle. The other ALD genes remain mainly constant with the exception of a 2-fold downregulation of ALD3 in the hxk2A strain.

Figure 2: Expression of individual genes within the respiratory chain. Transcriptome data from the early log phase of the mutants and the wild-type were normalized and expression ratios were calculated relative to the Wild-type. Shown here are the expression ratio’s of most of genes involved in the depicted pathways. The values are expressed as log2-ratio’s and are relative to the Wild-type. The first squares show the HAP4/g313/WT ratio, the middle squares show the hxk2/g39/WT ratio and the right squares show the hxk2/g39 HAP4/g313/WT ratio. The given values are the mean of two independent experiments.

In line with the physiological data, there is a huge increase in mRNA levels of genes encoding the TCA cycle enzymes. In each mutant many of these genes are strongly upregulated. Also in most cases it is a gene encoding a single iso-enzyme, which is strongly increased, whereas the other remains unchanged, due to different repressors and/or inductors interacting with a specific iso-enzyme only. In addition, a strong upregulation of almost all those genes that are involved in oxidative phosphorylation and expressed from the nucleus is observed. As shown in figure 2, although some specific genes remain expressed at the same level, the total expression levels of genes related to each individual component of the respiratory chain (NADH dehydrogenase, complex II, III and IV) and the ATP synthetase is increased. This correlates well with the increased
Chapter 2  
Physiological and transcriptional characterization

respiration and to the lowering of ethanol production. Also genes in the glyoxylate cycle were upregulated primarily in the hsk2Δ1 strains, most likely enabling use of fermentation products to re-enter the respiratory chain.

Regulatory Motifs (T-profiler)

A T-profiler analysis was carried out to score the activity of gene groups based on shared regulatory motifs. Essentially, this analysis quantifies the correlation between changes in expression of genes sharing common DNA motifs in their promoter region. Statistical analysis yields so-called t-scores to be assigned to such a group of genes with a positive value indicating that these genes are upregulated on average. A p-value is assigned by comparing the amount of genes in a group against the total amount of genes. A p-value below 0.05 is considered to be significant. A detailed description of the analysis can be found in Boorsma et al. 2005.

Table 2: Regulatory motifs identified using T-profiler.

<table>
<thead>
<tr>
<th>Consensus motif</th>
<th>TF</th>
<th>t</th>
<th>ORFs</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsk2Δ1</td>
<td>CATB</td>
<td>-5.8</td>
<td>139</td>
</tr>
<tr>
<td>ccggnnnnccg</td>
<td>MIG1</td>
<td>+5.05</td>
<td>157</td>
</tr>
<tr>
<td>ccaatca</td>
<td>HAP4</td>
<td>+4.15</td>
<td>126</td>
</tr>
<tr>
<td>cggggta</td>
<td>ORE</td>
<td>+4.13</td>
<td>124</td>
</tr>
<tr>
<td>caawwwwtag</td>
<td>RUM1</td>
<td>-4.19</td>
<td>97</td>
</tr>
<tr>
<td>tcggtta</td>
<td>UPC2</td>
<td>-4.56</td>
<td>245</td>
</tr>
<tr>
<td>niaccctd</td>
<td>RCS1</td>
<td>-4.61</td>
<td>453</td>
</tr>
<tr>
<td>acnnnnnggt</td>
<td>ZAP1</td>
<td>-5.38</td>
<td>195</td>
</tr>
<tr>
<td>hsk2Δ1</td>
<td>MIG1</td>
<td>+5.26</td>
<td>26</td>
</tr>
<tr>
<td>ccaatca</td>
<td>HAP4</td>
<td>+4.47</td>
<td>126</td>
</tr>
<tr>
<td>cgatgag</td>
<td>PAC</td>
<td>+4.35</td>
<td>245</td>
</tr>
<tr>
<td>cccnnnnwwwv</td>
<td>MIG1</td>
<td>+4.23</td>
<td>157</td>
</tr>
<tr>
<td>acnnnnnggt</td>
<td>ZAP1</td>
<td>-4.04</td>
<td>195</td>
</tr>
<tr>
<td>hsk2Δ1</td>
<td>UPC2</td>
<td>-4.08</td>
<td>245</td>
</tr>
<tr>
<td>HAP4</td>
<td>HAP4</td>
<td>+5.41</td>
<td>126</td>
</tr>
<tr>
<td>ccaatca</td>
<td>PAC</td>
<td>+5.3</td>
<td>245</td>
</tr>
<tr>
<td>acnnnnnggt</td>
<td>ZAP1</td>
<td>-7.48</td>
<td>195</td>
</tr>
</tbody>
</table>

Transcriptome data from the early log phase of the mutants and the wild-type were normalized and expression ratios were calculated relative to the Wild-type. These data were used in T-profiler to identify common motifs in promoter regions that appear to contribute more to expression than the mean. All the motifs that scored at least a t-score of -4 or 4, in at least one of the mutants tested are shown.
Using T-profiler, it was found that the $\text{hxk2}\Delta$ and $\text{HAP4}^\top$ mutant strains had increased levels of transcription of genes containing the general transcriptional activator motifs, known to be bound by PAC. The largest contribution to this increase was found to be due to genes needed for mitochondrial biogenesis and function. As expected we found the HAP4 (CCAATCA) motif in all the mutants and the MIG1 (CGGGGTA) motif was found in all strains, except in the $\text{HAP4}^\top$ strain (see table 2). Further, we found strong contributions of the CAT8 and ORE (oleate responsive element) motifs in the $\text{hxk2}\Delta\text{HAP4}^\top$ strain, but surprisingly these motifs are not significantly present in either the $\text{hxk2}\Delta$ or $\text{HAP4}^\top$ strains. These two motifs are associated with the derepressed status as found during growth on poor carbon sources or after the diauxic shift. Most surprising, the transcriptomes of the tested mutants showed a very strong negative contribution of the transcription factor of ZAP1, a transcription factor controlling zinc transport and homeostasis.

**Analysis of non-redundant functional categories**

A further analysis was carried out by using the GO Ontology categories in T-profiler. This methodology analyzes the extent to which genes within assigned functional categories are coregulated. Assignments may be based on the GO, MIPS or KEGG databases. A T-profiler analysis is presented based on the GO Ontology database in table 3. A strong positive upregulation for both the mitochondrial category and oxidative phosphorylation for all the strains was observed, again showing the direct involvement of HAP4 in this process. Similarly a strong upregulation of the hexose transporter category in all the strains (except the $\text{HAP4}^\top$ strain) was seen, mainly due to the induction of high-affinity hexose transporters ($\text{HXT6-7}$). Further, glucose catabolism is significantly downregulated as a whole in the $\text{hxk2}\Delta$ strains, but not in the other strains. This suggests that $\text{HAP4}^\top$ does not interfere with glucose catabolism as a functional group and that it is dependent on other regulators under the control of Hxk2p. Finally, it should be noted that the finding of downregulation of genes under control of Zap1p and Rcs1p, is supported by a significant downregulation of the categories involved in transition metal ion transport and siderochrome transport, respectively in the $\text{hxk2}\Delta$ and in the $\text{hxk2}\Delta\text{HAP}$ $^\top$ mutants.
Table 3: Functional categories according to GO Ontology contributing to the changes in expression identified using T-profiler.

<table>
<thead>
<tr>
<th>GO Ontology category</th>
<th>t</th>
<th>ORFs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondrion</td>
<td>+29.88</td>
<td>624</td>
</tr>
<tr>
<td>Organelar ribosome</td>
<td>+15.8</td>
<td>73</td>
</tr>
<tr>
<td>Nucleolus</td>
<td>+10.32</td>
<td>189</td>
</tr>
<tr>
<td>Oxidative phosphorylation</td>
<td>+10.18</td>
<td>27</td>
</tr>
<tr>
<td>Ribonucleoprotein complex</td>
<td>+4.85</td>
<td>367</td>
</tr>
<tr>
<td>Mitochondrial electron transport chain</td>
<td>+4.68</td>
<td>21</td>
</tr>
<tr>
<td>Cellular component unknown</td>
<td>-6.21</td>
<td>749</td>
</tr>
<tr>
<td>hox2.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondrion</td>
<td>+17.36</td>
<td>624</td>
</tr>
<tr>
<td>Organelar ribosome</td>
<td>-9.92</td>
<td>73</td>
</tr>
<tr>
<td>Nucleolus</td>
<td>-9.79</td>
<td>189</td>
</tr>
<tr>
<td>Hexose transport</td>
<td>-8.11</td>
<td>16</td>
</tr>
<tr>
<td>Oxidative phosphorylation</td>
<td>-6.99</td>
<td>27</td>
</tr>
<tr>
<td>Main pathways of carbohydrate metabolism</td>
<td>+4.86</td>
<td>65</td>
</tr>
<tr>
<td>Energy pathways</td>
<td>+4.83</td>
<td>180</td>
</tr>
<tr>
<td>Hydrolyase activity, hydrolyzing O-glycosyl compounds</td>
<td>-4.48</td>
<td>31</td>
</tr>
<tr>
<td>Glucose catabolism</td>
<td>-4.15</td>
<td>23</td>
</tr>
<tr>
<td>Amino acid and derivative metabolism</td>
<td>-6.42</td>
<td>186</td>
</tr>
<tr>
<td>Siderochrome transport</td>
<td>-6.89</td>
<td>9</td>
</tr>
<tr>
<td>hox2.3 : HAP4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondrion</td>
<td>+21.37</td>
<td>624</td>
</tr>
<tr>
<td>Organelar ribosome</td>
<td>-8.2</td>
<td>73</td>
</tr>
<tr>
<td>Peroxisomal matrix</td>
<td>+6.98</td>
<td>14</td>
</tr>
<tr>
<td>Hexose transport</td>
<td>-5.59</td>
<td>16</td>
</tr>
<tr>
<td>Cytoplasm organization and biogenesis</td>
<td>+5.09</td>
<td>670</td>
</tr>
<tr>
<td>Electron transport</td>
<td>+5.06</td>
<td>18</td>
</tr>
<tr>
<td>Carbohydrate metabolism</td>
<td>-4.62</td>
<td>182</td>
</tr>
<tr>
<td>Glucose catabolism</td>
<td>-4.32</td>
<td>23</td>
</tr>
<tr>
<td>Transition metal ion transport</td>
<td>-4.96</td>
<td>36</td>
</tr>
<tr>
<td>Siderochrome transport</td>
<td>-7.34</td>
<td>9</td>
</tr>
</tbody>
</table>

Transcriptome data from the early log phase of the mutants and the wild-type were normalized and expression ratios were calculated relative to the Wild-type. These data were used in Quontology to identify functional categories that appear to contribute more to expression than the mean. Shown are all the functional categories according to GO Ontology, which scored at least a t-score of -4 or 4, in at least one of the mutants tested. t-scores of 4 or more and -4 or less are considered significant.
Chapter 2  Physiological and transcriptional characterization

Discussion

The rather subtle regulation of glucose catabolism in *Saccharomyces cerevisiae* is brought about by a complex regulatory network that still is a challenge to understand, despite the fact that it has been a subject of many studies. Here we have quantified the effects of two important catabolic regulators, Hap4p and Hxk2p on the physiology of *S. cerevisiae*. Their overproduction, respectively absence, results in a major redistribution of the carbon flux over fermentative and respiratory pathways. Moreover, their effects are to some extent additive: both in the *hxk2Δ* and the *HAP4Δ* mutant a shift to respiratory catabolism is seen under excess glucose conditions and this is the case even more in the *hxk2ΔHAP4Δ* mutant. Accordingly, the increased energetic efficiency due to respiration results in considerable gain in the biomass yield on glucose (up to 3-fold). Increased yield values on the energy source may either indicate a more efficient mode of energy conservation or a lowered energetic demand for biosynthesis and maintenance. As there is no reason to assume the latter (cell composition presumably does not change and growth conditions being identical), the same Y_ATP value for the various strains should be obtained. Assuming a P/O ratio of 1.2 (Verduyn, et al., 1991), indeed, for all strains a value of 11.5 +/- 1 g•mol^{-1} was calculated.

Although under the conditions tested here, the growth rate in the *hxk2Δ* strain was significantly affected, it has also been shown that under other conditions (glucose-limited chemostat cultures with simple salt medium) this mutant can grow at a similar rate as the wild type. Apparently the mechanisms to maintain a high μ_max are less robust in this strain and it has been shown indeed that the μ_max of *hxk2Δ* strain depends on the external glucose concentration (Diderich, et al., 2002). In addition, *HAP4* overexpression appears to restore the growth rate to almost that of the wild-type. The *HAP4Δ* strain expresses an increased amount of respiratory genes, though this increase is not as massive under these conditions as in the *hxk2Δ* strain. The additive effect of overexpression of *HAP4* in the *hxk2Δ* background suggests that in the latter background the genes needed for mitochondrial biogenesis and function are not induced to their fullest extent, and thus can be increased even more by addition of more active *HAP4*. This increased respiratory capacity may cause the μ_max to increase.

Many of the physiological effects seen in the various strains are in good agreement with the analysis of the transcriptome profiles. Thus, the upregulation of gene expression related to the TCA cycle and oxidative phosphorylation corresponds with the increased respiration. More complex relations are seen with respect to the large changes in the glucose transport step, with a clear shift from low affinity transport to high affinity transport. The fact that this shift is absent in the *HAP4Δ* strain could be explained by induction of the high affinity transport system(s) being brought about by relieving the repression due to increased phosphorylation levels of Mig1p and Mig2p (indirectly caused by the absence of Hxk2p) (Treitel, et al., 1998). Downregulation of low-affinity transport could be interpreted as a response to the increased expression of high-affinity transporters. It can not be excluded though that the control of the glucose sensors Rgt2p/Snf3p may be altered or absent due to this mutation, resulting in a low glucose signal regardless of the extracellular glucose concentration.

It should be emphasized that observed changes in expression levels are not necessarily due to a direct (absence of) interaction of the regulator per se: if a regulator acts upstream
in the catabolic pathways, the resulting changes in metabolite concentrations may in turn affect expression of catabolic genes encoding enzymes anywhere in the pathway. For example, increased respiration may cause glycolysis to be somewhat downregulated in all mutants by the accompanying changes in the redox state (or the NADH pool) and the energy state (or the ATP and ADP pool).

Interestingly, both the absence of hxk2p and the overexpression of hap4p results in a very significant increase in genes associated with growth on non-fermentable carbon sources, e.g. \(ADH2\) and \(FBP1\). This suggests that in the \(hxk2\) mutants glycolysis and gluconeogenetic enzymes, the glyoxylate cycle and the TCA cycle are all present and may be active given proper intermediate levels. In this connection it should be mentioned that co-consumption of ethanol and glucose in the \(hxk2\) strain has been reported. (Raamsdonk, et al., 2001). The occurrence of simultaneous expression of genes involved in processes with opposing functions but considered to be in the same categories illustrates the need for proper defining functional categories (Koerkamp, et al., 2002).

With respect to the PP Pathway, only minor changes in expression were observed. Mass flux analyses showed that the flux through the PP pathway is stoichiometrically related to the growth rates due to the need for NADPH as a biosynthetic redox carrier (Maaheimo, et al., 2001) As there are minor changes in growth rate for the various strains tested, PP-fluxes should have changed according to this relationship, however gene expression of the PP Pathway has not significantly changed, suggesting that the necessary changes are either too small to be noted experimentally or they are not regulated at the transcriptional level. More drastic changes where seen for the glyoxylate cycle. Whereas in the wild-type this cycle seems almost non-active during growth on glucose, the \(hxk2Δ\) strains show an increased expression level of most of the genes involved. Moreover only in the \(hxk2ΔHAP4\) strain there’s a strong induction of \(ACS1\) which is needed for formation of Acetyl-CoA to be used either in the TCA-cycle or in the glyoxylate cycle, which also suggests this cycle is active in both strains and is actually both producing and consuming ethanol simultaneously, as has been reported earlier (Raamsdonk et al. 2001)

On a more global scale we found many DNA-motifs contained in promoter regions of genes changed in expression according to expectation. For instance genes with the CCAATCA (Hap2/3/4/5p) motif were mostly upregulated in all the mutants. On the other hand, genes containing the Mig1 specific CGGGGTA motif were mostly upregulated in all the strains, except the \(HAP4\) strain. These findings are well in agreement with the proposed regulatory network involving Mig1p and Hap4p.

A surprisingly strong negative contribution was seen for the motif known to be bound by the Zap1p regulator, which controls zinc transport and zinc homeostasis. This suggests that there is a much lower demand for Zn in strains that overexpress \(HAP4\). In accordance, it is observed that the zinc-dependent alcohol dehydrogenase \(ADH4\) is downregulated in this strain. In addition, the manganese dependent superoxide dismutase \(SOD2\) was upregulated, thereby diminishing the need for the zinc-dependent \(SOD1\), although it should be mentioned that the latter was not found to be downregulated.

In general, the results point towards an additive effect of the two regulators studied. Again specifically for the \(hxk2Δ\) and \(hxk2ΔHAP4\) strains we found categories associated with growth on alternative carbon sources, such as galactose and also maltose. All the above suggests that glucose repression, and preference for glucose as the carbon and energy source are completely abolished in strains lacking Hxk2p. In other words, the
HXK2 gene is crucial in maintaining glucose repression and its activity forms the basis for the Crabtree-positive phenotype. The absence of repression, respiratory activity and co-consumption of other carbon sources than glucose, combined with the maintenance of high affinity glucose transport characteristics under excess glucose conditions justifies the conclusion that a lack of Hxk2p renders *S. cerevisiae* rather inapt to respond appropriately to extracellular glucose and turns *S. cerevisiae* in a non-adaptive yeast. Our data indicate that the mechanism of control by Hap4p is quite different. Changed levels of this regulator do decrease the Crabtree-positive behavior but do not affect glucose sensitivity. The effect of increased energetic efficiency seems to be solely due to increased expression levels, and hence capacity, of oxidative catabolism.
Chapter 3

The effect of \textit{hxk2} deletion and \textit{HAP4} overexpression on fermentative capacity in \textit{S. cerevisiae}

J. Merijn Schuurmans$^1$, Sergio L Rossell$^2$, Arjen van Tuijl$^2$, Barbara M. Bakker$^2$, Klaas J. Hellingwerf$^1$ and M. Joost Teixeira de Mattos$^1$

$^1$Department of Molecular Microbial Physiology, Swammerdam Institute of Life Sciences, University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV Amsterdam, The Netherlands.

$^2$Department of Molecular Cell Physiology, Vrije Universiteit, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands

Published in FEMS Yeast Research (2008) 8: 195-203
Chapter 3  Effects on fermentative capacity

Abstract

To describe the fermentative potential of a yeast cell, the fermentative capacity (FC) has been defined as the specific rate of ethanol and CO₂ 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 CO₂ and/or ethanol are the preferred end products: this is the case e.g. during the raising of dough where the yeast should ferment sugars to CO₂ with minimal increase in biomass. To quantify the potential to produce CO₂ 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 (*HAP4Δ*). 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·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 CO₂ and O₂ 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 H₂SO₄ 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 N₂-gas at a flow rate of approximately 50 ml·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 MgCl₂ 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 °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 °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).
Chapter 3 Effects on fermentative capacity

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 °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 μmol·min⁻¹·(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 ¹⁴C 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, hck2Δ and HAP4 strains.

<table>
<thead>
<tr>
<th>Strains</th>
<th>qGlucose (mmol·g⁻¹·h⁻¹)</th>
<th>qEthanol (mmol·g⁻¹·h⁻¹)</th>
<th>qCO₂ (mmol·g⁻¹·h⁻¹)</th>
<th>qO₂ (mmol·g⁻¹·h⁻¹)</th>
<th>YGlucose (g/g)</th>
<th>C%</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.1</td>
<td>1.1</td>
<td>0</td>
<td>2.6</td>
<td>2.8</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>0.31</td>
<td>3.4</td>
<td>0</td>
<td>8.1</td>
<td>8.8</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>0.32</td>
<td>4.4</td>
<td>7.1</td>
<td>9.5</td>
<td>9.5</td>
<td>0.41</td>
</tr>
<tr>
<td>hck2Δ</td>
<td>0.1</td>
<td>1.1</td>
<td>0</td>
<td>2.2</td>
<td>2.5</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>0.31</td>
<td>3.2</td>
<td>0</td>
<td>6.2</td>
<td>6.2</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>0.32</td>
<td>4.4</td>
<td>8.5</td>
<td>8.9</td>
<td>8.9</td>
<td>0.53</td>
</tr>
<tr>
<td>HAP4</td>
<td>0.1</td>
<td>1.1</td>
<td>0</td>
<td>3.0</td>
<td>3.0</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>0.31</td>
<td>3.1</td>
<td>7.6</td>
<td>7.6</td>
<td>7.6</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>0.32</td>
<td>4.3</td>
<td>7.7</td>
<td>9.7</td>
<td>9.7</td>
<td>0.42</td>
</tr>
</tbody>
</table>

Strains were grown in glucose-limited aerobic chemostats at various dilution rates. Fluxes were determined for glucose, ethanol, CO₂ and O₂. The biomass yield on glucose and the carbon balance are also shown. Shown are the means of two independent experiments.
Chapter 3 Effects on fermentative capacity

The CEN.PK113-7D (wild-type), \( \text{hsk2} \Delta \) and \( \text{HAP4}^+ \) 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 \(\text{HAP4}^+\) turned out to be 0.32 h\(^{-1}\): both strains catabolize glucose at this growth rate respire-fermentatively with a concomitant drop in \(Y_{\text{glucose}}\) of 20%. The \(\text{hsk2} \Delta\) 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 \(\text{hsk2} \Delta\) 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\(_{\text{glucose}}\)) for both the wild-type and the \(\text{HAP4}^+\) strain hardly changed with changing growth rates whereas the FC\(_{\text{glucose}}\) of the \(\text{hsk2} \Delta\) 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, \(\text{hsk2} \Delta\) and \(\text{HAP4}^+\) strains. Left: FC\(_{\text{glucose}}\) of the wild-type (blue) \(\text{hsk2} \Delta\) (red) and \(\text{HAP4}^+\) (yellow) strains. Right: FC\(_{\text{maltose}}\) of the wild-type (blue) \(\text{hsk2} \Delta\) (red) and \(\text{HAP4}^+\) (yellow) strains. The results of at least two independent experiments are shown, and the SD is shown in error bars.]](image)
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 ratios 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 $\text{HAP4}^{\Delta}/\text{WT}$ ratio and the right squares show the $\text{hxk2}^{\Delta}/\text{WT}$ ratio. The given values are the mean of two independent experiments.
Chapter 3

Effects on fermentative capacity

Even more remarkable is the observation that deletion of \( hsk2 \) 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\textsubscript{maltose}) upon transfer from glucose-limited growth conditions to the FC assay with maltose whereas the \( hsk2 \Delta \) strain was able to catabolize this sugar immediately at a rate that was some 70\% of the FC\textsubscript{glucose} of the wild type. In addition, the FC\textsubscript{maltose} did not change with growth rate. In contrast, the behavior on maltose of the \( HAP4^+ \) strain was opposite to that of the \( hsk2 \Delta \) strain (figure 1) as that it expressed a lower FC\textsubscript{maltose} than the wild-type.

Further comparative studies were done with the wild-type and \( hsk2 \Delta \) 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\textsubscript{maltose} incubation, after 30 min. and after 60 min. of FC\textsubscript{maltose} incubation. To express the differences in FC\textsubscript{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 \( hsk2 \), the \( hsk2 \Delta \) 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 \( hsk2 \Delta \) strain, from alcohol as substrate, this cannot explain the difference in FC\textsubscript{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 \textit{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 \( \mu \text{mol}\text{ min}^{-1}\text{ mg DW}^{-1} \) opposed to a 0.1-3 \( \mu \text{mol}\text{ min}^{-1}\text{ mg DW}^{-1} \) 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 \textit{in situ} flux. e.g. for PFK (wild-type prior to FC incubation) the \( V_{max} \) is 0.22 \( \mu \text{mol}\text{ min}^{-1}\text{ mg DW}^{-1} \), which corresponds to 13.2 \( \mu \text{mol}\text{ h}^{-1}\text{ mg DW}^{-1} \), whereas the \textit{in situ} flux is 4.4 \( \mu \text{mol}\text{ h}^{-1}\text{ mg DW}^{-1} \). HXK activity is much lower in the \( hsk2 \Delta \) 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 \textit{in vivo} flux, (7.8 \( \mu \text{mol}\text{ h}^{-1}\text{ mg DW}^{-1} \) versus 3.4 \( \mu \text{mol}\text{ h}^{-1}\text{ mg DW}^{-1} \) 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.

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 \(V_{\text{max}}\) 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 \textit{hxk2\textgreek{g}39\textgreek{g}39} strain (b). Samples were obtained from glucose-limited aerobic cultures at a dilution rate of 0.32 h\(^{-1}\). Velocities are shown in nmol\(\text{min}^{-1}\cdot\text{mg prot.}^{-1}\), the affinity constants are shown in mM. SE (standard error) \textit{hxk2\textgreek{g}39\textgreek{g}39} \textit{V}_{\text{max}}1 = 35.9, \textit{V}_{\text{max}}2 = 32.5, \textit{K}_{\text{m}1} = 0.19, \textit{K}_{\text{m}2} = 8.2. \text{SE Wild Type} \textit{V}_{\text{max}}1 = 14.0, \textit{K}_{\text{m}} = 0.24

The overall transport is characterized as a high-affinity system (\textit{K}_{\text{m}, \text{glucose}} = 1.2 mM), which is most likely solely attributable to Hxt6p-Hxt7p. In contrast, the \textit{hxk2\textgreek{g}39\textgreek{g}39} strain shows a two-component system, with a \textit{K}_{\text{m}, \text{glucose}} of 14.1 mM, suggesting significant contribution of the Hxt2-transporter and a very high-affinity component with a \textit{K}_{\text{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 $V_{\text{max}}$ for glucose transport of both strains does not vary greatly, the different values for the $FC_{\text{glucose}}$ 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 $hxk2\Delta$ 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 $hxk2\Delta$ strain from the start of maltose incubation. These differences can account for the exceptional performance of the $hxk2\Delta$ 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 $hxk2\Delta$ 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 $hxk2\Delta$ and $HAP4\Delta$ 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 $hxk$ 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 \( \text{hxk2} \) strain was observed, mostly due to the deletion of one of the hexokinase iso-enzymes the alternative phosphorylating enzymes, \( \text{GLK1} \) and \( \text{HXK1} \), still can account for enough activity to sustain a higher glucose influx than observed \textit{in vivo}. This is true for all the other enzyme activities in the glycolytic path. Interestingly TPI activity was much higher than the other glycolytic 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-hydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP) in order to maintain redox neutrality. When the \( \text{hxk2} \) 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 \( \text{hxk2} \) 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 \( \text{hxk2} \) 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 \( \text{hxk2p} \) 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 \( \text{hxk2} \). 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 \( \text{hxk2} \) 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 \( \text{hxk2p} \) on the physiology of \textit{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.

\emph{Acknowledgment} We thank Alexander Lindenberg for his excellent technical support with the sugar transport assays
Chapter 4

A transcriptome analysis of an *HAP4* overexpression mutant under different physiological conditions

J. Merijn Schuurmans¹, Klaas J. Hellingwerf¹, Jack T. Pronk², Pascale Daran-Lapujade³, M. Joost Teixeira de Mattos⁴

¹Department of Molecular Microbial Physiology, Swammerdam Institute for Life Sciences, University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV Amsterdam, The Netherlands
²Department of Biotechnology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands
Abstract

In this chapter we analyze the effects of HAP4 overexpression on the transcriptome of Saccharomyces cerevisiae. Four specific conditions, namely batch growth ($\mu_{\max}$) nitrogen-limited growth ($\mu=0.1 \text{ h}^{-1}$) and glucose limited growth ($\mu=0.1$ and $0.32 \text{ h}^{-1}$) of both the wild-type and the HAP4 strain were analyzed. Besides the changes in central carbon metabolism, several target groups of genes under control of a specific regulator were identified. As expected the Hap4p element was overrepresented in conditions were cells were growing under glucose repressed conditions. The Mig1p element and the Cat8p elements were identified under conditions of glucose de-repressed growth. Both these elements are involved in regulation of transcription under conditions of glucose exhaustion whereas the Zap1p and Rcs1p elements (involved in zinc respectively iron homeostasis) were identified under quite varying conditions. The former was primarily present in batch grown wild-type cells but largely underrepresented in the HAP4 strain under the same conditions. The Rcs1p element was found in glucose limited HAP4 cultures at low growth rates and in the batch grown cultures compared to the other conditions. Additionally an Msn2-4p element was found under low-growth conditions ($\mu=0.1 \text{ h}^{-1}$). Together these results show an interesting overview of the specific effects on the transcriptome of Saccharomyces cerevisiae, when HAP4 is overexpressed.
Introduction

The ability of the yeast *Saccharomyces cerevisiae* to ferment sugars to ethanol and carbon dioxide even in the presence of oxygen has led to its wide use in applications, such as the brewing of beer and the raising of dough. However, this organism is also used in biotechnological processes concerned with the production of fine chemicals, specialties and other compounds, including proteins. In such processes, formation of ethanol is highly undesirable as it dramatically lowers the yield on glucose. Optimization of biomass yield has been attempted in many ways, mostly by searching for conditions or genetic modifications that lower ethanol production and increase the respiratory capacity of the organism. One way to achieve this is by altering the expression of genes encoding enzymes involved in respiration and fermentation. For instance Flikweert *et al.* (Flikweert, *et al.*, 1999) attempted to block flux towards ethanol by deleting the functional pyruvate decarboxylase genes. These strains however needed addition of acetate or ethanol to grow, which is not desirable in a production process. With the knowledge obtained the last decades with regard to the extent to which control over a metabolic pathway is distributed over the enzymes involved, it is to be foreseen that increasing the activity of a single enzyme in the pathway will hardly ever result in a similar increase in pathway fluxes due to redistribution of control (Schaaff, *et al.*, 1989). Indeed, studies on overexpressing glycolytic enzymes have shown this to be the case (Smits, *et al.*, 2000).

More promising is the approach where one alters the expression of global regulators (transcription factors) that control a certain pathway. In baker’s yeast, the HAP2/3/4/5 complex is such a global regulator controlling activation of the genes involved in respiration (de Winde and Grivell, 1993; Forsburg and Guarente, 1989). This occurs at very low levels of glucose or when glucose is depleted and respiration is the major catabolic mode and fermentation is virtually absent. Hap2p, Hap3p and Hap5p are members of this complex that are continuously expressed, and as such appear to be only structural components. Expression of the Hap4p activator, however, is controlled by the Mig1p and Mig2p repressors which respond negatively to the availability of glucose (Gancedo, 1998). Under high glucose conditions Hap4p is actively repressed and the protein is hardly present in the cell. When glucose availability becomes scarce, repression is relieved and Hap4p binds to the Hap2/3/5p components to form a complex, which activates genes that contain a CCAATCA like promoter element (Dang, *et al.*, 1994). These include both genes involved in the respiratory chain (e.g. Qcr8p) and in the TCA cycle (e.g. Mdh1p). This makes Hap4p the ideal candidate for overproduction as a tool to increase respiration as a whole, bypassing the stage of needing to adapt the levels of each glycolytic enzyme to relieve their control. Studies on a strain that overexpressed Hap4p indeed showed an increase in biomass yield on glucose and an increased respiratory capacity in batch cultures (Blom, *et al.*, 2000). Other studies showed changes in the respiratory capacity under various continuous conditions, but failed in relating this to a concomittant increase in biomass (van Maris, *et al.*, 2001). This is most likely due to strain and medium changes. Considering the complexity of regulation of glycolytic fluxes, of the expression levels of the genes involved therein and the kinetics of the glycolytic enzymes, it is to be expected that varying levels of Hap4p will have a severe effect on the transcriptome of the cell as a whole. Here we compare transcriptional profiles and
physiological data of a Hap4p overproducing strain with the wild-type under 4 different conditions in order to make a further step in the elucidation of the regulatory network underlying glycolysis in baker’s yeast.

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 in Van Maris et al. (van Maris, et al., 2001). For the data on a glucose-limited culture at a dilution rate of 0.32 h\(^{-1}\), strains were grown in laboratory fermenters (L.H. Engineering, Maidenhead, UK) under aerobic glucose-limited conditions. 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·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. The growth temperature was set at 30°C. 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 culture volume per minute. The cultures were stirred with double impellers (LH Engineering) at 750 rpm and were grown in continuous mode by steady addition of medium while the overflow medium was disposed as waste. After 6-7 volume refreshments, a steady-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 a paramagnetic oxygen analyzer type 1100 (Servomex, Crowborough, UK) and an IR gas analyzer type 4100 (Servomex, Crowborough, UK), respectively. For the batch cultivation of the CEN.PK113-7D and HAP4 overexpressing strain the following setup was used. The strains were precultured overnight at 30°C in 0.67% YNB 2% glucose 0.1 M phtalic acid, pH=5.0 using KOH. Cells were inoculated to an OD\(_{600}\) of 0.2 in the same medium in batch fermentors with a 0.5 liter working volume, aerated with 1 vvm and stirred at 500 rpm. Growth was followed in time both by measuring OD\(_{600}\) and biomass. For the glucose-limited and nitrogen-limited cultures standard conditions used in the Delft laboratory were used (Boer, et al., 2003). All results are typical results representing at least two identical of experimental setups.

Sample extraction and analysis

Samples taken for metabolite analysis were immediately treated with 35% cold (4°C) perchloric acid. Samples were 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 H₂SO₄ 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.

Preparation of total RNA and labeled cRNA

Samples for RNA isolation were collected each hour from a batch fermentor’s culture at appropriate time points, flash-frozen in liquid nitrogen and stored at –80°C. Total RNA was extracted using the method of Llinas, by the procedure described by Zakrzewska and co-workers (Zakrzewska, et al., 2005). The concentration and quality of RNA was 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). Twenty μg of total RNA 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 was tested using a Nanodrop spectrophotometer. Subsequently, the cRNA fragmentation reaction was carried out according to the manufacturer’s protocol. The degree of fragmentation was confirmed with RNA LabChip on a 2100 Bioanalyzer from Agilent Technologies. The samples were stored at –20°C prior to hybridization. For the continuous cultures at a dilution rate of 0.1 h⁻¹ the conditions described by Boer et al. 2003 were used (Boer, et al., 2003)

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 6400 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). For the continuous cultures at a dilution rate of 0.1 h⁻¹ the conditions described by Boer et al. were used (Boer, et al., 2003)
Data processing

DChip was used to normalize the raw data. This is a software package implementing model-based expression analysis of oligonucleotide arrays and several high-level analysis procedures (Li and Wong 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 by Zakrzewska et al. (Zakrzewska, et al., 2005). For the continuous cultures at a dilution rate of 0.1 h⁻¹ the conditions described by Boer et al. 2003 were used (Boer, et al., 2003).

T-profiler analysis of DNA microarray data

To assess the contribution of the expression of genes from specific gene classes to the total gene expression, T-profiler was used (Boorsma, et al., 2005). This algorithm uses an unpaired t test to classify a difference between the mean of a set of a specific class of genes and the mean of the remaining genes of the total gene expression profile. We have looked at classes of genes containing a similar promoter element. The elements used are within 600 basepairs of the start codon and are based on literature and computational analysis results. The experiments are separately analyzed and no arbitrary cutoffs were applied prior to T-profiler analysis. T-profiler and additional information can be found on http://www.t-profiler.org.

Results and Discussion

In this study four different cultivations were used to analyze the effects of HAP4 overexpression on the level of the transcriptome. Both wild type and the mutant were grown in batch culture and in chemostat culture under ammonium-limited respectively carbon-limited conditions at a specific growth rate of 0.1 h⁻¹. In addition, the mutant strains were grown glucose-limited at a specific growth rate of 0.32 h⁻¹ (being the critical growth rate where ethanol production begins).

Physiology

The most relevant physiological parameters of the strains grown under 4 different conditions are summarized in Table 1. The data, both physiological as transcriptional, presented for batch and glucose-limited at a dilution rate of 0.32 h⁻¹ are identical to Chapter 2 and Chapter 3 respectively. When wild-type and HAP4 are grown to steady-state in glucose-limited aerobic chemostat cultures at low dilution rate (0.1 h⁻¹), both strains show fully respiratory growth as can be deduced from the ratio of the specific CO₂ production rate and the specific O₂ consumption rate (qCO₂/qO₂, RQ) of approximately 1. This was confirmed by the absence of ethanol in the cultures. In accordance with the identical catabolic behaviour, under these conditions overproduction of hap4p has no
effect on the biomass yield on glucose ($Y_{glc}$). Under glucose limitation, both strains switched to respiro-fermentative catabolism at a dilution rate of 0.32 h\(^{-1}\) ($\mu_{\text{crit}}$ for the wild type is 0.31 h\(^{-1}\)) but with the wild type a significantly higher flux to ethanol, was seen. Surprisingly, this seemingly less efficient energy conservation catabolic mode was not reflected in the $Y_{glc}$ values (0.41 g•g\(^{-1}\) respectively 0.42 g•g\(^{-1}\)). A further anomaly was observed: even though the $q_{\text{ethanol}}$ of the wild type significantly exceeds that of the HAP4 strain, the RQ values are quite close despite the fact that carbon recoveries were close to 100%.

In nitrogen-limited aerobic steady-state chemostat cultures, large differences are seen between the behaviour of the strains. Now, the wild-type shows respiro-fermentative catabolism, shuttling pyruvate to both the respiratory pathway and the fermentative pathway, as indicated by an RQ value of 2.9, with more emphasis on the fermentative pathway. Here no such anomalies as mentioned above were found. The effects on yield value are in accordance with what would be expected. The lower ATP/glucose stoichiometry of the wild type is reflected by an increased glucose consumption rate to meet the energy demands for growth and maintenance and therefore a lower Yield value (0.11 g•g\(^{-1}\) compared to 0.21 g•g\(^{-1}\) in the HAP4 strain).

Table 1: Physiology of the wild-type and the HAP4 strain.

<table>
<thead>
<tr>
<th></th>
<th>$\mu$ (h(^{-1}))</th>
<th>$q_{\text{glucose}}$ (mmol•g(^{-1})dw•h(^{-1}))</th>
<th>$q_{\text{ethanol}}$ (mmol•g(^{-1})dw•h(^{-1}))</th>
<th>RQ (q_{CO2}/q_{O2})</th>
<th>$Y_{glc}$ (g•g(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CEN.PK113-7D</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Climited</td>
<td>0.1</td>
<td>1.1</td>
<td>0.1</td>
<td>1.1</td>
<td>0.51</td>
</tr>
<tr>
<td>Nlimited</td>
<td>0.1</td>
<td>5.3</td>
<td>6.2</td>
<td>2.9</td>
<td>0.11</td>
</tr>
<tr>
<td>Climited 0.32</td>
<td>0.32</td>
<td>4.4</td>
<td>0.1</td>
<td>1.3</td>
<td>0.41</td>
</tr>
<tr>
<td>Batch</td>
<td>0.36</td>
<td>10.13</td>
<td>17</td>
<td>3.4</td>
<td>0.18</td>
</tr>
<tr>
<td><strong>HAP4</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Climited</td>
<td>0.1</td>
<td>1.1</td>
<td>0.1</td>
<td>1.1</td>
<td>0.52</td>
</tr>
<tr>
<td>Nlimited</td>
<td>0.1</td>
<td>2.6</td>
<td>1.1</td>
<td>2.9</td>
<td>0.21</td>
</tr>
<tr>
<td>Climited 0.32</td>
<td>0.32</td>
<td>4.3</td>
<td>2.7</td>
<td>1.4</td>
<td>0.42</td>
</tr>
<tr>
<td>Batch</td>
<td>0.35</td>
<td>7.41</td>
<td>11.3</td>
<td>2.9</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Strains were grown in glucose-limited aerobic chemostats at two dilution rates, in nitrogen-limited aerobic chemostats and in batch. Fluxes were determined for glucose, ethanol, CO2 and O2. The biomass yield on glucose is also shown. $q_{\text{CO2}}$ and $q_{\text{O2}}$ are not shown, instead the respiratory quotient (RQ) is shown. The given values are the mean of two independent experiments in the cases of Batch and C-limited 0.32 and three independent experiments in the cases of Climited and Nlimited.

Grown in aerobic batch cultures, the same effect of hap4p overproduction was observed as outlined above for nitrogen-limited aerobic conditions, be it less outspoken. No effect of overproduction was observed with respect to the specific growth rate ($\mu_{\text{max}}$ of 0.36 h\(^{-1}\) for the wild-type and a $\mu_{\text{max}}$ of 0.35 h\(^{-1}\) for the HAP4 strain) but again the RQ of the HAP4 strain was reduced compared to the wild-type due to lower specific ethanol production rate and hence a higher yield value (0.24 g•g\(^{-1}\) versus 0.18 g•g\(^{-1}\)).
Transcriptome analysis

From all conditions tested RNA samples were taken during steady-state (in chemostat cultures) or early-mid log phase (OD$_{600}$ ~ 1, in the batch culture). These RNA samples were treated according to the Affymetrix™ protocol and hybridized to Affymetrix™ YG-S98 Saccharomyces cerevisiae chips. Instead of using individual expression of genes, we used T-profiler to get a global overview of the events going on in these cells under the conditions applied. This analysis resulted in a few specific groups of genes that all have a common promoter element, which is associated with a transcriptional activator or repressor. Although T-profiler recognizes several promoter elements for the same transcriptional activator or repressor, which are usually complementary, only one element is shown each time. The first two elements that were checked were the Hap4p element CCAATCA and the Mig1p element TWCCCCM. These results are shown in figure 1.

A positive T-score of the Hap4p element shows that genes that contain the CCAATCA element are upregulated more or less as a whole module but it should be mentioned here that individual expression of genes belonging to the module may differ. Taking into account that stringent glucose-limited conditions result in HAP4 expression, it is not unexpected to see that only aerobic batch cultures and nitrogen-limited chemostat cultures show a positive contribution. The other conditions do not show such changes in Hap4p mediated expression. This is consistent with the observation that these conditions resulted in significantly different physiology between the strains.

Figure 1: T-scores for the promoter elements Hap4p and Mig1p. Shown are the significant T-scores for the CCAATCA (Hap4p) binding element and the TWCCCCM (Mig1p) binding element. Only scores with a corresponding e-value of <0.05% are shown.
Other cross-comparisons can be made, now for one strain with the various conditions tested. Some conditions turn out to have no significant t-score for Hap4p, even though there is a large change in respiratory activity (e.g. comparing the glucose-limited aerobic culture with the nitrogen-limited aerobic culture with both wild-types or both HAP4 strains.) and despite the fact that the elements are present in these cases. Another indicator for the respiratory activity is the transcriptional repressor Mig1p, which represses expression of respiratory genes and HAP4 itself. And indeed in the conditions where a Hap4p element was expected a negative t-score for the Mig1p element was found, indicating repressing conditions caused by binding of Mig1p to the promoter element and reducing expression as a whole. This then might also explain why under these conditions the Hap4p element is not found significant as it is under control of Mig1p. The conditions where the Hap4p element was found are all comparisons of HAP4 over the wild-type, and in the HAP4 overexpression strain a second copy of HAP4 is present which is not under control of Mig1p.

Figure 2: T-scores for the promoter elements Cat8p and Msn2-4p. Shown are the significant T-scores for the CCGNNNNCCG (Cat8p) binding element and the CCCT (Msn2-4p) binding element. Only scores with a corresponding e-value of <0.05% are shown.

T-profiler also significantly scored the Cat8p promoter element CCGNNNNCCG and the Msn2-4p promoter element CCCT and the results are shown in figure 2. The Cat8p transcriptional activator controls gene expression of enzymes needed for gluconeogenesis and other processes which typically occur in glucose depleted environments. Cat8p is under control of both the Mig1p repressor and the Hap2/3/4/5 activator complex. Indeed the HAP4 strain grown under aerobic nitrogen-limited conditions scores significantly for the Cat8p relative to the wild type but does not so when grown in aerobic batch cultures. This might not be expected but it may be hypothesized that relatively high
respiratory activity per se triggers the cell to adapt to seemingly low glucose condition, despite the proper functioning of the glucose sensing machinery in this strain. The Cat8p element shows up in the wild type strain under the conditions tested as expected. For instance, in batch grown cells Cat8p controlled expression is much lower than under glucose-limited condition. The same is found when comparing a nitrogen-limitation to a glucose limitation. Comparing the HAP4 strains to each other under all condition revealed the same results.

The only comparison where a significant score for Cat8p lacks is between the batch cultures or nitrogen-limited cultures to the glucose-limited cultures at a dilution rate just over the μmax. Although these cultures are still glucose limited, they do ferment, implying that they are no longer fully glucose de-repressed. Of course, here the glucose limitation is less stringent, since the cells grow at a rate close to their maximal attainable growth rate. This causes the Mig1p repressor to block transcription of genes under control of Cat8p and as such the lack of significant scores is consistent.

A fair amount of significant Msn2-4p controlled elements were found. Msn2-4p is a transcriptional activator complex that is mostly associated with various kinds of stress, such as continuous heat stress (Mensonides, et al., 2002). In these series all the conditions were similar with respect to temperature and other environmental parameters (pH, stirring, aeration) (with the exception of 100 mM phthalic acid in the batch cultures). This finding rules out any responses of Msn2-4p to these parameters. Interestingly all the conditions in which Msn2-4p scores significantly are comparisons where the growth rate varies largely (e.g. batch wild-type (0.36 h^-1) versus glucose-limited cultures at a growth rate of 0.1 h^-1). It has been described that PKA activity and Msn2/4p expression antagonize one another controlling the cells between growth and non-growth under various conditions (starvation, heat stress, osmotic stress) (Smith, et al., 1998). This explanation would fit very well with the observed results of higher expression of genes under control of Msn2/4p under nutrient limiting conditions. The absence of this category in either of the comparisons between unrestricted growth or very low growth rate with the glucose-limited culture at 0.32 h^-1 seems contradictory to this explanation. It must be remarked here that at this growth rate the organisms have switched to respiro-fermentative metabolism, which suggests positive PKA signaling and as a consequence lower expression of Msn2/4p controlled genes.

Two other categories also scored significantly in this analysis, namely genes under control of the transcriptional activator Rcs1p with a common promoter element of RVACCCTD, and genes with the common promoter element ACCNNNNNGGT, which is recognized by the transcriptional activator Zap1p (shown in figure 3). Rsclp is a transcription factor, which is involved in iron homeostasis in yeast. As such the appearance of this category might not be surprising as increased respiration requires more iron containing haeme.

Yet, when comparing the HAP4 strain with the wild-type under glucose-limited conditions at a low growth rate (where both strains grow fully respiratory) this category shows up. Under these conditions, the two strains respire at the same rate, but it may well be that due to the Mig1p independent expression of HAP4 a signal is triggered which results in increased haeme synthesis, or even increased synthesis of other components of the respiratory chain. It would be interesting to investigate this hypothesis by a biochemical analysis of the haeme content of the overproducer and its respiratory
capacity. Noteworthy, the analysis showed a slight upregulation of the QCR genes in the HAP4 χ strain encoding the ubiquinol cytochrome c oxidoreductase proteins under glucose-limited conditions (data not shown).

Interesting results are obtained by comparing the Rcs1p element for the same strain grown under various conditions. In most analyses a higher expression of Rcs1p controlled genes was seen under the conditions where there is less respiration. Only for the HAP4 χ low versus high growth rate glucose-limited analysis this was not the case. This may indicate some opposing regulatory events: the strong and overruling glucose repression network results in low respiration rates that in turn give rise to changes in some cellular state parameter (e.g. the redox or energy state). These latter changes may be signals to the cell to enhance its respiration and thus to provide sufficient iron. Indeed, this seems in line with the absence of this relationship in the hap4p overproducing strain which, due to increased respiration activity under all circumstances, lacks the signal for Rcs1p controlled genes.

Throughout all analysis, it was surprising to find a significant impact of the category under control of Zap1p, controlling zinc homeostasis in S. cerevisiae. The results seem consistent with the idea that increased respiration results in more Reactive Oxygen Species being formed, and therefore more superoxide dismutase is needed. In yeast these are encoded by SOD1 and SOD2, of which Sod1p requires zinc to be functional. However, a further analysis showed no increased expression of both Sod proteins under enhanced respiration. An alternative explanation for the involvement of Zn-related processes may reside in the differential expression of ADH genes. ADH4 is the only
alcohol dehydrogenase which requires zinc. Indeed, significant upregulation of \textit{ADH4} and the Zap1 scores correlated in a number of analyses, but it must be noted that this correlation was not always found. This raises the question whether other Zn dependent proteins play some role in the increase in Zap1 controlled gene expression. It has been shown previously that increased levels of Hap4p result in a more oxidative catabolism in \textit{S. cerevisiae} (Schuurmans, et al., 2008[van Maris, 2001 #104]). Our analyses show that the major effect of the overproduction is on those genes that are involved in respiration rather than on those involved in glycolysis. Thus, the hap regulatory network seems to work on a different metabolic level, with an important difference with the physiological and genetic impact, that controlled by of \textit{hxk2} (Schuurmans, et al., 2008). Although the absence of the latter global regulator redistributes catabolism towards oxidative pathways, it has many other effects as well, including loss of glucose repression as a whole. This leads to a loss of response to both the concentration and nature of the carbohydrates in the environment. The respiration-specific effect of HAP4 overproduction is not absolute though, as has been shown here, and it remains to be seen whether the side effects are due to a completely other and as yet unknown role of the regulator or are somehow related to respiration and hence indirect consequences of the functioning of Hap4p.
Chapter 5

Control of Specific Growth Rate in *Saccharomyces cerevisiae*

J. L. Snoep\textsuperscript{1,2}, M. Mrwebi\textsuperscript{1}, J.M. Schuurmans\textsuperscript{2}, J.M. Rohwer\textsuperscript{1} and M. J. Teixeira de Mattos\textsuperscript{3}

\textsuperscript{1} Triple J Group for Molecular Cell Physiology, Department of Biochemistry, Stellenbosch University, Private Bag XI, Matieland 7602, South Africa

\textsuperscript{2} Manchester Centre for Integrative Systems Biology, Manchester Interdisciplinary Biocentre, The University of Manchester, 131 Princess Street, Manchester, M1 7DN, United Kingdom

\textsuperscript{3} Swammerdam Institute for Life Sciences, Department of Molecular and Microbial Physiology, University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV Amsterdam, The Netherlands

Submitted to Microbiology
Abstract

In this contribution we resolve the long standing dispute whether or not the Monod constant ($K_S$), describing the overall affinity of an organism for its growth limiting substrate, can be related to the affinity of the transporter for that substrate ($K_M$). We show that the two constants can be directly related to one another via the control of the transporter on the specific growth rate; they are identical if the transport step has full control. The analysis leads to the counter-intuitive result that it is to be expected that the affinity of an organism for its substrate is higher than the affinity of the enzyme that facilitates its transport. Experimentally we show this indeed to be the case for the yeast *Saccharomyces cerevisiae*, for which we determined in glucose limited chemostat cultures a $K_M$ value for glucose more than two times higher than the $K_S$ value. Moreover, we could calculate that at glucose concentrations of 0.03 and 0.29 mM the transport step controls the specific growth rate for respectively 78% and 49%. 

*Chapter 5*  
**Control of specific growth rate**

- 68 -

Abstract

In this contribution we resolve the long standing dispute whether or not the Monod constant ($K_S$), describing the overall affinity of an organism for its growth limiting substrate, can be related to the affinity of the transporter for that substrate ($K_M$). We show that the two constants can be directly related to one another via the control of the transporter on the specific growth rate; they are identical if the transport step has full control. The analysis leads to the counter-intuitive result that it is to be expected that the affinity of an organism for its substrate is higher than the affinity of the enzyme that facilitates its transport. Experimentally we show this indeed to be the case for the yeast *Saccharomyces cerevisiae*, for which we determined in glucose limited chemostat cultures a $K_M$ value for glucose more than two times higher than the $K_S$ value. Moreover, we could calculate that at glucose concentrations of 0.03 and 0.29 mM the transport step controls the specific growth rate for respectively 78% and 49%.
Chapter 5 Control of specific growth rate

Introduction

In the classic publication (Monod, 1949) in which Monod proposed his famous equation for the specific growth rate dependency on substrate concentration (eq. 1) he suggested, realizing the similarity with the Michaelis-Menten equation, that the $K_S$ value “should be expected to bear some more or less distant relation to the apparent dissociation constant of the enzyme involved in the first step of the breakdown of a given compound”.

\[ \mu = \frac{\mu_{\text{max}} \cdot S}{S + K_S} \]  

Monod appears to be very careful in this statement and rightly so for the mechanistic interpretation of an empirical constant that is dependent on the systemic behavior of thousands of reactions is not simple and lies at the core of the relatively new research field of Systems Biology.

Whereas the Michaelis-Menten equation was derived on the basis of an enzyme kinetic mechanism and its parameters have a mechanistic interpretation, the Monod equation is purely empirical. Thus the Monod constant describes the overall affinity for an organism for its growth limiting substrate, while the Michaelis-Menten constant can be directly expressed as a ratio of the elementary rate constants acting on the enzyme-substrate complex. In a description for growth under substrate limited conditions it would seem logical to assume the substrate transporter to play an important role as this is likely to be the enzyme with which the cell senses the substrate. As such, one could expect the affinity of the cell for the growth limiting substrate ($K_S$) to be related to the affinity of the substrate transporter ($K_M$).

In addition to the Monod equation, several other functional dependencies of the specific growth rate for a single limiting substrate have been proposed. For a historical review of different models for microbial growth kinetics see Jannasch (1993) (Jannasch and Egli, 1993), for a comparison of different growth models see Button (1993) (Button, 1993). Many of these models are very similar to the Monod model, but might contain additional parameters, such as the Moser (Moser, 1958) and Contois (Contois, 1959) models, while others are fundamentally different, such as the Blackman (Blackman, 1905), Teissier (Teissier, 1936), also known as the exponential model and the logarithmic model (Westerhoff, et al., 1982). While some attempts have been made to give a mechanistic interpretation to the Monod constant, these were never really successful (Liu, 2007). Systems Biology attempts to understand systemic behavior on the basis of the characteristics of the systems components. From such a perspective it would be extremely interesting to test whether it is possible to relate a systemic property such as the $K_S$ of an organism to the local property of an enzyme ($K_M$). We have addressed this question using a theoretical and an experimental approach. In a metabolic control analysis of the problem we could relate the two constants via the growth control of the substrate transporter. In glucose-limited chemostat cultures of the yeast Saccharomyces

- 69 -
cerevisiae we experimentally determined $K_S$ and $K_M$ and made the intriguing observation that the overall affinity of the organism is more than a factor 2 higher than the affinity of the transporter. In addition, from the two constants we could calculate the control of the specific growth rate by the glucose transporter.

Results

Theory

The chemostat is an ideal instrument for studying the relationship between specific growth rate and the concentration of a growth-limiting substrate. In a chemostat experiment the medium is composed such that one of the substrates will be growth-rate limiting. This makes it possible to set the specific growth rate via the influx rate of the medium. At steady state the specific growth rate ($\mu$) equals the dilution rate ($D$), defined as the medium flow rate/culture volume. As such it is possible to construct a plot of $\mu$ against the growth limiting substrate concentration ($S$) by varying the dilution rate and measuring the steady state residual substrate concentration. The Monod equation (eq. 1) can be fitted to such a data set, to estimate the maximal specific growth rate ($\mu_{max}$) and Monod constant $K_S$.

To understand how a change in medium influx rate ultimately leads to a change in the specific growth rate, it is useful to first analyze a minimal chemostat system, consisting of four variables, $S$, $S_i$, $X$ and $B$ (Fig. 1).

![Figure 1. Scheme for the simple chemostat set-up as used in the theory and model sections. The chemostat consists of two external variables (in extracellular volume $V_o$): the growth limiting substrate concentration ($S$) and the biomass concentration ($B$). The biological system consists of two variables (in intracellular volume $V_i$): $S_i$ the internal substrate concentration and $B$, a precursor for biomass formation. The microorganism consists of three enzyme catalyzed reactions: $v_{tr}$ the substrate transporter, $v_{in}$ an internal reaction producing $X$, and $v_{b}$ a biosynthetic reaction forming biomass from $X$. The chemostat instrument consists of pump reactions (p) leading to the influx of $S$ and efflux of effluent from the culture (containing $S$ and $B$).](image)

For our analysis we use the metabolic control analysis framework, originally developed in Kacser and Burns (Kacser and Burns, 1973) and Heinrich and Rapoport (Heinrich and Rapoport, 1974), which has been expanded extensively since then ((Fell, 1996; Fell, 1992; Heinrich, 1996)), and has also been applied to chemostat cultures (Small, 1994;
Chapter 5 Control of specific growth rate

Snoep, et al., 1994). Any effect of the pump rate on the specific growth rate must have been mediated via either of the two external variables, $S$ or $B$:

$$G_p^\mu = G_p^S \cdot R_p^S + G_p^B \cdot R_p^B$$

$$= G_p^S \cdot \varepsilon_{tr} \cdot C_{tr}^{\mu} + G_p^B \cdot \varepsilon_{b} \cdot C_{b}^{\mu} + \varepsilon_{tr} \cdot C_{tr}^{\mu} + \varepsilon_{b} \cdot C_{b}^{\mu}$$

where $G_p^\mu$ is a global control coefficient, defined as $\frac{d\mu}{dp}$, and describes the percentage change in specific growth rate $\mu$ upon a 1 percentage change in pump rate $p$. Since $\mu$ is proportional to the dilution rate (and thus also to $p$), $G_p^\mu=1$. The change in steady state concentrations of $S$ and $B$ by $p$ is quantified by $G_p^S$ and $G_p^B$ respectively and their effect on the local response of the microorganism is given by $R_p^S$ and $R_p^B$ respectively. These local responses are defined at the steady state concentration of $S$ and $B$ and can be interpreted as the change in specific growth rate of the isolated microorganism upon a change in $S$ or $B$ (e.g. $R_p^S = \frac{d\mu}{dS}$ with * denoting the steady state concentrations of the respective variables). Assuming that $S$ is only sensed by its transporter $tr$, the effect of a change in $S$ is mediated via its effect on the transporter activity $v_{tr}$, quantified by its elasticity coefficient ($\varepsilon_{tr}^S = \frac{\partial v_{tr}}{\partial S} \cdot \frac{S}{v_{tr}}$). The resulting effect of the change in transporter activity on steady state $\mu$ is given by the control coefficient of the transporter on the specific growth rate ($C_{tr}^\mu = \frac{d\mu}{dv_{tr}} \cdot \frac{v_{tr}}{\mu}$). In a similar way eq. 3 describes the effects of $p$ on biomass $B$, where both reactions $v_{b}$ and $v_{b}$ are sensitive to changes in the biomass concentration.

The steady state biomass concentration is equal to the biomass yield on substrate $Y_{s}$ times the substrate that is consumed by the organisms: $B = Y_{s} \cdot (S_f - S)$, with $S_f$ the substrate concentration in the feed. Assuming that $Y_{s}$ is relatively constant and that the growth limiting substrate concentration will generally be low and insignificant compared to $S_f$ (at low specific growth rates), we can derive:
Thus, at low specific growth rates, changing the pump rate does not affect the biomass concentration. Then, eq. 3 reduces significantly, and substituting $G_p^* = 1$ leads to:

$$G_p^* = \frac{1}{\epsilon_{SP}^* \cdot c_{vr}} = \frac{1}{R_S}$$  \hspace{1cm} (7)

Eq. 7 relates the change in substrate concentration after changing the pump rate to the local elasticity and control coefficients of the substrate transporter. This equation is general, i.e. not restricted to a specific growth rate function or transport rate equation and holds if the residual substrate concentration is much lower than the medium substrate concentration, i.e. at low dilution rates.

From the Monod equation the response coefficient of $\mu$ with respect to $S$ can be derived:

$$R_S^* = \frac{d\mu}{dS} \cdot \frac{S}{\mu} = \frac{K_S}{S + K_S}$$  \hspace{1cm} (8)

If we substitute a specific rate equation for the substrate transporter we can derive a relationship between the Monod constant $K_S$ and the Michaelis Menten constant $K_M$. For instance in the case of Michaelis Menten kinetics for the transporter:

$$v_{tr} = \frac{V_{MAX} \cdot S}{K_M + S}$$  \hspace{1cm} (9)

its elasticity equals:

$$\epsilon_{SP}^* = \frac{\partial v_{tr}}{\partial S} \cdot \frac{S}{v_{tr}} = \frac{K_M}{K_M + S}$$  \hspace{1cm} (10)
Substituting eq. 10 and eq. 8 into eq. 7 and rearranging:

\[ C_{tr}^K = \frac{1 + \frac{S}{K_M}}{1 + \frac{S}{K_S}} \]  

When Michaelis Menten kinetics (eq. 9) are used to describe the transport step for the growth limiting substrate, this step will have full control on growth rate, i.e. \( C_{tr}^p = 1 \), and from eq. 11 it follows that in that case \( K_M \) equals \( K_S \).

The Michaelis Menten equation is well known and widely used in studies on isolated enzymes but its applicability for the analysis of systems of linked reactions is very restricted, as it assumes the absence of product. However, in a system of linked reactions, product concentrations can never be zero, as they function as substrates for the next reaction. Thus, the effect of product on the enzyme activity must be taken into account. This effect can be of a kinetic (competition with the substrate for binding to the active site) or a thermodynamic type (with increasing product concentration the Gibbs free energy of a reaction becomes less negative).

If we first consider the kinetic effect of product on the enzyme activity, eq. 9 can be extended to:

\[ v_{tr} = \frac{V_{MAX} \cdot \frac{S}{K_{MI}}}{1 + \frac{S}{K_{MI}} \cdot \frac{P}{K_{MP}}} \]  

and its elasticity equals:

\[ e_{tr}^{S} = \frac{K_{MS}(1 + \frac{P}{K_{MP}})}{K_{MS}(1 + \frac{P}{K_{MP}}) + S} \]  

Thus, if one would assay the sensitivity of the transporter for its substrate, while keeping the internal product concentration constant at the steady state concentration, eq. 13 is equivalent to eq. 10 with \( K'_{MS} = K_{MS}(1 + \frac{P}{K_{MP}}) \), and eq. 11 holds, with \( K'_{MS} \) replacing \( K_M \).

An example of such a system could be the PTS glucose transport system in *E. coli* which is an active transport system, effectively irreversible but still product sensitive.

For a reversible reaction, e.g. assuming a symmetrical transporter for the facilitated transport of glucose in yeast, the following rate equation can be used:
When eq. 15 (instead of eq. 10) is substituted in eq. 7, it is not possible to express $K_S$ in terms of $K_M$ without knowing the internal glucose concentration. If we consider the two extreme cases: $S_{in} = 0$ and $S_{in} = S$, it can be shown that in the latter case there is no relation between $K_S$ and $K_M$, and the elasticity of the transporter approaches infinity, while in the first case equation 15 reduces to eq. 10 (as does eq. 13).

Importantly, when using product sensitive rate equations such as eq. 12 and 14, the transporter will not automatically have a control on growth rate of 1. More specifically, unless the transporter is completely limiting growth, its flux control coefficient will be smaller than 1. In the cases where the transporter is far away from equilibrium (as is to be expected for the uptake of the growth limiting component), it can then be derived that $K_S$ will be smaller than $K_M$. This is a rather counter-intuitive result; the affinity of an organism for its growth limiting substrate is greater than the affinity of the enzyme with which the organism senses (and transports) the substrate.

Core models

To illustrate the theoretical findings core models simulating different transport kinetics were constructed. Each of the core models has the same network structure (as shown in Fig. 1), but uses a different kinetic type for the transporter. Parameter values for the different models vary, and were chosen such that the steady state behavior of the models follows Monod kinetics. The core models have four variables, $S$, $S_{in}$, $X$, and $B$, described by the following ordinary differential equations:

\[
\frac{dS}{dt} = D \cdot (S_f - S) - v_{tr} \tag{16}
\]

\[
\frac{dS_{in}}{dt} = v_{tr} - v_{in} \tag{17}
\]

\[
\frac{dX}{dt} = v_{in} - v_h \tag{18}
\]

\[
\frac{dB}{dt} = v_h - D \cdot B \tag{19}
\]
The first core model uses irreversible but product sensitive kinetics for the transporter with \( v_{tr} \) as in eq. 12, \( \mu_{max} = 9 \text{ mM/h}, K_{MS} = K_{MP} = 0.1 \text{ mM} \), converting internal substrate to intermediate \( X \), following reversible kinetics as in eq. 14, \( \mu_{max} = 10 \text{ mM/h}, K_{MS} = K_{MP} = 1 \text{ mM}, K_{eq} = 100 \) and \( v_b \) modeled with eq. 9, \( \mu_{max} = 0.52 \text{ mM/h}, K_{MS} = 2 \text{ mM} \). Note that the internal reactions, i.e. \( v_{tr}, v_{in} \) and \( v_b \) are multiplied by \( B \), as their rates are proportional to the biomass in the culture. In addition the internal volume was for reasons of simplicity modeled as constant \( \frac{V_i}{V_o} = 0.0001 \). The medium substrate concentration \( S_f \) was chosen as 20 mM. The dilution rate \( (D) \) was scanned over a range from 0.01 to 0.45 1/h and the steady state variable values were calculated. The Monod equation gave a good description of the steady state relation between \( D \) and \( S \), and a \( \mu_{max} \) of 0.50 h\(^{-1} \) and a \( K_S \) of 0.0054 mM were determined. At a \( D \) of 0.1 h\(^{-1} \) a steady state concentration for \( S \) of 0.0013 mM was obtained and \( B \) equalled 19.998 mM, with an internal concentration of \( S \) in equal to 0.0197 mM. The elasticity of the transporter with respect to \( S \), \( \frac{v_{tr}}{g_{72}} \), calculated from eq. 13 was 0.989. The control of the pump on \( S \), \( G_p \), equals 1.23. From the \( v_{tr}^e \) and \( G_p^e \) using eq. 7 the control of the transporter on the specific growth rate, \( C_{tr}^e \) can be calculated to be 0.82. The same value for \( C_{tr}^e \) was also obtained directly when the “bacterium” in the core model was analyzed at the steady state values of \( S \) and \( B \). Importantly, this value is close to the value estimated from the model data using eq. 11, i.e. \( (1 + 0.00134/(0.1 + 0.0197))/(1 + 0.00134/0.0054) \)= 0.81, which indicates that in the case where the transporter kinetics can be described with eq. 12, \( C_{tr}^e \) can be estimated from \( K_{MS}, K_3 \) and \( S \). Note also that in the model the \( K_3 \) value is much smaller than the \( K_{MS} \) value, i.e. the organism has a higher affinity for its substrate than the transporter of the growth limiting substrate.

For the core model simulating a reversible transporter, the same parameter values were chosen, except for the transport kinetics, for which a \( K_{eq} = 1 \) and a \( K_{MP} = 0.9 \text{ mM} \) for both external and internal substrate was chosen (i.e. non-active, symmetrical transporter, eq. 14). The dilution rate was scanned from 0.01 to 0.45 h\(^{-1} \) and the Monod equation was fitted on the steady state solutions, which yielded a good description with a \( K_S \) value of 0.126 mM and a \( \mu_{max} \) value of 0.51 h\(^{-1} \).At a dilution rate of 0.1 h\(^{-1} \) an external substrate concentration of 0.030 mM and an internal concentration of 0.0197 mM was obtained, with \( v_{tr}^e = 2.8364 \) and \( G_p^e = 1.265 \), yielding (using eq. 7) a \( C_{tr}^e \) of 0.28. The same value was obtained when the external variables \( (S \) and \( B \) were clamped at their steady state values, and \( C_{tr}^e \) was determined directly. The control of the transporter on the specific growth rate was also calculated from the \( K_{MS}, K_3, S \) and \( S_m \) values using eqs. (15, 8 and 7), resulting in a value of 0.28, indicating that indeed a good estimation for this control can be obtained from the steady state chemostat data.

These core models only serve to illustrate the theoretical analysis section with numerical examples, the chosen parameter values are not realistic.

**Experimental results**

The Monod equation describes how the specific growth rate of an organism is dependent on the concentration of a single substrate. We have used chemostat cultures to achieve a single substrate limitation while having control over the specific growth rate via setting
of the dilution rate. At steady state, $\mu$ equals $D$ and by determining the steady state residual substrate concentration at various dilution rates, the relationship between the limiting substrate concentration and the specific growth rate can be studied. Measurement of the growth limiting substrate concentration is usually difficult because the concentrations are low and likely to change during sampling unless a method is used that rapidly stops metabolism. For these reasons we have used glucose-limited chemostat cultures of the yeast *Saccharomyces cerevisiae*, which is known to have a low affinity for this substrate and we operated the chemostat at low culture densities and stopped metabolism by rapid sampling in acid. 

*S. cerevisiae* VIN13 was grown at a range of dilution rates between 0.05 and 0.48 h\(^{-1}\) and the specific growth rate as a function of the residual glucose concentration is plotted in Figure 2. Fitting the Monod equation on the data set yields a $K_S$ estimate of 0.12 mM (Asymptotic SE 0.009) and a $\mu_{\text{max}}$ value of 0.50 h\(^{-1}\) (Asymptotic SE 0.011). In the chemostat the steady state glycolytic flux ($q_{\text{glc}}$) can be calculated from the medium glucose concentration ($Glc_f$), the residual glucose concentration ($Glc$), the dilution rate ($D$) and the biomass concentration, using: 

$$q_{\text{glc}} = \frac{Glc_f - Glc}{\text{biomass}} \cdot D.$$ 

Steady state uptake rates of glucose in the chemostat were determined over the dilution range 0.05 to 0.48 h\(^{-1}\), Figure 3. At low dilution rates, a linear relation was observed between $q_{\text{glc}}$ and $D$, indicating that the $Y_{\text{Glc}^{-1}}$ is constant. 

![Graph of specific growth rate as a function of residual glucose concentration.](image)

**Figure 2.** Specific growth rate as a function of the residual substrate concentration. *S. cerevisiae* was grown over a range of dilution rates and steady state residual glucose concentrations were determined. The Monod curve is fitted through the data points.
In addition, up to a dilution rate of 0.15 h\(^{-1}\) the residual glucose concentration is < 5% of the medium glucose concentration, and no significant change in biomass concentration was observed between dilution rates 0.05 and 0.15 h\(^{-1}\) (in agreement with the assumptions on the basis of which eq. 6 was derived).

At \(D\) values higher than 0.4 h\(^{-1}\) a dramatic increase in the slope of \(q_{\text{glc}}\) against \(D\) is observed (Figure 3), indicating a decrease in \(\nu_{\text{glc}}^{\text{Ymax}}\). This phenomenon is well known as the Crabtree effect, and shows the shift from a purely oxidative metabolism, to a fermentative metabolism with ethanol produced.

To be used as a growth substrate, glucose first needs to be taken up, for which yeast is known to have several transporters. To investigate the relationship between the affinity of the glucose transport step (\(K_M\)) and the overall affinity of the cell for glucose (\(K_S\)) we harvested yeast cells at two dilution rates and characterized the glucose transport step. In Figure 4 the glucose uptake kinetics for yeast grown at a dilution rate of 0.1 h\(^{-1}\) are shown in an Eadie-Hofstee plot, where the data were fitted, assuming zero trans influx kinetics, to a two component transport system, consisting of a high affinity (\(K_M = 0.75\) (mM), \(v_{\text{max}} = 506\) (nmol/mg prot/min)), and a low affinity transporter (\(K_M = 201\) (mM), \(v_{\text{max}} = 787\) (nmol/mg prot/min)). At a dilution rate of 0.35 h\(^{-1}\) similar results were obtained (\(K_M = 0.72\) (mM), \(v_{\text{max1}} = 632\) (nmol/mg prot/min), \(K_M = 93\) (mM), \(v_{\text{max2}} = 569\) (nmol/mg prot/min)).

![Figure 3. Steady state glycolytic flux as a function of dilution rate. S. cerevisiae was grown in glucose limited chemostat cultures over a range of dilution ranges and the steady state uptake rate of glucose was determined. The uptake rate is expressed per unit OD 600 nm; due to the low densities at which the cells were grown it was not possible to get an accurate dry weight measurement.](image-url)
The steady state uptake rate of glucose in the chemostat equals 40 and 282 nmol Glc/min/(mg protein) at $D = 0.1$ and 0.35 h$^{-1}$ respectively, (assuming 50% of the dry weight is protein). With the very low residual glucose concentration in the chemostat (0.03 and 0.29 mM at dilution rates of 0.1 and 0.35 h$^{-1}$ respectively) the measured uptake kinetics cannot explain the measured steady state glucose uptake rates, even if the internal glucose concentration were zero. For the chemostat grown cells the internal glucose cannot be zero, otherwise there would be no glycolytic flux. An estimate for the internal glucose concentration can be made on the basis of the steady state flux and the kinetics of the glucokinase. Using the kinetic information on the glucokinase, and the steady state metabolite concentrations from the kinetic model as published by Teusink et al. (Teusink, et al., 2000) together with the expression level as determined in Daran-Lapujade et al. (Daran-Lapujade, et al., 2007) for glucose limited chemostat culture at $D = 0.1$ h$^{-1}$, an internal glucose concentration of 0.003 mM was calculated, which is close to the 1μM concentration suggested in Postma et al. (Postma, et al., 1989), (for $D = 0.35$ h$^{-1}$ an internal glucose concentration of 0.025 mM was calculated, see Materials and Methods for details).

**Figure 4. Eadie-Hofstee plot for glucose transport kinetics.** Transport rates were determined using $^{14}$C-labeled glucose in zero-trans influx assays for *S. cerevisiae*, grown in glucose limited chemostat cultures at a dilution rate of 0.1 h$^{-1}$. The line shows the best fit to a two component transport system.

If this non-zero glucose concentration is taken into account for the predicted glucose uptake rate on the basis of the measured in vitro kinetics then an even greater difference between measured and predicted glucose uptake rate is observed. The most likely explanation for this discrepancy is that the internal glucose concentration in the in vitro uptake experiments is not negligible and leads to an overestimation of the $K_M$ for glucose. An objective function that sums the squared differences between the experimental data and the model prediction was constructed (allowing for a non-zero...
internal glucose concentration in the glucose assay and adding as additional constraint the glucose uptake rate in the chemostat, see Material and Methods for details). Minimizing the objective function resulted in the following parameter values for the cells grown at \( D = 0.1 \text{ h}^{-1} \): for the high affinity transporter, \( K_M = 0.30 \text{ mM} \), and \( v_{\text{max}} = 494 \text{ (nmol/mg prot/min)} \) and for the low affinity system, \( K_M = 187 \text{ mM} \) and \( v_{\text{max}} = 786 \text{ (nmol/mg prot/min)} \), with an internal glucose concentration in the in vitro assay of 0.15 mM. At a dilution rate of 0.35 h\(^{-1}\) an identical internal glucose concentration was obtained with the following kinetic parameters: \( K_M^1 = 0.27 \text{ mM} \), \( K_M^2 = 87 \text{ mM} \), \( v_{\text{max}1} = 619 \text{ (nmol/mg prot/min)} \) and \( v_{\text{max}2} = 578 \text{ (nmol/mg prot/min)} \).

Applying eqs. 8 and 15, a response coefficient of yeast for glucose of 0.8 and an elasticity of the transporter for glucose of 1.02 can be calculated at a dilution rate of 0.1 h\(^{-1}\); at \( D = 0.35 \text{ h}^{-1} \) response and elasticity coefficients of respectively 0.29 and 0.60 were obtained. From these, using eq. 7 a control of the transporter on the specific growth rate of 0.78 and 0.49 can be calculated at \( D = 0.1 \) and \( 0.35 \text{ h}^{-1} \) respectively. This means that at the low external glucose concentration of 0.03 mM, the glucose transporter holds 78 % of the growth control (note that this is the control on growth rate for a bacterium that is isolated from the chemostat with the external metabolites clamped at the steady state values). Similarly a control of 49 % could be calculated at an external glucose concentration of 0.29 mM.

**Discussion**

Growth and reproduction are essential functions of living organisms. In growing cells, thousands of reactions are coordinated to build all cellular components necessary for the production of daughter cells. Despite its importance in many diseases (e.g. cancer), control and (dys-) regulation of growth rate is ill-understood, i.e. it is not known to what extend the different reactions in the metabolic network are limiting the specific growth rate (\( \mu \)).

The growth rate dependency on external parameters is much better studied, especially for uni-cellular organisms limited in growth by the availability of a single substrate (S), where typically a hyperbolic relationship is observed between \( \mu \) and S. We focused on the description of that hyperbolic relation using the Monod equation (eq. 1), which fitted our data well. Intriguingly the Monod equation, describing the coordinated response of thousands of reactions, is identical to the Michaelis-Menten equation, describing the activity of an isolated enzyme, and although Monod has stated that \( K_S \) is not equal to \( K_M \) he did suggest that there may be some relationship between the two parameters (Monod, 1949). In the chemostat the pump rate controls the specific growth rate via its effect on the growth limiting substrate concentration, \( S_G^* \). This control can be related to the elasticity for glucose and the control on growth rate of the substrate transporter via eq. 7. This equation is general, but when a specific growth model, such as the Monod model, and a kinetic type for the transporter are inserted, it is possible to relate the \( K_M \) of the transporter to the \( K_S \) of the organism. An important constraint for the relationship is that it is dependent on whether the elasticity of the transporter can be expressed as a function of its \( K_M \). For irreversible enzymes this is always possible (although the value may also be dependent on the internal substrate concentration), but for reversible enzymes close to equilibrium the importance of the \( K_M \) value for the elasticity coefficient becomes small.
Then it will in general not be possible to relate the $K_M$ of the transporter to the $K_S$ of the organism. In addition under such conditions the control of the transporter on the specific growth rate will be very small.

An important finding of our study is that from the relation between $K_S$ and $K_M$ it is possible to calculate the control of the transporter on the specific growth rate, if the residual substrate concentration is known (in case of reversible kinetics also the internal concentration needs to be known if it is not negligible). Thus for *S. cerevisiae* it was estimated that the transporter has 78% of the growth control, at a clamped glucose concentration of 0.03 mM. The experimental approach we followed is similar to the one in Postma et al. (Postma, et al., 1989), where a $K_S$ of 0.11 to 0.39 mM was determined (although the data could not be described very well by Monod kinetics), with a high affinity $K_M$ of 1 mM. In addition, a residual glucose concentration at $D = 0.1$ h$^{-1}$ of 0.11 mM was measured and an internal glucose concentration of 0.001 mM was calculated (Postma, et al., 1989). Using our theoretical analysis, it can be calculated from these data that the glucose transporter controls the specific growth rate for 55 to 85% (depending on the $K_S$ value).

The analysis we have given is general and can be applied to experimental data of other organisms as well. Good experimental data is available for another model organism, *Escherichia coli*, for which a $K_S$ value of 0.41 μM was obtained with a residual glucose concentration of 0.20 μM at a dilution rate of 0.3 h$^{-1}$ (Senn, et al., 1994). From these data a response coefficient of glucose for the specific growth rate of 0.67 can be calculated using eq. 8. A $K_M$ value for glucose of the PTS of 20 μM has been reported (Stock, et al., 1982). In a first assumption to estimate the elasticity of the PTS for glucose a random order rapid equilibrium type of kinetics was used for the overall reaction catalyzed by the PTS. The elasticity for such a rate equation is equal to the one in eq. 12. From the elementary rate constants given in Rohwer et al. (Rohwer, et al., 2000) it can be calculated that the effect of the internal glucose-6-phosphate concentration is negligible due to the low affinity of the enzyme for this product. Thus an elasticity of the PTS for glucose of 0.99 can be calculated at an external glucose concentration of 0.2 μM. The elasticity of the PTS system for glucose can also be estimated directly from the detailed kinetic model (Rohwer, et al., 2000), as 0.97. These data indicate that in *E. coli* the glucose transporter controls the specific growth rate for 67% (or 69% if the elasticity of 0.97 is used) at the low glucose concentrations as obtained in the glucose limited chemostat cultures at $D = 0.3$ h$^{-1}$.

In this contribution we have shown that the Monod constant, although in itself purely empirical, can be related to the Michaelis Menten constant of the transporter for the growth limiting substrate. Key to the analysis is to use the Monod equation to express the sensitivity of the specific growth rate in terms of the Monod constant. Subsequently this sensitivity was related to the elasticity of the transporter for the substrate and the control of the transporter on the specific growth rate. Thus, $K_S$ and $K_M$ values can be related to each other via the control of the transporter on the specific growth rate. This leads to the interesting result that this control, which is often difficult to measure directly, can be estimated from the Monod and Michaelis Menten constants, as we have shown for *S. cerevisiae* and *E. coli*.

From a systems biology perspective these results have a wider implication than just finding the relation between a phenomenological and a mechanistic constant. For a field...
Chapter 5 Control of specific growth rate

that has as one of its aims to understand systemic behavior on the basis of the characteristics of its components (Snoep, 2005), it has far stretching implications that it is possible to express a systemic property, describing the overall sensitivity of an organism for its growth limiting substrate, as a function of the characteristics of the transporter for the substrate. Crucial in the analysis was to link the importance of the component, as expressed by its growth control, to its sensitivity for the growth limiting substrate. Under the specific conditions as prevailing in the glucose-limited chemostat cultures, the glucose transport step holds more than 50% of the growth control, which is quite amazing when considering that growth is a concerted action of several thousands of enzymes. The interplay between a good theoretical framework such as MCA, modeling and experiment, as used in this study, is illustrative for the type of approaches that we think will be essential for addressing systems biology problems.

Materials and Methods

Strain and cultivation

*Saccharomyces cerevisiae* strain VIN13, kindly provided by the Institute of Wine Biotechnology, Stellenbosch University, was used in all the experiments. Cells were grown over a range of dilution rates in glucose-limited chemostat cultures (Bioflo 110 fermentors, New Brunswick Scientific Co., Inc, New Jersey). The working volume was approximately 650 ml and pH was controlled at 5.5 ± 0.1 by automatic addition of 1M NaOH. The culture was sparged with air at a flow rate of 20 L/h and the culture was stirred at 250 r.p.m. The temperature was controlled at 30°C. The dissolved oxygen (O₃) was monitored with a DO₂ electrode Model InPro6110/160 (New Brunswick Scientific Co., Inc, New Jersey) and kept above 60% saturation. Medium composition was as described in Verduyn et al. (Verduyn, et al., 1992) and glucose concentration in the feed was either 1 or 2 mM, ensuring glucose limiting conditions and a low biomass concentration. No effect of medium glucose concentration on steady state residual glucose concentration was observed.

For determination of the residual glucose concentration, samples were extracted in an equal volume of cold (4°C) PCA (10%) utilizing under-pressure, to improve sampling rate. Samples were neutralized using 2M K₂CO₃ and left on ice for 15 minutes. To remove precipitated salts and proteins, the samples were centrifuged at 20800 g at 4°C for 10 minutes. Residual glucose concentrations were determined using the method described in Senn et al. (Senn, et al., 1994).

Cells to be used for glucose uptake experiments were grown at higher biomass concentrations in Applikon fermentors with a working volume of 1 L, using the same mineral medium but with a glucose concentration in the medium of 42 mM. The culture was stirred at 750 r.p.m. and sparged with air at a flow rate of 60 L/h, culture pH was maintained at pH 5.0 ± 0.1.

For the zero trans-influx experiments, samples were rapidly taken and washed twice in mineral medium lacking a carbon source, by centrifugation at 3000 g at 4°C (SS-34 rotor, Sorvall) for 10 minutes, and stored on ice for further use in the zero-trans influx experiments. Dry weight was determined by taking 2x 10 ml samples in preweighed tubes, centrifuged at 3000 g at 4°C (SS-34 rotor, Sorvall) for 10 minutes, washed twice with dH₂O and dried overnight at 100°C. Tubes were then weighed again and the
difference was used to calculate the dry-weight. 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. Metabolite concentrations were measured by HPLC (LKB, Bromma, Sweden) using a Rezex organic acid analysis column with an 8-mm particle size, 8% cross-linking and a hydrogen ionic form (Phenomenex, Torrance, CA) at a temperature of 45°C and with 7.2mM H2SO4 as an eluent. Detection was performed with an RI1530 refractive index detector (Jasco, Tokyo, Japan). Peak integration and data processing were performed with BORWIN CHROMATOGRAPHY software (Le Fontanil, France).

Transport assay
Zero trans-influx rates of sugars were determined in a 5 second assay according to Walsh et al. (Walsh, et al., 1994) at 30°C with the modification that growth medium was used instead of phosphate buffer. Cold glucose concentrations were verified by using a glucose oxidase assay, using glucose oxidase (Roche Diagnostics GmbH, Mannheim, Germany), peroxidase and ABTS in 0.5M tris-HCl, mixed with standard or sample and incubated at 37°C for 1 hour under slow agitation after which the absorbance at 415 nm was measured using a SPECTRAnax PLUS384 Microplate Spectrophotometer (Molecular Devices Corporation, Sunnyvale, California).

Calculations
All calculations and model simulations were performed with Mathematica version 6 (Inc, 2007). For the calculation of the internal glucose concentration in the steady state chemostat cultures the following equation was used for the glucokinase reaction

\[ v_{GLK} = \frac{V_M}{K_{ATP}K_{M_{GLC}i}} \left( GLC_i \cdot ATP - \frac{G6P \cdot ADP}{R_{eq}} \right) \cdot \left( 1 + \frac{ADP}{K_{M_{ADP}} + \frac{G6P}{K_{M_{G6P}}}} \right) \cdot \left( 1 + \frac{ATP}{K_{M_{ATP}} + \frac{ADP}{K_{M_{ADP}}}} \right) \] (20)

(Deusink, et al., 2000): with: \( R_{eq} = 3800 \), \( K_{M_{GLC}i} = 0.08 \) mM, \( K_{M_{G6P}} = 30 \) mM, \( K_{M_{ATP}} = 0.15 \) mM, \( K_{M_{ADP}} = 0.23 \) mM, \( G6P = 2.5 \) mM, \( ATP = 2.5 \) mM, \( ADP = 1.3 \) mM as the parameter values and steady state metabolite concentrations taken from Deusink et al. (Deusink, et al., 2000). For the \( V_M \) of the glucokinase a value of 1779 nmol/mg prot/min was used, as determined by Daran-Lapujade et al. (Daran-Lapujade, et al., 2007) for aerobic glucose limited grown S. cerevisiae at a dilution rate of 0.1 h⁻¹, which is in good agreement with the 2 U/mg protein as reported in Postma et al. (Postma, et al., 1989). Using this equation the internal glucose concentration was solved at a glycolytic flux of 40 and 282 nmol/mg prot/min, as was measured in this study at \( D = 0.1 \) and 0.35 h⁻¹ respectively.
For fitting of the kinetic parameters of the glucose transport kinetics the following objective function was minimized:

\[
\sum \left( \frac{V_{M1}(GLC(n) - GLC_i)}{K_{M1} + GLC(n) + GLC_i} + \frac{V_{M2}(GLC(n) - GLC_i)}{K_{M2} + GLC(n) + GLC_i} \right)^2 + \\
w \cdot \left( \frac{J_{GLC} - \frac{V_{M1}(GLC_c - GLC_{ic})}{K_{M1} + GLC_c + GLC_{ic}}}{\frac{V_{M2}(GLC_c - GLC_{ic})}{K_{M2} + GLC_c + GLC_{ic}}} \right)^2
\]

with \(data(n)\) the transport activity data for \(n\) different glucose concentrations \((GLC(n))\), \(GLC_i\) the internal glucose concentration during the transport assay, \(J_{GLC}\) the steady state glucose uptake rate, \(GLC_c\) the residual glucose concentration in the chemostat, \(GLC_{ic}\) the steady state internal glucose concentration in the chemostat, and \(w\) a factor to give more weight to the steady state chemostat uptake rate, which is a single average value, as opposed to the large number of kinetic data points (a value of 5 was used for \(w\), increasing to higher values did not significantly influence the outcome of the optimization).
Chapter 6

General Discussion
Retrospect

In this thesis several aspects of central carbon catabolism have been addressed in relation to changes in expression of various catabolic regulators, with a focus on fermentative and respiratory capacity. Although this research started with characterizing strains with altered expression of GCR (Glycolytic Regulator) genes (GCR1 and GCR2), it turned out that strains harboring deletions of these genes were not easily obtained, and were even harder to work with. However work on GCR deletion strains has been continued by other researchers. Particularly the work of H. Uemura and co-workers has shown the function, by analysis of deletion mutants of GCR1 and GCR2, in maintaining proper glycolytic activity and their effects on other metabolic pathways (Sasaki and Uemura, 2005). GCR1 also functions in activating transcription of ribosomal genes and supporting maximum expression of G1 cyclins (Barbara, et al., 2007a) and is part of the yeast “coiome”, a protein complex which is involved in transcriptional regulation (Barbara, et al., 2007b). Additionally, unequivocal and often irreproducible results have been obtained with strains modified in GCR1 gene expression, (J.T. Pronk, personal communication).

For these reasons, we decided to focus our studies on the characterization of strains with increased respiratory capacity under glucose excess and aerobic conditions. During the research sometimes different media or strain backgrounds were used, so care should be taken when directly comparing results. A first transcriptional characterization of three such strains in batch culture is described in chapter 2. In this chapter some earlier findings on the hxk2/g39 strain and the HAP4χ strain are confirmed and several new aspects are identified. Thus it is shown that there are synergistic effects when modified expression of both regulators is introduced in one and the same strain: an hxk2 deletion strain with overexpression of HAP4 shows an even larger increase in respiration than both single mutations separately. This experiment illustrates that additional activator protein (HAP4) can enhance the respiratory chain activity even further. Indeed, biomass yield of this mutant was increased with respect to the wild-type, to such an extent that the hxk2ΔHAP4 strain nearly reached the theoretical maximal biomass yield for baker’s yeast. In addition to confirming the findings of increased respiration and lack of glucose repression for the hxk2/g39 strains (Diderich, et al., 2001a; Hohmann, et al., 1999), several additional results were obtained, i.e. a change in the transcription of the genes needed for zinc and iron homeostasis.

The hxk2Δ and HAP4χ strains were then investigated for their performance under anaerobic conditions, as described in Chapter 3. With respect to fermentative capacity it was found that the HAP4χ strain does not significantly differ from the wild-type. This again illustrates that the Hap-system functions at the physiological level of respiration, or possibly at that of the tri-carboxylic acid cycle, but not at that of glycolysis. In contrast, our studies on the fermentative capacity of the hxk2Δ strain support the idea that this regulatory system is involved in the upper part of glycolysis and even at the level of glucose sensing and carbon source uptake: deletion of HXK2 results in a large decrease in fermentative performance on glucose, but an increase in performance on maltose. The cause of this difference was investigated at the level of transcription profiles, activity of glycolytic enzymes and rate of glucose/maltose transport.
To investigate the transcriptional changes in the HAP4 χ strain in more detail, an extensive analysis of transcript profiles of the HAP4 χ strain grown under nutrient-limited as well as unlimited growth conditions was carried out and is presented in Chapter 4. Clearly, the effects of Hap4p on the biochemistry of the cell turn out to be complex but as a common feature it is concluded that Hap4p regulation is related to oxygen metabolism.

Chapter 5 describes a study we have carried out in collaboration with our colleagues from the University of Stellenbosch on the functional interpretation of the Michaelis-Menten affinity constant (Kₘ) of the glucose transporters and the overall affinity of the organism for growth on glucose (Kₛ) in terms of the growth rate control of the glucose transport machinery of the cell.

In conclusion, this thesis describes several new findings on the physiology of yeast strains with an increased respiratory catabolism; nevertheless many questions in this field are still unanswered and new questions have emerged.

Hexokinase II signaling

Even though the phenotype of the hx2kΔ strain was identified back in 1977 (Zimmermann and Scheel, 1977), the details of the changes this mutation causes, and how these changes are brought about, is still not fully understood. It was reported that approximately 14% of Hxk2p is localized in the nucleus in glucose-grown cells (Herrero, et al., 1998), and that it is there Hxk2p fulfills its regulatory function. Although many now believe that the catalytic activity of Hxk2p in glucose phosphorylation does not participate in transmitting the signal for glucose repression, a strain with a chimaeric hexose transporter protein (Otterstedt, et al., 2004) shows in fact a similar phenotype as a hxk2Δ strain, indicating that at least a part of the signal is created there because the glucose sensing Snf3p-Rgt2p pathways is still present in both strains. The chimaeric hexose transporter strain (Otterstedt, et al., 2004), is a transporting system made up from parts of the low affinity hexose transporter Hxt1p and parts of the high affinity hexose transporter Hxt7p and the constructed strain expresses only this single chimaeric protein as a hexose transporter. Presumably, the Hxt7p part is located partly in the cytosol, which could possibly signal a low-glucose condition. Although this could be mediated by Mth1p (which can bind to the C-terminal tail of the glucose sensors and is part of the Snf3p-Rgt2p signaling pathway), mutants in the Snf3-Rgt2 pathway do not show such a large shift towards respiration-supported growth, suggesting that Hxk2p is indeed involved in transferring a signal from the surface of the cell to the nucleus. Additionally, it has been shown that intracellular glucose can reduce transport rates up to 50% (Teusink, et al., 1998), which would imply that it is imperative for a cell to keep intracellular glucose levels as low as possible, which requires glucose-phosphorylation. These observations could still support a mechanism of action of Hxk2p near the cell surface. On the mode of action of Hxk2p in the nucleus much more progress has been made. Several interactors of Hxk2p have been identified, namely Med8p (de la Cera, et al., 2002) and the transcriptional repressor Mig1p (Ahuatzi, et al., 2007). These results, together with the action of the Snf1p kinase in response to activation of the PKA
pathway, show that glucose repression converges at the Mig1p repressor, as both Snf1p and Hxk2p regulate the phosphorylation level of Mig1p. However, it then still remains unanswered as to why only hsk2 deletion results in a nearly completely de-repressed cell, and not the mig1Δmig2Δ strain. Therefore, it would be interesting to resolve whether the hsk2Δ phenotype can be re-created by the combined mutation of several downstream factors, as deletion of only the MIG proteins is not enough.

**Changes in transcription of genes, required for iron and zinc homeostasis**

Specifically for the HAP4Δ strains, and to a lesser extent in the hsk2Δ strain, large changes in the expression of genes for zinc and iron homeostasis were found (chapter 4). For iron homeostasis, exemplified by the Rcs1p element, an increase in genes involved in iron uptake and utilization was found under carbon-limited growth conditions. Under these conditions the respiration rate of both strains is the same in vivo, but it should be realized that the actual the maximal respiratory capacity of the HAP4Δ strain could very well be higher than that of the wild-type. As such, the cells would have an increased need for haeme. If a cell is poised for increased respiratory capacity in some way, in the case studied here by changes in the regulatory network, upregulation of the synthetic capacity of components of the respiratory chain should follow, in accordance with the observation of increased expression of genes controlled by Rcs1p.

We found that a HAP4Δ strain also downregulates genes needed for transport of zinc, which suggests altered requirements in zinc homeostasis. At first, a relation with the zinc-dependent superoxide dismutase and a manganese-dependent superoxide dismutase was thought to be involved, by reasoning that the increased amounts of mitochondria would result in higher levels of Radical Oxygen Species (ROS). However, changes in expression of these genes were not observed. Alternatively, a link between the different ADH iso-enzymes was proposed. During growth on glucose ADH1 and ADH3 are the most abundant alcohol dehydrogenases, which both require 4 zinc molecules, whereas ADH4 only requires 2. Other reports showed that indeed when cells are zinc-deprived ADH1 and ADH3 are repressed and ADH4 expression is increased to overcome the low availability of zinc (Bird, et al., 2006). And indeed large changes of the expression of ADH4 were found under the conditions tested in Chapter 4, as well as changes in the expression of the ZRT (zinc transport) genes. Interestingly, the effects of zinc at the physiological and molecular level are still largely unknown and much work is currently performed to further elucidate various problems associated with zinc availability in for instance beer fermentations, where - if zinc ions become limiting - the fermentation process becomes sluggish. A recent publication on zinc availability showed that the response to a zinc limitation is very pleiotropic, including physiological changes related to oxygen availability, respiration and ethanol formation (De Nicola, et al., 2007). The role of zinc in mitochondrial biogenesis is far from elucidated; its functional role in respiration has been demonstrated (e.g. it is reported that cytochrome c oxidase is inhibited by zinc), but scarcely characterized. Therefore, we can at this point not draw any conclusions as to how Hap4p and zinc metabolism are related but we suggest that the
observed decrease in zinc regulatory genes in the HAP4\(\ne\) mutant is connected to better functioning of the respiratory chain and the increased amount of functional mitochondria in this mutant.

**Improving fermentative capacity**

The ultimate goal of the research performed in this thesis was to improve our knowledge as to how the balance between fermentation and respiration is regulated, in order to improve the fermentative capacity of yeast cells under anaerobic conditions. Ideally, improved strains should retain their ability to grow fully respiratory when needed, e.g. during their industrial production process. Many approaches have been used to increase this fermentative capacity, for instance by overexpressing the enzymes involved in the lower part of glycolysis (Smits, et al., 2000). This resulted in an increase of the glycolytic flux under several specific conditions. However, generally it shifted rate control in this pathway from one enzyme to another. As the HAP4\(\ne\) strain showed, changing the expression level of the transcriptional regulator which controls the pathway, leads to an increase of virtually all enzymes involved and as such increases the flux through the pathway as a whole. Increasing the glycolytic performance could perhaps also be achieved with the following approach: By changing key signals in the cell (e.g. PKA activity), via mutation of key proteins in these signaling pathways, a response could be achieved, which is independent of the environment in which the cells reside. As the hsk2A strain showed, omission of the signal transmitted by Hsk2p, completely changed cell metabolism and made these cells fairly unresponsive to varying glucose concentrations. To increase the fermentative capacity perhaps a similar removal or increase of signals might accomplish a high fermentative capacity. At high growth rates, industrial baker’s yeast has a much higher fermentative capacity than at low growth rates (Van Hoek, et al., 1998). The trick should then be to “fool” a slowly growing cell into sensing that all the necessary signals are there to conclude that it grows fast. A first start would be to investigate further the role of the PKA pathway and its interaction with the Msn2-4p pathway, in response to nutrient starvation. Some effects of the Msn2-4p pathway were observed in nutrient-limited chemostat cultures. If one could decouple the PKA pathway from the Msn2-4p dependent starvation response (a way to start would be a msn2-4A mutant), and combine this with a hyperactive PKA mutation (ira1A and ira2A), a cell might be tricked into a high fermentative output, even at near carbon source depletion. Although this will most likely raise problems in parts of intermediary metabolism, for a high glycolytic flux this could be an option worthy to investigate.

**Systems Biology – towards a virtual cell and its synthetic descendants**

Baker’s yeast has been a very important research object for many years now, for many reasons, such as improving its quality for the baker, improving growth characteristics as a cell factory, and as a model organism for better understanding of higher eukaryotes. Research in the eighties and nineties mostly focused on the isolation and characterization
of single genes of interest. However, nowadays many tools are available to perform large scale analyses, such as genome-wide transcription assays, highlighting changes in transcription of every gene available in yeast upon selected perturbation, whether these are nutrient pulses, stress conditions, or the effect of a gene deletion. In addition, results of more and more different types of these large scale analyses are becoming available, such as quantifying wide ranges of proteins (de Groot, et al., 2007; Kolkman, et al., 2006) or metabolites (van der Werf, et al., 2007; Wu, et al., 2005) of the cell under a certain condition. More and more tools are designed to dissect the various aspects of regulation on different levels. For instance, regulation analysis can be used to dissect the overall physiological changes in a pathway into those brought about by transcriptional events and those due to metabolic events such as kinetics, feed back loops, product inhibition etc. (for more information about regulation analysis see (Rossell, et al., 2005). One aspect of all these large scale assays is the enormous amount of data they lead to. Fortunately, now with more computer power, these data-sets can be more easily analyzed. Proper design of data handling, allowing for exchange between research groups, could lead to a valuable and manageable data set that may form the basis for modelling cellular aspects such as regulation, growth and physiological performance. Using this approach of integrating every data point available, allows for much greater understanding of the underlying mechanisms of how cells work, than by just characterizing every gene as a single unit, as it incorporates the effects of interactions and functioning of networks within the cell. This can be exemplified by our study (chapter 3) with respect to the cause as to why an hfk2Δ mutant performs so much worse than the wild-type under anaerobic conditions. Although many levels (transcriptome, enzyme activity, transport), have been studied, none showed a clue as to why an hfk2Δ mutant performs so poor. Integrating data on the metabolomics of wild type and mutant strains would be the proper approach to further understanding of the events going on inside the mutant cells.

The studies presented in this thesis show once again that it is not justified to consider the yeast cell as a simple model system. Its glycolysis is one of the longest and most intensively studied subjects in the life sciences and still has many secrets for us. This thesis illustrates the complexity of glycolytic regulation, as well as the intricate interaction between glycolysis and fermentation, respectively respiration. Only a concerted effort of many disciplines, from genetics through enzymology and physiology to data analysis and mathematical modelling can resolve the functioning and regulation of this central and essential catabolic module.
References


References


---

References


References


References


List of publications


Summary

The yeast *S. cerevisiae*, also known as baker’s yeast is a unicellular fungus that naturally occurs on various decomposing fruits. It is widely used in beer, bread and wine fermentations and serves as a model organism for higher eukaryotes. As such, the organism is widely studied and since 1996 the entire genome has been sequenced. A lot of research has been focused on the primary sugar metabolism and its regulation as well as on how this can be employed to our benefit. In the introduction (Chapter 1) the complex network of hexose transporters and signal transduction routes is described. *S. cerevisiae* has a preference for the hexose glucose. In order to effectively catabolize this sugar various mechanisms are used, which are all intricately linked, resulting in a complex network. Since *S. cerevisiae* is a Crabtree positive yeast, it will produce ethanol under aerobic conditions, whenever glucose is present in excess. Although advantageous in many applications, this has the disadvantage that the biomass that can be produced per unit of glucose is low due to the low energetic efficiency of the organism’s catabolism.

The research described throughout this thesis (Chapter 2, 3 and 4) characterizes two major transcriptional regulators which are important in maintaining the balance between fermentation and respiration in baker’s yeast. Our knowledge as to the mechanism and the quantitative impact of this regulation may provide us with tools to improve the performance of the organism with respect to ethanol formation on the one hand and biomass formation on the other.

To begin with, non-limited batch conditions are investigated on both the physiological and transcriptional level (Chapter 2). There it is shown that by deleting the gene *HXK2* a large shift towards respiratory metabolism is obtained. On the physiological level, this mutant grows slower than the wildtype, but expresses a reduction in fermentation rate and an improved biomass yield on glucose. In accordance with these findings, genome-wide expression analysis shows an increase in genes involved in the TCA-cycle and oxidative phosphorylation. Additionally, many other genes that are normally not expressed under glucose excess conditions are shown to be expressed. These included genes in the glyoxylate cycle and genes needed for utilization of other carbon sources. Taken together, the analysis reveals that deletion of *HXK2* results in a strain that is relieved of glucose repression. Analysis of the *HAP4* overexpression mutant also shows that catabolism shifts towards respiration. On the physiological level the strain grows at a similar rate as the wild-type, with a slightly increased yield on biomass. Gene expression is increased with respect to genes involved in the TCA-cycle and oxidative phosphorylation, however not as strong as was seen in the *HXK2* deletion mutant. Unexpectedly, a downshift in expression of genes needed for maintaining proper zinc content in a cell is observed. Analysis of the double mutant, in which both *HXK2* was deleted and *HAP4* was overexpressed, reveals an even larger shift towards oxidative metabolism. The growth rate in this strain was higher than in the *HXK2* deletion mutant, and a near theoretical maximum biomass yield on glucose is achieved. Genome-wide expression analysis shows an even larger increase in expression of genes needed for the TCA-cycle and oxidative phosphorylation and again a downshift in genes needed for zinc homeostasis.

Thus we conclude that the regulators Hap4p and Hxk2p function to some extent...
synergistically: the double mutant expresses a physiological behaviour of enhanced respiratory catabolism and hence yield optimization.

Since these strains show an increase in respiration it was then investigated whether their ability to ferment was affected under conditions where respiration is not repressed (Chapter 3). In this chapter the mutants are tested for their fermentative capacity (the ability to ferment sugars to ethanol and CO\textsubscript{2} under anaerobic conditions), an important industrial parameter that indicates the dough-raising power of a yeast strain. The strains were grown continuously under aerobic glucose-limited conditions and subsequently transferred to an anaerobic environment. For the \textit{HAP4} overexpression mutant no changes in behaviour upon transfer to anaerobic conditions are observed and nor did changes in growth rate affect this behaviour. However, the \textit{HXK2} deletion mutant showed completely different behaviour, when transferred from the aerobic chemostat to an anaerobic environment. At low growth rates their ability to ferment over a short time is not affected compared to the wild-type. In contrast, at higher growth rates, this ability was significantly impaired. To investigate their reduced fermentative capacity on glucose at higher growth rates, gene expression, glycolytic enzyme activity and \textit{in vitro} sugar uptake was measured. On the gene expression level hardly any changes in glycolytic gene expression during aerobic cultivation are observed. On the enzyme level, there is a reduction in maximum hexokinase activity in the mutant, but this could not account for the observed reduction in fermentation rate. Additionally, glucose uptake over a short time shows no clear difference. Another carbon source, maltose, which is abundant in bread dough, was tested. The \textit{HXK2} deletion mutant was able to ferment this sugar effectively compared to the wild-type, irregardless of the growth rate.

The \textit{HAP4} overexpression mutant was studied in more detail by comparing genome-wide expression profiles under four different physiological conditions (Chapter 4). The data used for the analysis where obtained by our colleagues in Delft and merged with those presented in chapter 2 and 3. Two glucose excess conditions (batch and nitrogen-limitation) and two glucose limited conditions ($\mu$=0.1 and 0.32 h\textsuperscript{-1}) were analysed. The analysis shows an increase in genes under control of Hap4p under glucose repressed conditions. Besides the Mig1p and Cat8p elements being identified under glucose de-repressed conditions, two additional regulators were identified under varying conditions, namely Rsc1p and Zap1p. Both are involved in cation homeostasis: Rsc1p is a regulator for proper maintenance of iron content in the cell and Zap1p for zinc. The changes in genes under control of Zap1p are analysed in more detail. As changes in \textit{ADH} expression were observed, a possible explanation of the changes in expression of Zap1p controlled genes emerged. \textit{ADH4} is the only alcohol dehydrogenase which requires zinc. And indeed, it is shown that \textit{ADH4} expression is correlated in most cases with up- or downregulation of Zap1p controlled genes. However, many other alternatives remain that can explain the changes in zinc regulation in the \textit{HAP4} overexpression strain.

Overexpression of \textit{HAP4} shows the major effects on those genes involved specifically in respiration, which is a clear difference to what was seen in the \textit{HXK2} deletion mutant, which shows a whole array of changes, including loss of glucose repression.

In Chapter 5, a theoretical framework is developed that relates the \textit{overall} affinity constant for a substrate ($K_s$), effectively controlling the specific growth rate, to the \textit{specific} affinity for that substrate of the transporter ($K_m$). This framework, based on metabolic control analysis was used to interpret data obtained from glucose-limited
chemostat cultures. If the transporter were to have full control, it follows that these two constants are identical. If, however, the transport step does not have full control, it leads to the counter-intuitive result that the affinity of the organism for a substrate can be higher than the affinity of the transporter. Indeed, it followed from that experimental data on chemostat cultures and *in vitro* glucose uptake assays that the affinity of the organism can be more than two times higher than the affinity of the transporter. Additionally, at specific glucose concentrations the control of the transporter on the specific growth rate could be calculated.
Samenvatting

*S. cerevisiae*, beter bekend als bakkersgist, is een eencellige schimmel die in de natuur groeit op rottend fruit. Deze gist wordt veel gebruikt bij de bereiding van bijvoorbeeld bier, brood (vandaar de naam) en wijn. Ook wordt deze gist veel gebruikt als model organisme voor andere eukaryote cellen, zoals dierlijke cellen. Sinds 1996 is het hele genoom van deze gist bekend, wat het mogelijk maakte om gebruik te maken van informatie op DNA en RNA niveau. Bij de productie van bier, brood en wijn is het belangrijk dat bakkersgist voldoende alcohol en koolstofdioxide produceert. Om deze redenen is er veel onderzoek gedaan naar het primaire suiker metabolisme en de regulatie hiervan, vooral ook om zo de productie te verbeteren. Hierdoor is er al veel bekend over het complexe netwerk dat de opname en het gebruik van bepaalde suikers reguleert. In de introductie (Hoofdstuk 1) wordt uitgelegd wat er bekend is over dit complexe netwerk. Bakkersgist heeft een voorkeur voor glucose (druivesuiker). Als er glucose aanwezig is zorgt dit voor een reactie in de gist zodat eerst de glucose wordt geconsumeerd, dit mechanisme wordt glucose repressie genoemd. Bakkersgist is ook een Crabtree positieve gist, dit houdt in dat zelfs in de aanwezigheid van voldoende zuurstof, de gist bij hoge glucose hoeveelheden alcohol maakt. Dit is handig voor de produktie van bijvoorbeeld bier, maar zorgt er wel voor dat de biomassa opbrengst niet optimaal is. Het onderzoek dat beschreven wordt in dit proefschrift (Hoofdstuk 2, 3 en 4) karakteriseert in detail de effecten van twee specifieke regulatoren die betrokken zijn bij de balans tussen fermentatie van suikers enerzijds en respiratie van suikers anderzijds. Kennis van het mechanisme en het kwantitatieve effect van de regulatie hiervan kan ons manieren verschaffen om de prestaties van gist te verbeteren.

Eerst is er gekeken naar een *HXK2* deletie, een *HAP4* overexpressie mutant en een dubbelmutant die beide mutaties bevat, onder niet gelimiteerde batch conditions (Hoofdstuk 2). Op fysiologisch niveau tonen beide stammen en de dubbelmutant (een combinatie van de twee) allereerst een verschuiving naar meer respiratie. De *HX2* deletie mutant groeit langzamer, maar de biomassa opbrengst is een stuk hoger, door een reductie in fermentatie snelheid. Op gen expressie niveau zijn er veel veranderingen opgetreden in de expressie van genen, die nodig zijn voor de citroenzuur cyclus en de oxidatieve fosforylering (de ademhalingsketen). Ook waren er een aantal veranderingen in de expressie van suiker transporters en veranderingen in genen die nodig zijn voor het gebruik van andere suikers. In de *HAP4* overexpressie mutant was ook een toename in de biomassa opbrengst te zien, en deze stam groeide met vrijwel dezelfde snelheid als het wild-type. De veranderingen op gen expressie niveau waren minder wijd verspreid, voornamelijk genen in de citroenzuur cyclus en de ademhalingsketen kwamen meer tot expressie. Ook werd hier een categorie genen gevonden die met de zink huishouding in de cel te maken hebben. Deze kwamen lager tot expressie. De dubbelmutant toonde een combinatie van de twee stammen, met een iets verhoogde groeisnelheid ten opzichte van de *HXK2* deletie stam en een biomassa opbrengst die bijna het theoretische maximum bereikt.
Aangezien deze stammen een verhoogde ademhaling vertonen, kon het zijn dat ze hierdoor minder goed konden fermenteren onder condities waar ademhaling niet gerepresseerd is. Hiervoor werden de stammen getest op hun fermentative capaciteit, of anders gezegd hoe goed de gisten alcohol en koolstofdioxide onder anaerobe condities kunnen maken. De twee stammen en het wild-type werden gegroeid in glucose gelimiteerde chemostaat cultures en bij steady-state overgebracht naar anaerobe mini-fermentors waar de snelheid van alcohol produktie, met glucose als substraat, gemeten werd (Hoofdstuk 3). In de HAP4 overexpressie mutant werden geen verschillen met het wild-type gevonden, maar in de HXK2 deletie mutant nam de snelheid van alcohol produktion af naarmate de groeisnelheid in de chemostaat hoger was. Daarentegen was de snelheid van alcoholproduktie met maltose als substraat in deze mutant, ongeacht de groeisnelheid, hoger dan het wild-type. Om uit te zoeken hoe deze verschillen tot stand komen, is er naar gen-expressie niveau, naar glycolytische enzym activiteit en in vitro glucose transport activiteit gekeken. Op gen-expressie niveau was er weinig verschil te zien. Bij de enzym activiteit was er een verlaging van maximale activiteit van hexokinase te zien. Echter de hoeveelheid activiteit die over was in de mutant zou nog steeds voldoende zijn om wild-type fermentatie toe te staan. Ook in suikertransport was er weinig verschil met het wild-type.

De HAP4 overexpressie mutant is nog verder in detail bekeken onder vier verschillende fysiologische condities (Hoofdstuk 4). Deze condities zijn twee glucose overmaat condities, namelijk batch en stikstof gelimiteerd en twee glucose gelimiteerde condities bij twee verschillende groeisnelheden. Van elk van deze condities is gekeken naar het transcriptie profiel. Bij de glucose overmaat condities werden genen die direct onder controle staan van Hap4p gevonden. Bij glucose gelimiteerde condities werden elementen gevonden die onder controle staan van Mig1p en Cat8p, twee regulatoren in de glucose repressie. Ook werden onder veel condities twee categorieën met genen gevonden die onder controle staan van Zap1p en Rsc1p. Rsc1p is een regulator eiwit dat de ijzer huishouding in de cel controleert. Zap1p controleert de zink huishouding. Naar waarom deze categorieën in de analyse naar boven kwamen in veel condities gekeken. Het zou te maken kunnen hebben met de expressie van ADH4, de enige alcohol dehydrogenase die zink bevat, aangezien de expressie hiervan correlaat met in welke mate het Zap1p element werd opgepikt.

Als laatste is de relatie tussen de affiniteit van een organismie voor een bepaald substraat (de Monod constante) en de affiniteit van het substraat transportende eiwit gekeken (Hoofdstuk 5). Door middel van een theoretisch model, metabole controle analyse en chemostaat experimenten wordt aangetoond dat deze twee constantes direct aan elkaar gerelateerd kunnen worden. Als het transportende eiwit volledige controle heeft over het proces dan zijn deze twee constantes identiek. Wanneer er daarentegen geen volledige controle is dan kan dat leiden tot het resultaat dat de affiniteit van het organismie hoger is dan die van het transportende eiwit. Door gebruik van glucose gelimiteerde chemostaat en glucose opname experimenten, is hier aangetoond dat de affiniteit van het organismie meer dan twee keer zo hoog als de affiniteit van de transporter kan zijn. Ook kon de controle van de transporter op de specifieke groeisnelheid bepaald worden.
Dankwoord

Zo, dan zijn we nu aangekomen bij misschien wel het best gelezen gedeelte van het proefschrift, namelijk het dankwoord. Hierbij wil ik beginnen met hoe ik eigenlijk in de biologie terecht gekomen ben. Voor vele in de familie, zal deze keuze niet zo vreemd zijn (of misschien juist heel vreemd), aangezien mijn beide ouders biologie hebben gestudeerd en vele andere in de familie (ooms, tantes, neefjes en nichtjes) ook. Toen het op de middelbare school tijd werd om maar eens te gaan kiezen voor een studievak heb ik altijd drie mogelijkheden voor ogen gehad, medicijnen, geschiedenis en biologie. Om uit te zoeken of ik medicijnen wat vond heb ik na de middelbare school een korte tijd bij de eerste hulp van het AMC gewerkt. Hoewel de mensen op die afdeling erg aardig waren, zonk de moed mij al gauw in de schoenen zodra ik de poliklinieken en verpleegafdelingen betrad. Zo was deze keuze al gauw verkeerd. Er bleven over geschiedenis en biologie. Al sinds de vierde of vijfde klas stond ik met (nu ook gepromoveerd) Bastiaan te babbelen over onze interesse in de biologie. Samen naar de UvA en biologie studeren, dat leek ons wel wat. Vanaf 1996 kon de pret beginnen en hebben we op de Anna’s Hoeve in de Watergraafsmeer veel geleerd en lol getrapt. Eind 1998 was het toen tijd voor de eerste wetenschappelijke stage. Dat ik onderzoek wilde doen was voor mij al duidelijk, maar in welk vakgebied van de biologie dat dan zou moeten, ik had geen idee. Gelukkig kwam ik bij Karel van Dam terecht, die ervoor zorgde dat ik al gauw verkocht was aan het onderzoek naar bakkersgist. Het organisme! Je maakt er brood, bier en wijn mee, wat wil je nog meer. Zo ben ik van gist onderzoek naar gistonderzoek gerold en ben ik na mijn afstuderen dit AIO project begonnen. Daarvan staan nu de vruchten beschreven in de hiervoorgaande delen (die jullie lezers natuurlijk uitvoerig bestudeerd hebben).

Als eerste wil ik graag Joost bedanken (Texairia da Mottas toch?), voor alle begeleiding, het doorstaan van al mijn vragen (hoe reken je de q in batches ook weer uit?)en het geduld bij mijn ongelooflijke schrijfkunst, maar ook zeker voor de gezelligheid. Lekker een shaggy roken op de kamer van Joost en babbelvuren over van alles en nog wat, totdat de hele kamer blauw stond en er fronzende blikken van buiten kwamen over hoe we het konden overleven daarbinnen. Veel vrijheid om je onderzoek zelf te sturen was er ook, dit leidde tot een voor mij zeer prettige manier van werken. Ook Klaas wil ik ontzettend bedanken voor het in het gareel houden als ik in mijn vrijheid weer eens wat minder interessante resultaten produceerde. Ook tijdens werkbesprekingen en rapportages konden we altijd goed discussiëren om zo toch het onderste uit de dek te halen. Op deze manier kwam alles toch steeds weer op zijn pootjes terecht. Dan wil ik natuurlijk ook alle collega’s waar ik al die jaren mee heb samengewerkt hier op het microbiologie lab bedanken en dat zijn er over de jaren velen geweest. Laat ik maar beginnen met Arthur (waar ben je nou?) en Frank. Zonder jullie had ik nog altijd met drie slecht functionerende chemostaten en een kapotte HPLC gezeten. Dan Jos, Jocelyne, Alex en Remco, mijn kamergenoten voor het grootste gedeelte van de periode dat ik aan dit project heb gewerkt. Jos, bleek sinds het BCP al, zelfs mijn vader te kennen uit vroegere tijden. Jocelyne zat altijd achter de computer, maar als er gepraat werd over de wetenschap dan wist zij door handbewegingen de eiwitten “precies” na te bootsen. Alex
en Remco (bedankt voor de Bacillus) voor de gezellige uurtjes op de kamer en in de kroeg. Toendertijd werkte ik met Femke, Ania, Andre, Catarina, Piet (Pietje!!) en later ook Grażyana aan de gistjes en door ons toedoen zijn er vele gistcellen gesneuveld. Andre ook nog speciaal bedankt voor de uitleg bij je T-profiler tool, want in het begin snapte ik er de ballen van. Janelle (labmommy) en Andrea, thanks for all the fun in the lab, the bars (Irish pubs yay) and on the lab days. Martijn (al dat shifty x-en en y-en) en Michael (nog een keertje long island ice tea in Berlijn?), ik denk dat we de nieuwe filmzaal in Kriterion wel gefinancieerd hebben over de jaren. In het onderzoek gedurende die tijd nooit veel met elkaar betrokken geweest, maar dat maakte we ruimtjesschool goed in Kriterion, Abe Veneto of een andere drinkgelegenheid. Isil (yes, in Dutch), jij ook bedankt, je kwam er wat later bij maar ondanks dat hebben toch prettig samen aan gistjes kunnen werken en de nieuwe stroming het fermentatisme opgericht (met 10 geboden en al). Marcela, Gert-Jan, Pjotr, Ronald (ook al mede oprichter van het fermentatisme) en Merel, Kriterion zal ons nog lang zien. Alle mensen van de groep van Stanley (eerst dezelfde groep, later opgesplitst) wil ik bedanken voor discussies tijdens besprekingen en natuurlijk meer in het oude vertrouwde Kriterion. Stanley, Gertien, Hans, Jarne, Rick, Alex, Muus, Marian, Marcel en Richard bedankt. Ook wil ik alle mensen op de VU bedanken voor de samenwerking, en het prettige verblijf aldaar. Barbara, Sergio (did we ever make an exact 5.00sec assay?), Arjen, Alex, Karen, Jildau, Willem (wordt er nog geschaakt?) en Coen en Karin (de Stelling zal wel verlaten zijn tegenwoordig). Jack en Pascale uit Delft bedankt voor de prettige samenwerking. De studenten die het toch aan durfden bij dat langharig tuig stage te gaan lopen, Lennart, Ulrika en Rik ook bedankt. De plantenfysiologen Bastiaan (wat is je petje blauw), Julian (wel rechtuit op de dijk fietsten), Bas en Kai (ik moet wat eten), met jullie op zonnige dagen ’s avonds laat op zoek naar bier was altijd een welkome afwisseling met binnenzitten in Kriterion. Ook die andere vaak aanwezigen, Teun, Chris, Steven, Sergio, Wilfried, Gerben, Ringo, Lotje bedankt voor de gezellige borrels. Al was het voor dit project toch wil ik ook Karel (dankzij jou zit (zat) ik in de gisten), Jasper, Arle (let’s do an experiment at 3.00 a.m.) en Leonie bedanken om mij enthousiast te houden voor de gistfysiologie. Frans en Edwin (we motten maar weer eens jammern) ook bedankt voor het gist tijdperk bij de plantfysiologen en Melle, toch wel zo’n 8 jaar mijn huisgenoot geweest, de Erik de Roodstraat dat was een tijd om nooit te vergeten (omgekeerde kerstboom en beugelverzameling, Bastiaan als meubilair). Lekker chillen daar op de bank, in het Vondelpark, luisteren naar Pantera of Public Enemy, een welkomse afwisseling na een dagje in het lab. Tom, Ria, Stijn en Phinneo, het was altijd gezellig in de Willy Sluiterstraat. Helaas in Soundgarden of gewoon bij jullie in de tuin is het ook altijd heel gezellig (ik moet maar weer eens langskomen).

Pa en Ma, zuslief Milou (ook al biolooge) altijd was het fijn om thuis in Muiden te zijn, als jullie gewoon in Amsterdam waren blijven wonen, dan woonde ik er nu misschien wel nog. Opa’s en oma’s ook bedankt! Dan als laatste, Ester, mijn lief, mijn vrouw nu zelfs, dankzij jou geduld met mijn humeur na een dagje stress, is er nu hier een mooi boekje en zijn we nog samen. We hebben nu een mooie kleine jongen, Jacob, die dit boekje nog wel voor zijn neus krijgt, maar voor nu voor jullie allebei een dikke zoen! Allen bedankt!!

Merijn