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Identification and regulation of genes involved in anaerobic growth of *Saccharomyces cerevisiae*

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Ishtar Snoek

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“Identification and regulation of genes involved in anaerobic growth of
Saccharomyces cerevisiae.”

by Ishtar Snoek

For my father and my mother,
without whom I would never have done this.

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Chapter 1

Factors involved in anaerobic growth of *Saccharomyces cerevisiae*



I.S. Ishtar Snoek and H. Yde Steensma, 2007.
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Introduction

Since the introduction of molecular oxygen in the atmosphere, a multitude of organisms has evolved that need this compound to survive. However, there are still organisms that can grow anaerobically, and even those that can survive under both conditions. The question is what the difference is between these organisms. Why can some grow only in the presence of molecular oxygen, some only in the absence, and are some able to withstand both conditions? The yeast *Saccharomyces cerevisiae* is one of the few yeasts with the capacity to grow rapidly both under aerobic and anaerobic conditions (Visser *et al.*, 1990). This property has made it one of the most abundantly used yeasts in industry. Anaerobic incubation of *S. cerevisiae* plays a major part in the production of both alcoholic beverages and of bread.

Another industrial interest in anaerobic growth arises because of the problems with oxygen gradients encountered in voluminous aerobic fermentations. High cell densities required for the production of heterologous proteins may lead to gradients in the oxygen concentration as a result of imperfect mixing. In general, full levels of oxygenation are almost impossible to maintain in large-scale fermenters. Local and transient hypoxic or anaerobic conditions will trigger transcriptional and metabolic changes in the cells, which could lead to fermentation and thus disturb the production process. Manipulating the activity of a transcription factor that controls key enzymes of specific metabolic pathways, could be a solution. For example, over-expression of Hap4 resulted in partial relieve of glucose repression of respiration (Blom, Texeira de Mattos, and Grivell, 2000), and disruption of *MIG1*, alone or in combination with *MIG2* resulted in the partial alleviation of glucose control of sucrose and galactose metabolism (Klein *et al.*, 1999). Because other mechanisms may also control the intended pathway, the effects are often only partial.

Yet another possible industrial application of anaerobic growth lies in the transfer of this ability to other organisms. For example, the yeast *Kluyveromyces lactis* can utilize lactose as a sole carbon source. This sugar is the major component of whey, which is a waste product of cheese industry. Conversion of whey to ethanol would greatly reduce the costs and environmental strain of this industry. *K. lactis* is able to ferment, but can not grow under anaerobic conditions (Breunig and Steensma, 2003). Transfer of the genetic information

for anaerobic growth from *S. cerevisiae* could be a solution to this problem. A similar case can be made for the bioethanol production from lignocellulosic hydrolysates, which mainly contain xylose. In this case the organism that would be subjected to a transplantation of the ability for anaerobic growth, is *Pichia stipitis* (Shi and Jeffries, 1998).

Bioethanol is most commonly produced by anaerobic fermentations with *S. cerevisiae*. Many attempts have been made to increase the overall conversion yield from glucose to ethanol. Recently, Bro *et al* (2005) have used a genome-scale metabolic network model in order to find target genes for metabolic engineering (Bro *et al.*, 2005; Bro *et al.*, 2005).

Apart from being fundamentally interesting, insights in the processes that are important for anaerobic growth in *S. cerevisiae* and in the mechanisms that control them can help to solve problems industry is facing with respect to the anaerobic growth of organisms.

Fermentation

In the absence of molecular oxygen, the enzymes pyruvate decarboxylase and alcohol dehydrogenase convert pyruvate into ethanol and carbon dioxide to reoxidize the two molecules of NADH which were produced in glycolysis (Barnett, 2003). This process is known as alcoholic fermentation. As a consequence only 2 ATP molecules are formed from one molecule of glucose.

The ability to ferment sugars is a necessity for growth under anaerobic conditions. Although few yeast species are able to grow without oxygen (Visser *et al.*, 1990), most of them are able to ferment (van Dijken *et al.*, 1986; van Dijken *et al.*, 1986). When a hexose is imported into the cell, it is broken down by glycolysis into two molecules of pyruvate. During glycolysis there is a net production of two molecules of ATP and two molecules of NADH.

Under aerobic conditions NAD⁺ is regenerated by transfer of the electrons of NADH to the first protein of the respiratory chain. In *S. cerevisiae* the main entry point of NADH in the respiratory chain is the NADH-Q oxidoreductase Ndi1p, which faces the matrix of the mitochondria (Yagi *et al.*, 2001; Yagi *et al.*, 2001). The subsequent process of respiration results in the reduction of molecular oxygen to water and to the generation of a proton gradient along

the mitochondrial membrane. This gradient, which is also called the proton-motive force is then used to drive ATP-synthase, a mitochondrial-membrane enzyme complex (Mitchell, 1966). Also, the pyruvate produced by glycolysis can be further dissimilated to carbon dioxide and water via the pyruvate dehydrogenase complex and the tricarboxylic acid cycle, which results in an additional ATP molecule as well as five redox equivalents. In total, the complete respiratory dissimilation of one molecule of glucose results in 16 ATP molecules (van Maris, 2004).

Oxygen may be a key factor in the regulation of pyruvate decarboxylase activity. In Crabtree-negative (see below) yeasts like *Candida utilis* and *K. lactis* the levels increase only under oxygen-limited conditions, while in Crabtree positive yeasts, such as *S. cerevisiae*, high levels of this enzyme are present also under aerobic conditions (Kiers *et al.*, 1998; Weusthuis *et al.*, 1994). Thus, fermentation would likely be a response to oxygen limitation, which indeed it is in many cases. Interestingly, *K. lactis* could be turned into a Crabtree positive yeast by inactivation of the pyruvate dehydrogenase complex (Zeeman *et al.*, 1998).

When alcoholic fermentation occurs under aerobic conditions, this is called the Crabtree effect (de Deken, 1966). The long term Crabtree effect is the occurrence of aerobic fermentation under fully adapted, steady-state conditions at high growth rates, which has been explained in terms of a limited respiratory capacity of the yeast (Fiechter, Fuhrmann, and Kappeli, 1981; Kappeli, 1986), and an uncoupling effect of acetate, formed at high growth rates (Postma *et al.*, 1989). The short-term Crabtree effect is the sudden fermentative response under fully aerobic conditions upon addition of excess sugar to yeasts that did not ferment before this addition (Verduyn *et al.*, 1984). The increased flux of sugar entering the cell results in an increased production of NADH, which cannot be completely oxidized by the respiratory chain. Thus, the production of ethanol and acetate by fermentation is needed to remove the excess NADH (Kappeli, 1986; Kolberg *et al.*, 2004). Crabtree positive yeasts, such as *S. cerevisiae* and *K. lactis*, have facilitated-diffusion glucose-transport systems with much higher K_m values for glucose than the high-affinity proton-symport mechanisms that are common in Crabtree negative yeasts (van Dijken, Weusthuis, and Pronk, 1993).

A related phenomenon is the Pasteur effect, which is defined as the inhibition of the sugar consumption rate by aerobiosis. The common

explanation of this phenomenon is that fermentation cannot effectively compete with respiration, in terms of ATP yield, and that this in turn leads to a reduced fermentation rate under aerobic conditions (Lagunas, 1986). In *S. cerevisiae* the Pasteur effect occurs in aerobic sugar-limited chemostat cultures, and in resting-cells suspensions, because of low sugar consumption rates (Weusthuis, 1994).

The Kluyver effect is widespread among yeasts and is the phenomenon that any given yeast may be able to ferment certain sugars, but not others (Sims and Barnett, 1991). There are several factors that may cause this effect: oxygen requirement for sugar transport, activity of the pyruvate decarboxylase (Barnett, 1992), and product inhibition (Weusthuis *et al.*, 1994).

Even when a particular yeast species is capable of fermenting different sugars, the results of these fermentations may be different. For example, in *S. cerevisiae*, maltose is co-transported with protons in a one to one stoichiometry: proton-symport. This import requires the hydrolysis of 1 molecule of ATP per molecule maltose imported. Therefore, the anaerobic growth on maltose yields a higher specific ethanol production as compared to the fermentation of glucose (Weusthuis *et al.*, 1993).

Fermentation is a redox neutral process and any redox equivalents produced in other processes, should be reoxidized by the production of glycerol or other highly reduced compounds. The Custers effect occurs in the *Brettanomyces*, *Dekkera* and *Eeniella* genera. These yeasts show an anaerobic inhibition of fermentation of glucose to ethanol and acetate, which is thought to be the result of redox problems (Scheffers, 1996).

Non-respiratory oxygen-utilizing pathways

Molecular oxygen is not only essential for respiration, but is also required in several biosynthetic pathways, like those for heme, sterols, unsaturated fatty acids, pyrimidines and deoxyribonucleotides (Andreasen and Stier, 1953; Chabes *et al.*, 2000; Nagy, Lacroute, and Thomas, 1992). These reactions have been reviewed recently (Snoek and Steensma, 2006) but are briefly summarized here for completeness.

The synthesis of heme is dependent on traces of molecular oxygen and there is no known way to eliminate this requirement. It has been suggested that

in anaerobically growing cells, the heme released by degradation of respiratory cytochromes, is recycled in the cytoplasm (Clarkson *et al.*, 1991; Kwast *et al.*, 2002). The dependency of the biosynthesis of heme on oxygen also implies that production of hemeoproteins, most of which are cytochromes, requires oxygen. There may be anaerobic alternatives for these proteins (Dunn *et al.*, 1998; Kwast *et al.*, 2002; Stukey, McDonough, and Martin, 1990). However, these proteins still need heme and thus oxygen. If the cells are growing, recycled heme cannot account for it all and cells should have alternative solutions to this problem.

A second pathway that requires oxygen is the biosynthesis of sterols (figure 1). Sterols are produced in an oxygen-dependent way, through the activities of six Erg enzymes. For the synthesis of one molecule of ergosterol, twelve molecules of molecular oxygen are needed (Rosenfeld and Beauvoit, 2003). Under anaerobic conditions the cells no longer synthesize sterols, but instead import them. This sterol uptake is essential under anaerobic conditions (Wilcox *et al.*, 2002) and depends on the cellular levels of ergosterol and oleate (Burke *et al.*, 1997; Ness *et al.*, 1998). Oleate is added to media for anaerobic growth in the form of Tween 80, and can be used as a source for unsaturated fatty acids (UFA's), the production of which is also oxygen dependent. The transport might be a result of the permeability of the membrane, combined with specific transporters (Alimardani *et al.*, 2004; Faergeman *et al.*, 1997; Ness *et al.*, 2001; Tinkelenberg *et al.*, 2000; Trotter, Hagerman, and Voelker, 1999).

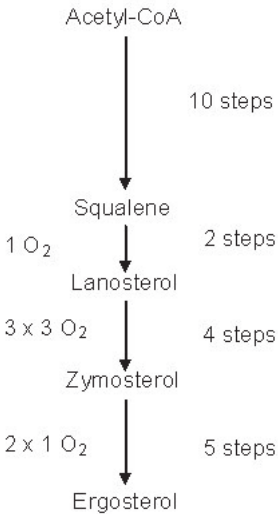


Figure 1: Molecular oxygen-requiring steps in the ergosterol biosynthesis pathway (Rosenfeld and Beauvoit, 2003).

Synthesis of pyrimidines is also oxygen dependent. The fourth step in the process, the conversion of dihydroorotate to orotate is catalyzed by dihydroorotate dehydrogenase (DHDODase), which is a respiratory chain-dependent mitochondrial protein in most yeasts. However, *S. cerevisiae*, which is able to grow anaerobically, has a cytosolic DHDODase. This enzyme is not dependent on the functionality of the respiratory chain (Gojkovic *et al.*, 2005). Indeed, transfer of the *S. cerevisiae* DHODase gene (encoded by *URA1*) into *Pichia stipitis* transformed this yeast into a facultative anaerobe (Shi and Jeffries, 1998).

Biosynthesis of deoxyribonucleotides is catalyzed by ribonucleotide reductases (RNR's) (Kolberg *et al.*, 2004). These enzymes convert the ribonucleotides into their deoxyribonucleotide counterparts. There are three major classes of RNR's. Members of class I are dependent on the presence of oxygen, members of class III function in the absence of oxygen and members of class II can reduce ribonucleotides under both conditions. Until now only class I RNR's have been found in yeast species. However, since the 3D structures of the three classes are quite similar, while the sequence homology is very low, it could be that a class II or III RNR is present in the yeasts that are able to grow without oxygen.

Nicotinic acid is required for the synthesis of NAD⁺ and *S. cerevisiae* can synthesize it from tryptophan via the kynurenine pathway. The nicotinate moiety can also be recycled and be incorporated in NAD⁺ directly by the activity of nicotinate phosphoribosyl transferase (Npt1). Only the second pathway is oxygen-independent. Since there is no other way to synthesize NAD⁺, the *NPT1* gene is essential under anaerobic conditions (Panozzo *et al.*, 2002).

Under aerobic conditions the reoxidation of NADH formed during glycolysis occurs through the respiratory chain, transferring the reducing equivalents to oxygen. This is not possible during anaerobiosis. Several ways to reoxidize NADH are known in *S. cerevisiae*. Apart from alcoholic fermentation, the genes *FRDS* and *OSM1* encode fumarate reductases, which irreversibly catalyze the reduction of fumarate to succinate, thereby reoxidizing NADH. Other ways to reoxidize excess NADH are through the actions of Gpd2, which is a glycerol-3-phosphate dehydrogenase and produces glycerol, and Adh3, which is a mitochondrial alcohol dehydrogenase (Ansell *et al.*, 1997; Bakker *et al.*, 2000).



Transcriptional, translational and post-translational control

The adaptation of *S. cerevisiae* to an anaerobic environment, as compared to conditions in which oxygen is present, takes place at different levels in the cell. First, there is the evolutionary adaptation. Since this yeast has been used in anaerobic processes for centuries, it has adapted to living without oxygen more

than any other known yeast strain. The ability to grow anaerobically is believed to originate from the whole genome duplication around one hundred million years ago (Piskur and Langkjaer, 2004; Wolfe and Shields, 1997). Species such as *K. lactis*, which diverged from a common ancestor before this event, are not able to grow without oxygen. Today the evolutionary favoring of a predominantly fermentative metabolism, which is an essential part of the ability to grow anaerobically, of *S. cerevisiae* in the wild can still be seen in its codon bias, and it is therefore termed a translationally biased organism (Carbone and Madden, 2005).

Adaptation of the yeast cell to an anaerobic environment requires transcriptional changes of genes that are differentially needed under anaerobic and aerobic conditions. Several factors for transcriptional regulation of anaerobic metabolism have been proposed (Zitomer and Lowry, 1992). *ROX1*, which is one of the targets of Hap1 (Zhang and Guarente, 1995) (Hach, Hon, and Zhang, 1999), together with the Tup1/Ssn6 complex, represses hypoxic genes in the presence of oxygen (Deckert *et al.*, 1995). In another regulatory system *UPC2* and *ECM22* are implicated in a dual role in the induction of anaerobic sterol import (Crowley *et al.*, 1998; Shianna *et al.*, 2001; Ter Linde, 2003; Davies, Wang, and Rine, 2005). The induction of *UPC2* upon anaerobiosis appears to be the result of heme-depletion. Another factor that has been implicated in the sterol import system, needed under anaerobic conditions, is Sut1. Sut1, and perhaps also Sut2, has a regulatory effect on the permeability of the membrane (Alimardani *et al.*, 2004). The expression of Sut1 increased following a shift to anaerobic conditions. Other genes have also been implicated in anaerobic regulation either because of their effect on transcriptional levels or because of their heme-dependency, such as Mot3, Mox1, Mox2 (Abramova *et al.*, 2001), Ord1 (Lambert JR, Bilanchone VW, and Cumsy MG, 1994), and Hap2/3/4/5 (Zitomer and Lowry, 1992). All of these genes together regulate the expression of aerobically and anaerobically specific genes in a complex way.

However, the transcriptional responses to anaerobiosis of many genes are still unexplained, such as the *PAU* genes, which are genes of unknown function that have a strong and consistent higher transcription level under anaerobic conditions (Tai *et al.*, 2005). Also, the transcriptional changes of the cell wall proteins Dan1 and Tir1 when aerobic conditions are compared to anaerobic ones, cannot be explained by the alleviation of aerobic repression by Rox1 alone

(Kitagaki H, Shimoi H, and Itoh K, 1997; Ter Linde and Steensma, 2002). It has been shown that for the *DAN/TIR* genes activation through Upc2 is necessary. Repression seems to be mediated by Rox1, Mot3, Mox1, Mox2 and the Tup1/Ssn6 complex (Abramova *et al.*, 2001). Repression of *ANB1* is not completely abolished by deletion of *ROX1*, suggesting that in this case activation is also needed (Ter Linde and Steensma, 2002). Furthermore, the promoter of the anaerobically higher expressed *YML083C* gene does carry a Rox1 binding site, but deletion of these bases has no effect on transcription levels (Ter Linde and Steensma, 2003). It thus appears that alleviation of repression is not enough for a gene to be anaerobically activated. To achieve this, activators are necessary as well.

Not always can transcription alone account for the observed changes in protein activity, as was demonstrated for the presence of active catalases under anaerobic conditions (Hortner *et al.*, 1982). The third level of regulation is the formation of active protein. This is dependent on several processes, such as the mRNA stability, mRNA export, translation of the mRNA into protein, protein folding and stability and finally protein activation. For example, transcription of the anaerobic gene *ANB1* is regulated by oxygen and heme via Rox1p. *ANB1* is probably the yeast homologue of the eukaryotic translation initiation factor eIF-4D. Apart from influencing translational initiation, the protein itself undergoes a post-translational modification of the Lys-50 residue to the amino acid hypusine (Mehta *et al.*, 1990). Another example is *SOD1*, which is posttranslationally activated through the delivery of copper to the enzyme by the copper chaperone for *SOD1* (CCS) to accommodate a fast response to a sudden elevation of oxygen availability (Brown *et al.*, 2004).



Plasma membrane and cell wall modulation

The plasma membrane forms a relatively impermeable barrier for hydrophilic molecules. It consists of a bilayer of polar lipids and proteins. These proteins are often associated with other proteins in the plasma membrane or with the cytoskeleton. They can be either intrinsic, spanning the whole membrane, or extrinsic, embedded in part of the membrane and protruding from one side. Functions of these proteins vary from amino acid transporters,

sugar transporters and ATPases, to proteins involved in cell wall synthesis and signal transduction. Some proteins that are part of the cytoskeleton are also located in the cell wall. The lipids are disposed asymmetrically across the bilayer and vary greatly in size and composition, which is tightly regulated. They probably also play a role in the activity of the embedded proteins. Some membrane-associated processes, such as amino acid transport and membrane ATPase activity, are affected by a changed lipid composition. The rigidity of the membrane is largely determined by the sterol content. This may affect the lateral movement and activity of membrane proteins. Alternatively, sterols may also create patches into which polypeptides can insert (van der Rest *et al.*, 1995).

The lipid composition of the membrane under anaerobic conditions is different from that of cells grown under aerobic conditions. Anaerobically, the plasma membrane contains less unsaturated fatty acids, less sterol, less ergosterol and less squalene (Nurminen, Konttinen, and Suomalainen, 1975). These differences can be explained by the inability of the cell to synthesize these compounds without oxygen.

The cell wall is a rigid structure that surrounds the cell and gives it its shape. It protects the cell from the effects of outside conditions such as heat, cold and osmotic stress. It also works as a selection filter for the entrance of substances into the cell.

The cell wall is composed of several layers, the first of which contains β 1,3-glucan and chitin. These compounds are responsible for the mechanical strength of the cell wall. The outer layer consists of heavily glycosylated mannoproteins. These make the inner layer less accessible to cell wall-degrading enzymes. The porosity of the cell wall is mainly determined by this outer layer, because of the long and highly branched carbohydrate side chains linked to asparagine residues. The inner layer is highly porous and limits only the passage through of very large molecules. The way in which the mannoproteins are linked to the inner layer divides them in two groups. GPI-dependent cell wall proteins (GPI-CWPs) are linked indirectly through a β 1,6-glucan moiety. Pir proteins (Pir-CWPs) are directly linked to β 1,3-glucan. The cell seems to be able to repair cell wall damage, among others through the Slt2 MAP kinase pathway, which is rapidly induced upon stress. Sensing of the damage is probably the result of plasma membrane stretch. The sensors, such as Mid2 are linked to the β 1,3-

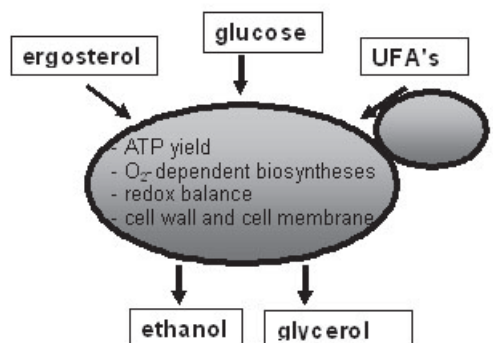
glucan network in a Pir-like fashion. Generally the activation of the Slt2 MAP kinase pathway leads to the activation of several cell wall reinforcing reactions, one of which is the elevation of chitin levels. Another MAP kinase pathway, the Hog1 pathway, is also implicated in the cell wall construction, both under stress and non-stress conditions (Klis *et al.*, 2002).

Upon anaerobiosis there is a general remodeling activity associated with the cell wall and plasma membrane. This remodeling is required, in part, for the efficient import and processing of the supplements needed under these conditions, such as oleate and ergosterol, in order to combat the compromised ability to regulate membrane fluidity (Kwast *et al.*, 2002). However, these changes are slow to occur and take several generations for completion (Lai *et al.*, 2005). Generally, transcript levels of *CWP1* and *CWP2* decrease, while those of the seripauperin family genes, such as the *DAN*, *TIR* and *PAU* genes, increase (Klis *et al.*, 2002). These changes are quite drastic and suggest a complete switch from one set of GPI-CWP's to another. It is not known how this change facilitates the import of supplements and if perhaps it has some additional functions.

Concluding remarks

Growth in the absence of molecular oxygen requires adaptation of the cell for at least three reasons. First, energy yield is usually much lower than under aerobic conditions, second several biosynthetic pathways require molecular oxygen and third, different molecules have to be transported into and out of the cell (figure 2).

Figure 2: Major changes under anaerobic conditions in comparison to aerobic conditions. The lower ATP yield and maintenance of redox balance require increased uptake of glucose and lead to the excretion of ethanol and glycerol. The inability to synthesize sterols and unsaturated fatty acids may induce cell wall and cell membrane changes to allow uptake of these substances.



○ Outline of this thesis

Yeasts are among the few eukaryotic organisms that can grow under anaerobic conditions, and not even all yeast species can do that. It has been known which genes are essential for *S. cerevisiae* to grow aerobically. In chapter 2 a systematic screen for anaerobically essential genes is described. As it turned out, almost all anaerobically essential genes are also aerobically essential. Only a few genes are essential specifically under anaerobic conditions as compared to aerobic ones. Also, none of the anaerobically essential genes has a higher transcription level under anaerobic conditions. In chapter 3 a competitive fitness experiment is described in which deletion strains of several genes that have a consistent higher transcription level under anaerobic conditions have to compete with a wild type strain under anaerobic chemostat conditions. Upregulation of these genes under anaerobic conditions only contributes marginally to fitness under the conditions tested.

Several studies have demonstrated that more than 300 genes are changed in transcriptional expression levels when aerobically grown cells are compared to anaerobically grown cells (Ter Linde *et al.*, 1999) (Piper *et al.*, 2004). However, not all of these genes are regulated by the known regulatory pathways, such as the Hap1/Rox1 pathway, or the Upc2/Ecm22 pathway (Kwast *et al.*, 2002) (Ter Linde and Steensma, 2003). This PhD project set out to find more regulatory elements specific for anaerobic conditions. This is described in chapter 4. Four putative upregulators were identified. Unfortunately the transcriptomics data showed that the identified putative transcription factors were not anaerobically specific. However, the data from the *spt3* deletion strain, described in chapter 5, showed that although the activity of the protein this gene encodes is not anaerobically specific, the set of genes that responds to the absence of Spt3 is. A model is proposed in which SAGA, of which Spt3 is a component, integrates the environmental conditions the cell is facing to come to a transcriptome profile that ensures optimal adjustment to this set of conditions.

In chapter 6 the results of the experiments done on the *snf7* deletion strain are reported. Regulation by the Snf7 protein did not show anaerobic specificity per se, but specificity for cell wall and plasma membrane proteins

was observed, some of which are expressed only under anaerobic conditions. It is hypothesized that Snf7 is a general remodeling factor that regulates modulation of the cell wall and the plasma membrane in response to several environmental changes, of which anaerobicity is one.

Chapter 2

Why does *Kluyveromyces lactis* not grow under anaerobic conditions?

Comparison of essential anaerobic genes of *Saccharomyces cerevisiae* with the *Kluyveromyces lactis* genome

○ Abstract

While some yeast species, e.g. *Saccharomyces cerevisiae*, can grow under anaerobic conditions, *Kluyveromyces lactis* can not. In a systematic study we have determined which *S. cerevisiae* genes are required for growth without oxygen. This has been done by using the yeast deletion library. Both aerobically essential and non-essential genes have been tested for their necessity for anaerobic growth. By comparison of the *K. lactis* genome with the genes found to be anaerobically important in *S. cerevisiae*, which yielded 20 genes that are missing in *K. lactis*., we hypothesize that import of sterols might be one of the more important reasons that *K. lactis* cannot grow in the absence of oxygen.

Introduction

The yeast *Kluyveromyces lactis* is industrially interesting because it is able to grow on lactose as a sole carbon source (Breunig and Steensma, 2003). This sugar is one of the main components of whey, which is a waste product of the production of cheese. If the lactose in whey could be converted to ethanol, the costs and environmental strain of waste disposal in this industry could be greatly reduced. The respiro-fermentative nature of metabolism in *K. lactis*, however, is limiting the efficiency of this process. Anaerobic growth could lead to full fermentation and thus higher production of ethanol by this yeast. Attempts have been made to transfer the ability of *K. lactis* to utilize lactose as a carbon source to *Saccharomyces cerevisiae*, but so far no industrially applicable yeast strain has emerged from this approach (Rubio-Teixeira *et al.*, 1998).

Yeast species differ in the ability to grow under anaerobic conditions. Only a few species can grow as successfully under anaerobic as under aerobic conditions, as was demonstrated by Visser *et al.* (Visser *et al.*, 1990). Molecular di-oxygen is needed as the terminal oxidator in the respiratory pathway, leading to the production of energy. Oxygen is also required in several biosynthetic pathways, like those for heme, sterols, unsaturated fatty acids, pyrimidines and deoxyribonucleotides (Andreasen and Stier, 1953; Chabes *et al.*, 2000; Nagy, Lacroute, and Thomas, 1992). Cells growing under anaerobic conditions obviously found ways to circumvent the oxygen dependency of these pathways. Without oxygen, energy can be produced by switching to fermentation. Although *K. lactis* is able to ferment, it cannot grow under anaerobic conditions (Kiers *et al.*, 1998). The problem may lie in the oxygen dependency of biosynthetic pathways. In the following paragraphs the different problems arising from the absence of oxygen will be discussed briefly, in relation to what is known in other organisms, in particular *S. cerevisiae*.

The synthesis of heme is dependent on traces of molecular oxygen and there is no known way to eliminate this requirement. It has been suggested that in anaerobically growing cells, the heme released by degradation of respiratory cytochromes, is recycled in the cytoplasm. In *S. cerevisiae* Mdl1 is a putative mitochondrial heme carrier that is upregulated under anaerobic conditions. This protein may be responsible for the transport of heme from the mitochondrial matrix to the cytoplasm (Clarkson *et al.*, 1991; Kwast *et al.*,

2002). The dependency of the biosynthesis of heme on oxygen also implies that production of hemeoproteins, most of which are cytochromes, requires oxygen. There may be anaerobic alternatives for these proteins. One study in *S. cerevisiae* showed that the hemoproteins Erg11, Cyc7, Ole1 and Scs7 are all upregulated under anaerobic batch culture conditions (Kwast *et al.*, 2002). However, only Scs7 was induced under anaerobic glucose-limited chemostat culture conditions (Ter Linde *et al.*, 1999). *ERG11* and *CYC7* are known to code for cytochrome P450 and cytochrome *c* respectively. Ole1 on the other hand is a fatty acid desaturase, required for monounsaturated fatty acid synthesis (Stukey, McDonough, and Martin, 1990), while Scs7 is a desaturase/hydroxylase, required for the hydroxylation of very long chain fatty acids (VLCFA) (Dunn *et al.*, 1998). These proteins still need heme and thus oxygen. If the cells are growing, recycled heme cannot account for it all and cells should have alternative solutions to this problem.

A second pathway that requires oxygen is the biosynthesis of sterols. Under aerobic circumstances sterols are produced in an oxygen-dependent way, through the activities of six Erg enzymes. For the synthesis of one molecule of ergosterol, twelve molecules of molecular oxygen are needed (Rosenfeld and Beauvoit, 2003). Under anaerobic conditions the cells no longer synthesize sterols, but instead import them. This sterol uptake is essential under anaerobic conditions (Wilcox *et al.*, 2002). Transfer depends on the cellular levels of ergosterol and oleate (Burke *et al.*, 1997; Ness *et al.*, 1998). The transport might be a result of the permeability of the membrane. The transcription factor Sut1, and perhaps also Sut2, has a regulatory effect on this permeability (Ness *et al.*, 2001). The expression of *SUT1* increased following a shift to anaerobic conditions. The transcription factor *UPC2* is also involved in sterol uptake (Wilcox *et al.*, 2002). Together these transcription factors upregulate transcription of *AUS1*, *PDR11* and *DAN1*, the products of which work in synergy to mediate sterol uptake (Wilcox *et al.*, 2002; Alimardani *et al.*, 2004). In another study, *ARV1* was identified as being required for sterol uptake and distribution. Strains having a deletion in this gene were unable to grow anaerobically (Tinkelenberg *et al.*, 2000).

Since the production of unsaturated fatty acids (UFA's) is oxygen dependent, the medium for growing cells anaerobically is usually supplemented with Tween80, which is a source of oleate. The presence of this compound

represses the transcription of *OLE1*, which encodes the Acyl-CoA desaturase, which is involved in the biosynthesis of palmitoleate and oleate. *FAT1* may encode a transporter involved in oleate uptake, which is required for anaerobic growth (Faergeman *et al.*, 1997). The mitochondrial protein Rml2 may also participate in the assimilation (Trotter, Hagerman, and Voelker, 1999).

Synthesis of pyrimidines is also oxygen dependent. The fourth step in the process, the conversion of dihydroorotate to orotate is catalyzed by dihydroorotate dehydrogenase (DHDODase), which is a respiratory chain-dependent mitochondrial protein in most yeasts. However, *S. cerevisiae*, which is able to grow anaerobically, has a cytosolic DHDODase. This enzyme is not dependent on the functionality of the respiratory chain (Gojkovic *et al.*, 2005). Indeed, transfer of the *S. cerevisiae* DHODase gene (encoded by *URA1*) into *Pichia stipitis* transformed this yeast into a facultative anaerobe (Shi and Jeffries, 1998).

Biosynthesis of deoxyribonucleotides is catalyzed by ribonucleotide reductases (RNR's) (Kolberg *et al.*, 2004). These enzymes convert the ribonucleotides into their deoxyribonucleotide counterparts. There are three major classes of RNR's. Members of class I are dependent on the presence of oxygen, members of class III function in the absence of oxygen and members of class II can reduce ribonucleotides under both conditions. Until now only class I RNR's have been found in yeast species. However, since the 3D structures of the three classes are quite similar, while the sequence homology is very low, it could be that a class II or III RNR is present in the yeasts that is able to grow without oxygen.

Nicotinic acid is required for the synthesis of NAD⁺ and *S. cerevisiae* can synthesize it from tryptophan via the kynurenine pathway. The nicotinate moiety can also be recycled and be incorporated in NAD⁺ directly by the activity of nicotinate phosphoribosyl transferase (Npt1). Only the second pathway is oxygen-independent. Since there is no other way to synthesize NAD⁺, the *NPT1* gene is essential under anaerobic conditions (Panozzo *et al.*, 2002).

Under aerobic conditions the reoxidation of NADH formed during glycolysis occurs through the respiratory chain, transferring the reducing equivalents to oxygen. This is not possible during anaerobiosis. Several ways to reoxidize NADH are known in *S. cerevisiae*. The genes *FRDS* and *OSM1* encode fumarate reductases, which irreversibly catalyze the reduction of fumarate to

succinate, thereby reoxidizing NADH. *FRDS1* (encoded by *FRDS*) is present in the cytosol and *FRDS2* (encoded by *OSM1*) in the promitochondria, which lack an integrated electron transfer chain and a functional oxidative phosphorylation system and therefore are considered to be inactive for energy production. A mutant with a deletion in both the *FRDS* and the *OSM1* genes is not able to grow under anaerobic conditions (Arikawa *et al.*, 1998; Enomoto, Arikawa, and Muratsubaki, 2002). Other ways to reoxidize excess NADH are through the actions of the *Gpd2*, which is a glycerol-3-phosphate dehydrogenase, and *Adh3*, which is a mitochondrial alcohol dehydrogenase. However, deletion of these genes only reduced the growth rate, but did not abolish growth under anaerobic conditions (Ansell *et al.*, 1997; Bakker *et al.*, 2000).

ADP/ATP carriers function in aerobic cells to exchange cytoplasmic ADP for intramitochondrially synthesized ATP. Under anaerobic conditions the same proteins work in opposite direction, exchanging ATP from glycolysis to the mitochondria. In *S. cerevisiae* three genes encode for these transporters, *AAC1*, *AAC2* and *AAC3*, all of which are transcribed in an oxygen dependent manner (Betina *et al.*, 1995; Sabova *et al.*, 1993; Gavurnikova *et al.*, 1996). Deletion of *AAC2* and *AAC3* was anaerobically lethal (Drgon *et al.*, 1991; Kolarov, Kolarova, and Nelson, 1990).

In addition to the presence of genes essential for anaerobic growth in the genome, metabolism must be redirected. For instance, due to the lower yield of fermentation in comparison to respiration a higher glycolytic flux and a higher uptake rate of sugars is necessary to maintain a high growth rate. Therefore the proper regulatory mechanisms must be present as well. In *S. cerevisiae* several transcription factors are involved. Hap1 is a factor that has been implicated in the regulation of transcription in response to the availability of oxygen. The protein forms a homodimer in response to heme-binding. This complex upregulates transcription of aerobic genes. One of those genes is *ROX1*, which represses the transcription of anaerobic genes (Deckert *et al.*, 1995). Both *UPC2* and *ECM22* are implicated in the induction of an anaerobic sterol import system (Crowley *et al.*, 1998; Shianna *et al.*, 2001). Other proteins that have been reported to influence transcription levels of anaerobic genes are *Sut1*, *Ord1*, and the *Hap2/3/4/5* complex (Ness *et al.*, 2001; Lambert JR, Bilanchone VW, and Cumsky MG, 1994; Zitomer and Lowry, 1992). In our laboratory also *SPT3*, *SPT4*, *SAC3* and *SNF7* have been found to encode anaerobic transcription factors (I.S.I.

Snoek, unpublished data). The absence of one or more of these genes may also result in the inability to grow anaerobically.

To answer the question why *K. lactis* cannot grow without oxygen while other yeast strains, like *S. cerevisiae* can (figure 1), we wished to determine whether *S. cerevisiae* has genes that are important for anaerobic growth, that *K. lactis* has not. We made use of the collection of *S. cerevisiae* gene-deletion mutants in strain BY4743 that was created by substituting each known ORF by a KanMX-cassette (Giaever *et al.*, 2002). We used the diploid parts of the collection. We tested each strain for its ability to grow anaerobically. This resulted in a list of anaerobically essential genes. In line with the definition by Giaever *et al.* (Giaever *et al.*, 2002) we have defined anaerobically essential genes as necessary for growth in YPD, supplemented with ergosterol and Tween 80. By comparing this list with the genome of *K. lactis* (www-archbac.u-psud.fr/genomes/r_klactis/klactis.html), we were able to identify several genes with little or no similarity in *K. lactis*. We discuss whether the absence of these genes may explain why *K. lactis* is not able to grow without oxygen.

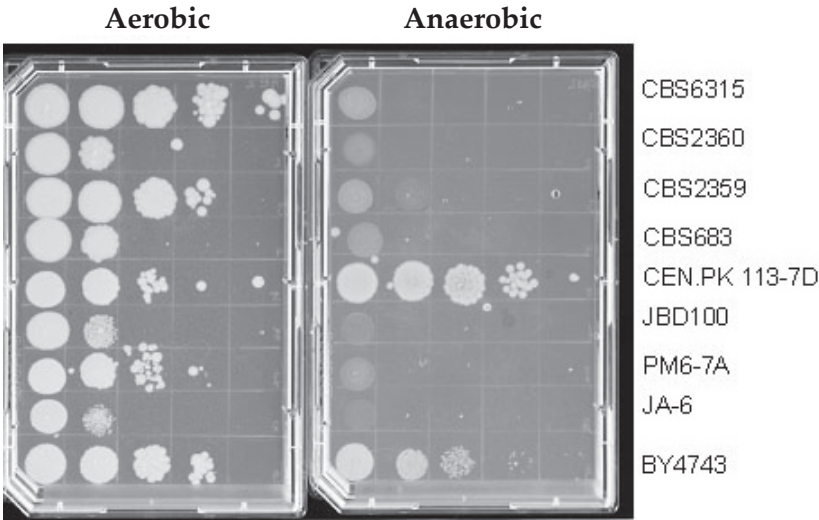


Figure 1: *S. cerevisiae* strains CEN.PK 113-7D and BY4743 and *K. lactis* strains CBS6315, CBS2360, CBS2359, CBS683, JBD100, PM6-7A and JA-6 grown under anaerobic and aerobic conditions. 4 µl of 10-fold dilutions were spotted onto two MYplus plates. Plates were photographed after four days incubation, either aerobically or anaerobically, at 30°C.



Materials and methods

Strains

Strains used are listed in Table 1. The *S. cerevisiae* mutant gene deletion collections 95401.H1 (homozygous diploids) and 95401.H4 (heterozygous diploids, essential genes only) were purchased from Research Genetics.

Media

Yeast cells were grown in YPD (Difco peptone 2%, Difco yeast extract 1%, glucose 2%), MY (Zonneveld, 1986), or MYplus. MYplus is MY with 1% casamino acids, adenine, uracil and L-tryptophan at 30 µg/ml and 10 µg/ml

Table1: Yeast strains used in this study

strain	Genotype	Source
<i>K. lactis</i> CBS6315	<i>Mata</i>	CBS, Utrecht, The Netherlands
<i>K. lactis</i> CBS2360	<i>Mata</i>	CBS, Utrecht, The Netherlands
<i>K. lactis</i> CBS2359	<i>Mata</i>	CBS, Utrecht, The Netherlands
<i>K. lactis</i> CBS683	-	CBS, Utrecht, The Netherlands
<i>K. lactis</i> JBD100	<i>MATa HO lac4-1 trp1 ara3-100</i>	(Heus <i>et al.</i> , 1990)
<i>K. lactis</i> PM6-7A	<i>uraA1-1 adeT-600</i>	(Wesolowski-Louvel <i>et al.</i> , 1992)
<i>K. lactis</i> JA-6	<i>MATa ade1-600 adeT-600 trp1-11 ura 3-12 KHT1 KHT2</i>	(Ter Linde and Steensma, 2002)
CEN.PK 113-7D	<i>Mata</i>	P. Kötter (J.-W. Goethe Universität, Frankfurt, Germany)
BY4743	<i>MATa/α his3Δ1/his3Δ1 leu2Δ0 / leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0 /ura3Δ0)</i>	Euroscarf, Frankfurt, Germany
<i>Lipomyces starkeyi</i> CBS1807		CBS, Utrecht, The Netherlands

ergosterol and 420 µg/ml Tween80. For anaerobic growth in YPD, 10 µg/ml ergosterol and 420 µg/ml Tween 80 were added, giving YPDET. When necessary, 150 µg/ml G418 was added. Sporulation medium contained 0.1% Difco yeast extract, 1% potassium acetate, 0.05% glucose. Media were solidified by adding 1.5 % agar (Sphero).

Anaerobic incubation

For anaerobic incubation of Petridishes the Anaerocult IS system (Merck) was used. Anaerobicity was monitored both by an indicator strip (Anaerotest, Merck) and by using a *Lypomyces starkeyi* strain, which cannot grow under anaerobic conditions. Liquid cultures were shaken at 150 rpm in an anaerobic cabinet (Bactron Anaerobic Chamber, Sheldon Inc.).

Anaerobic growth assay of *K. lactis* and *S. cerevisiae*

Strains were shaken overnight in 2 ml YPD medium at 30°C. The next day, the strains were used to inoculate 10 ml fresh YPD medium to an A_{655} of 0.2. After shaking at 30°C for another 4 h, the cells were diluted in water to an A_{655} of 0.2 and 4 µl of a 10-fold dilution series in water were spotted onto two MYplus plates. One of the plates was incubated aerobically for 4 days at 30°C, the other anaerobically also for 4 days at 30°C.

Identification of anaerobically essential genes in *S. cerevisiae*

The collection of homozygous and heterozygous deletion-strains obtained from Research Genetics was used. This collection consists of mutants of the strain BY4743 in which each ORF has been replaced by a KanMX-cassette as described by Giaever *et al.* (Giaever *et al.*, 2002).

The 95401.H1 version of the collection of homozygous deletion strains were grown overnight aerobically in 140 µl of YPD with G418 in flat-bottom 96-wells plates (Greiner, Germany). About 1-2 µl of culture was transferred with a pin replicator (Nunc, USA) to a new plate containing fresh YPD medium with G418. The cultures were incubated at 30°C for 72 hours. Duplicate plates were incubated anaerobically using Anaerocult IS (Merck, Germany) for the same period, also at 30°C. Absorbance was then measured at 655 nm in a microtiterplate reader (model 3550, Biorad, USA).

The collection of BY4743 derived heterozygous diploid strains (95401.

H4) with mutations in essential genes were used to inoculate 200 μ l YPD. After o/n incubation at 30°C, a fresh microtiterplate with 200 μ l YPD per well was inoculated using a 96-pin replicator. The next day 2 μ l of the cultures were spotted onto sporulation agar in a microtiterplate-sized Petridish (Nunc). After 3-5 days at 30°C sporulation reached a maximum of only 1-10% for strains derived from BY4743. For other strains this value was 70-90%. Plates stored at 4°C could be used for at least a month. For dissection a small aliquot of the sporulated culture was resuspended in one drop of a lyticase solution (1 mg lyticase (Sigma) in 1 ml of water). After 3-5 min at room temperature the suspension was diluted 10-fold with water and used directly or kept on ice. For each strain 4-6 asci were dissected using a Singer MSM system dissection microscope on two YPDET plates, one was incubated aerobically, the other anaerobically both at 30°C. Of the strains that did not segregate 2:2 for both anaerobic and aerobic growth another 10 tetrads were dissected. The entire collection was screened twice in this way, starting from the original Genetic Research microtiterplates. The few discrepancies between the first and the second round were tested a third time.

Results

Anaerobically essential genes

While it is generally accepted that *K. lactis* is not able to grow under anaerobic conditions, data to support this notion are hard to find. We therefore tested several frequently used *K. lactis* strains for their ability to grow anaerobically. Figure 1 shows the results on mineral medium supplemented with Tween 80 and ergosterol, but similar results were obtained on rich medium (YPD) with the same supplements. Whereas the two *S. cerevisiae* strains grew abundantly, all seven *K. lactis* strains only showed some residual growth, probably caused by the initially present oxygen which would allow growth until essential components are exhausted. Similar effects are observed when *S. cerevisiae* is incubated anaerobically without Tween 80 or ergosterol. It thus appears that *K. lactis*, at least the seven strains tested, is not able to sustain growth in the absence of molecular oxygen.

The energy yield on glucose during fermentation is much lower than during respiration. Therefore strains need a high fermentation capacity. Several

K. lactis strains, including CBS2360, have the so-called Rag-phenotype, they cannot grow on glucose in the presence of the respiration inhibitor antimycin A due to a mutation in the *RAG1* gene encoding the only low-affinity glucose transporter in this strain (Goffrini *et al.*, 1989; Goffrini *et al.*, 1990). Obviously the fermentation rate is too low to support growth. Several other strains, like JA-6, have two tandemly arranged glucose transporter genes, *KHT1* and *KHT2*, at the *RAG1* locus. In these strains fermentation is enhanced (Breunig *et al.*, 2000). The lack of sufficient fermentation capacity may contribute but can not to be the only explanation for the inability of *K. lactis* to grow anaerobically as there was no difference in anaerobic growth between the seven *K. lactis* strains, including CBS2360 and JA-6. Since *S. cerevisiae* can grow under anaerobiosis other factors might be present in *S. cerevisiae* which are lacking from *K. lactis*. As a first approach we investigated which genes are important for anaerobic growth in *S. cerevisiae* and then determined the presence of these genes in *K. lactis*.

Circa 1300 *S. cerevisiae* genes are essential for aerobic growth on rich medium. It was unknown however, how many of these are also necessary for anaerobic growth. We therefore sporulated and dissected the 1166 heterozygous diploids with deletions in the essential genes (collection 95401.H4). This test showed that the aerobically essential genes indeed segregated 2:2 under aerobic conditions. Most of these genes were in fact also needed for growth under anaerobic conditions. Only 33 genes were not required for anaerobic growth, giving four normal sized colonies per tetrad, two of which did not grow when restreaked and incubated aerobically. In 32 strains anaerobic growth was retarded, with two normal and two small (< 0.5 mm diameter) to very small (< 100 cells per colony) colonies per tetrad, making the deleted genes in these strains necessary for optimal anaerobic growth. The results are listed in tables 2A and 2B.

**Table 2A: ORF's essential for aerobic growth,
but not for anaerobic growth**

ORF	Gene	Function / localization
YGR082w	<i>TOM20</i>	Transport outer mitochondrial membrane *
YGL055w	<i>OLE1</i>	Stearoyl-CoA desaturase, mitochondrial inheritance, ER
YGL018c	<i>JAC1</i>	Aerobic respiration, Iron sulfur cluster assembly, Mitochondrion
YMR134w		Iron homeostasis
YDL120w	<i>YFH1</i>	Yeast Frataxin Homologue, Iron homeostasis, mitochondrion *°
YDR353w	<i>TRR1</i>	Thioredoxin reductase (NADPH) Regulation of redox homeostasis
YBR167c	<i>POP7</i>	Ribonuclease P, mitochondrial RNA processing complex
YPL231w	<i>FAS2</i>	3-oxoacyl (acyl carrier protein) reductase/synthetase
YBR192w	<i>RIM2</i>	Mitochondrial genome maintenance, Transporter °
YGL001c	<i>ERG26</i>	Ergosterol biosynthesis
YGR175c	<i>ERG1</i>	Ergosterol biosynthesis
YHR072w	<i>ERG7</i>	Ergosterol biosynthesis
YHR190w	<i>ERG9</i>	Ergosterol biosynthesis
YLR100w	<i>ERG27</i>	Ergosterol biosynthesis
YLR101c		
YGR280c	<i>PXR1</i>	Possible telomerase regulator or RNA-binding protein
YIR008c	<i>PR11</i>	Alpha DNA polymerase, DNA replication initiation
YIL118w	<i>RHO3</i>	Rho small monomeric GTPase, signal transduction
YBR061c	<i>TRM7</i>	t RNA methyl transferase *
YDL212w	<i>SHR3</i>	Amino acid transport, ER *
YEL034w	<i>HYP2</i>	Translation elongation factor, homologous to ANB1 *
YER008c	<i>SEC3</i>	Golgi to plasmamembrane transport
YDR427w	<i>RPN9</i>	19 S proteasome regulatory particle *
YER107c	<i>GLE2</i>	Nuclear pore organization and biogenesis *
YKR038c	<i>KAE1</i>	Kinase associated endopeptidase
YMR239c	<i>RNT1</i>	Ribonuclease III
YBR190w		Unknown °
YDR412w		Unknown
YEL035c	<i>UTR5</i>	Unknown
YFR003c		Unknown
YGL069c		Unknown °
YIL083c		Unknown
YJR067c	<i>YAE1</i>	Unknown

* Considered viable in the most recent version of SGD

° Gave aerobically 2+ : 2 very small colonies

**Table 2B: ORF's essential for aerobic growth,
but with retarded anaerobic growth**

ORF	Gene	Function / localization
YBL030c	PET9/ AAC2	ATP/ADP antiporter, mitochondrial innermembrane *
YGR029w	ERV1	Sulhydryl oxidase, iron homeostasis, mitochondrion organisation and biogenesis
YML091c	RPM2	Ribonuclease P, mitochondrial organization and biogenesis *
YMR301c	ATM1	Mitochondrial ABC transporter protein
YER043c	SAH1	Methionine metabolism
YMR113w	FOL3	Dihydrofolate synthase *
YDR499w	LCD1	DNA damage checkpoint, telomere maintenance
YER146w	LSM5	mRNA splicing, snRP *
YER159c	BUR6	Transcription co-repressor *
YGL150c	INO80	ATPase, chromatin remodelling complex *
YPR104c	FHL1	POL III transcription factor *
YBL092w	RPL32	Ribosomal protein
YGL169w	SUA5	Translation initiation *
YNL007c	SIS1	Chaperone, translational initiation
YDR166c	SEC5	Exocytosis
YER036c	KRE30	ABC transporter
YDR376w	ARH1	Heme a biosynthesis, Iron homeostasis, Mitochondrial innermembrane
YLR259c	HSP60	Heat shock protein, mitochondrial translocation
YKL192c	ACP1	Fatty acid biosynthesis, cytosol *
YNL103w	MET4	Transcription co-activator, Methionine auxotroph *
YHR005c	GPA1	Pheromone respons in mating type *
YPL020c	ULP1	SUMO specific protease, G2/M transition
YLR022c		Unknown
YHR083w		Unknown
YOR218c		Unknown
YKL195w		Unknown
YLR140w		Unknown °
YML023c		Unknown
YNL026w		Unknown
YNL171c		Unknown *
YNL260c		Unknown
YNR046w		Unknown

* Considered viable in the most recent version of SGD

° Gave aerobically 2+ : 2 very small colonies

As expected, genes involved in ergosterol synthesis are not necessary when this compound is present in the medium. Similarly, the finding of several mitochondrial genes is not surprising either. However, for almost all other genes in the list, even those to which a function has been attributed, it is not clear why they are essential for aerobic but not for anaerobic growth.

We next tested the homozygous deletion mutants that could grow aerobically in YPD for growth in YPDET in the absence of molecular oxygen. While some residual growth to varying degrees was observed, the 23 strains listed in table 3 consistently did not grow beyond the background.

Table 3: Genes essential for anaerobic growth and not essential for aerobic growth

Systematic name	Gene	Function
YAL026C	DRS2	Integral membrane Ca(2+)-ATPase
YPL254W	HFI1	Subunit of SAGA
YBR179C	FZO1	Mitochondrial integral membrane protein
YDR138W	HPR1	Subunit of THO/TREX
YDR364C	CDC40	Splicing Factor
YOR209C	NPT1	Nicotinate phosphoribosyl transferase
YLR242C	ARV1	Sterol metabolism/ transport
YLR322W	VPS65	Unknown
YDR149C		Unknown
YDR173C	ARG82	Transcription factor
YPL069C	BTS1	Terpenoid biosynthesis
YPR135W	CTF4	Chromatin-associated protein
YGL025C	PGD1	Subunit of Mediator
YGL045W/ YGL046W	RIM8	Unknown
YGL084C	GUP1	Glycerol transporter
YNL236W	SIN4	Subunit of Mediator
YNL225C	CNM67	Cytoskeleton
YNL215W	IES2	Associates with INO80
YKR024C	DBP7	ATP-dependent RNA helicase
YGR036C	CAX4	(Pyro)phosphatase
YDR477W	SNF1	Protein serine/threonine kinase
YNL284C	MRPL10	Protein synthesis
YOL148C	SPT20	Subunit of SAGA

It was expected that at least some of the genes that are reported in the literature to be of importance for anaerobic growth (see introduction) would come up in this screen. Therefore, we took a more careful look at the results for the strains lacking these genes. The results are listed in table 4.

Table 4: Growth of strains lacking the genes described to be important for anaerobic growth in literature as described in the introduction

Systematic name	Gene	Aerobic growth	Anaerobic growth
YHR007C	ERG11	-	-
YEL039C	CYC7	+	+
YGL055W	OLE1	-	+
YMR272C	SCS7	+	+
YGL162W	SUT1	+	+
YPR009W	SUT2	+	+
YJR150C	DAN1	+	+
YOR011W	AUS1	+	+
YIL013C	PDR11	+	+
YBR041W	FAT1	+	+
YEL050C	RML2	+/-	Not done
YKL216W	URA1	+	+
YLR188W	MDL1	+	+
YOR209C	NPT1	+	-
YEL047C	FRDS	+	+
YJR051W	OSM1	+	+
YBL030C	AAC2	-	-
YBR085W	AAC3	-	-
YLR256W	HAP1	+	+
YPR065W	ROX1	+	+
YDR213W	UPC2	+	+/-
YLR228C	ECM22	+	+
YDR392W	SPT3	+	+
YGR063C	SPT4	+	+
YDR159W	SAC3	+	+
YLR025W	SNF7	+	+

Of all the genes found in the literature to be connected to anaerobic metabolism, only two, *NPT1* and *ARV1*, were found to be anaerobically essential. A possible explanation for this apparent discrepancy could be the redundancy of the genes in question (see Discussion). In contrast we did find 21 genes to be important for anaerobic growth, which were not previously implicated. Indeed, analysis of the list shows no logical reason for these genes to be anaerobically essential. The genes do not belong to any pathway or functional group linked to anaerobic growth.

Search for anaerobically important genes in the *K. lactis* genome

A search for the genes listed in table 3 and 4 with the genome of *K. lactis* resulted in identification of 20 genes for which a homologue could not be found. In this comparison we also included the regulatory genes that we identified in our group and that have an anaerobically upregulating activity. These are listed in table 5.

The 20 genes listed in table 5 are anaerobically essential genes, genes that were linked to anaerobic growth in literature and transcription factors for this function. This suggests that *K. lactis* has deficits both in the regulation of anaerobic genes and in the presence of these genes itself. Since not all genes found to be missing are active in the same process, it could very well be that the inability of this strain to grow anaerobically has multiple causes.

Discussion

Most of the genes that are essential for aerobic growth have an equally important role under anaerobic conditions, since only 33 of them are not needed at all and 32 are necessary for optimal growth in YPD supplemented with Tween and ergosterol when oxygen is absent. This figure is much smaller than anticipated, given the large number of genes encoding mitochondrial proteins. However, our data confirm that apart from respiration mitochondria have many other metabolic functions even under anaerobic conditions, also illustrated by the presence of (pro-)mitochondria in anaerobically grown cells (Plattner and Schatz, 1969) It is also remarkable that the transcription level of none of these genes changes significantly when aerobic versus anaerobic cells are compared

Table 5: Genes that have a role in anaerobic growth but for which no homologue could be found in the genome of *K. lactis*

Systematic name	gene	<i>K. lactis</i> ORF	Swiss prot qualification	Similarity to <i>S. cerevisiae</i>	PFAM database qualification
YPL254W	HFI1	-	-	-	-
YBR179C	FZO1	-	-	-	-
YLR242C	ARV1	-	-	-	-
YDR173C	ARG82	-	-	-	-
YGL045W	RIM8	-	-	-	-
YNL215W	IES2	-	-	-	-
YGR036C	CAX4	-	-	-	-
YDR149C	-	-	-	-	-
YGL025C	PGD1	V2688	high	medium	low
YNL225C	CNM67	IV0280	medium	low	low
YLR322W	VPS65	-	-	-	-
YEL047C	FRDS	VI4423	high	high(OSM1)	high
YJR150C	DAN1	-	-	-	-
YOR011W	AUS1	IV091	high	high(PDR5)	high
YIL013C	PDR11	II2419	high	high(PDR12)	high
YGL162W	SUT1	IV0417	-	-	-
YPR009W	SUT2	-	-	-	-
YGR063C	SPT4	V1285 VI3656	-	low low	-
YDR213W	UPC2	III4511	-	low	-
YPR065W	ROX1	II2917	high	medium	medium

(Ter Linde *et al.*, 1999;Piper *et al.*, 2004) or when mutants in the Hap1 or Rox1

anaerobic transcription factors mutants are compared to wild type strains (Ter Linde and Steensma, 2002). The remaining, just over a thousand, essential genes probably represent the minimum number of household genes that are necessary for growth under a wide variety of conditions. For this reason and the close relationship between the two strains, we have not included them in the comparison of the genomes (Bolotin-Fukuhara *et al.*, 2000). There is a possibility, however, that these genes may have evolved in a different way, leaving them non-functional for the anaerobic tasks their counterparts in *S. cerevisiae* perform.

The number of genes which we identified as being essential for anaerobic growth in *S. cerevisiae* is also small. We found 23 genes which are specifically needed for anaerobic growth of which only two were previously described as important for anaerobic growth. Given the limitations of our screen this is not unexpected. First, many genes involved in anaerobic growth are present in one or more copies. For instance *UPC2* and *ECM22* can partially complement each other. Due to our stringent criteria for growth we did not consider small differences in growth to be significant. Similarly, the *DAN*, *PAU* and *TIR* genes are all present in multiple copies. Second, cells were grown in YPD with Tween and ergosterol. Genes involved in the synthesis of components present in the medium thus could not be detected.

The two genes that were previously described in literature as being important for anaerobic growth were *NPT1* and *ARV1*. The *NPT1* gene was the only one found in an extensive screen for essential anaerobic genes (Panozzo *et al.*, 2002). However, the authors considered the anaerobic conditions used questionable and thus the screen was termed hypoxic rather than anaerobic. Our study confirms the importance of *NPT1* for anaerobic growth.

From the results in Table 3 it is clear that the genes are involved in various functions. It is remarkable though that two genes of the SAGA complex, *HFI1* and *SPT20*, two of the Mediator complex, *PGD1* and *SIN4*, and several other transcription(-related) factors, i.e. *HPR1*, *ARG82*, *CTF4* and *SNF11* have come up in our screen. Since these complexes and factors are also present and functional under aerobic conditions, it is not clear why they are essential for anaerobic growth. Possibly the combination of low ATP levels, caused by the lower yield under anaerobiosis, and impaired protein synthesis causes some sort of synthetic lethality. *GUP1* is designated to be coding for a glycerol uptake protein. This protein could under anaerobic conditions be functioning as a glycerol export protein to expel the excess glycerol that is produced. For the other genes it is

unclear why their disruption would lead to the inability to grow under anaerobic conditions.

In addition to the 23 genes that we found essential for anaerobic growth we included genes that have a (potential) regulating role upon anaerobiosis and genes that are known from literature to play a role when cells are growing under anaerobic conditions (Table 4).

The comparison revealed 20 genes that are anaerobically active in *S. cerevisiae* and missing from *K. lactis*. Of the 23 genes that showed to be essential under anaerobic conditions only, 11 can not directly be assigned a homologue in *K. lactis*. Also, the sequences of 5 genes that act as regulators in the absence of oxygen are not present with high similarity. Three *S. cerevisiae* genes in table 5, namely *PGD1*, *CNM67* and *ROX1* do seem to have a possible homologue in *K. lactis*, according to the homology of the structures as predicted by Swissprot (column 4 in Table 5), but the comparisons of the sequences in the other columns is not designated 'high', so they were included in the list. Three *S. cerevisiae* genes gave high similarity with *K. lactis* ORFs, which gave different *S. cerevisiae* genes when used as probes to search the *S. cerevisiae* genome. For example, when the *FRDS* sequence was used against the *K. lactis* genome, the ORF *klact_VI4423* was a significant hit. When this ORF is used to search Swissprot, known yeast annotations, PFAM and KOGG databases, the ORF is identified as a homologue of the *OSM1* gene. The *FRDS* and *OSM1* genes in *S. cerevisiae* are highly homologous. The *OSM1* sequence is widely accepted as a gene and annotated as such. The *FRDS* sequence however, is not. Therefore it was not present in the databases used to compare the *K. lactis* ORF with. For this particular case it was clear that *K. lactis* has only one fumarate reductase enzyme, while *S. cerevisiae* has two, which are highly similar. The other two cases, *AUS1* and *PDR11*, are less clear. Although *AUS1*, *PDR11*, *PDR5* and *PDR12* are all members of the ABC transporters, only the first two have been implicated in sterol uptake. At this moment it is not possible to draw a conclusion about the specific function of these *K. lactis* genes.

Saccharomyces kluyveri is another yeast that can grow under anaerobic conditions (Moller, Olsson, and Piskur, 2001). To validate our data we have checked the presence or absence in this yeast of the 20 genes in Table 5. In the BLAST search, for which the web page www.genetics.wustl.edu/saccharomycesgenomes/ was used, all but three were found to be present in

S.kluyveri with a high similarity and a p-value of less than 10^{-4} . The other three, *PGD1*, *CNM67* and *VPS65*, were also present at a high similarity but their p-values were 0.97, 0.10 and 0.81 respectively. This comparison supports the conclusion that the genes in Table 5 might be a key to understand why *K.lactis* cannot grow under anaerobic conditions.

Four of the genes for which a *K. lactis* homologue could not be found, notably *ARV1*, *DAN1*, *AUS1* and *PDR11*, are related to sterol uptake. Moreover, three of the missing transcription factors are also involved in sterol uptake, namely *SUT1*, *SUT2* and *UPC2*. Import of sterols under anaerobic conditions is essential since their biosynthesis requires oxygen. Therefore, we would like to hypothesize that *K. lactis* can not import sterols. The lack of sterol import thus would be one factor that contributes to the inability of *K. lactis* to grow under anaerobic conditions. Since 14 more anaerobic genes are absent in *K. lactis* it appears unlikely that sterol uptake is the only factor. For example, the *S. cerevisiae* gene *ARV1*, which was described earlier as essential for anaerobic growth and which came up in our screen for such genes, is absent in the *K. lactis* genome.

In addition, regulation might also play a role. For example, a single functional homologue of the AAC genes is present in *K. lactis* (named *KIAAC*), but this gene is downregulated under anaerobic conditions, leaving *K. lactis* with low levels of a functional ADP/ATP carrier when oxygen is absent (Trezeguet *et al.*, 1999).

Since the number of anaerobically important genes missing in *K. lactis* is extensive, it is probable that several of these genes will be needed to allow *K. lactis* to grow under anaerobic conditions. Both complementation assays and transcriptome analysis would be needed to explore this issue further. By supplying the cells with the proper genes, either encoding transcription factors or anaerobically essential proteins, *K. lactis* could become less dependent on the availability of oxygen, if not able to grow under completely anaerobic conditions. Experiments to test this hypothesis are in progress.

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Chapter 3

Oxygen-dependent transcription levels have only limited predictive value for the contribution of the gene to the fitness under anaerobic conditions.

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○ Abstract

The applicability of transcriptomics as a tool to identify gene function rests on the assumption that global information on gene function can be inferred from transcriptional regulation patterns. This study investigates whether *S. cerevisiae* genes that are consistently transcriptionally upregulated under anaerobic conditions, regardless of the nutrient limitation, do indeed contribute to fitness in the absence of oxygen. Tagged deletion mutants were constructed in 27 *Saccharomyces cerevisiae* genes that showed a strong and consistent transcriptional upregulation under anaerobic conditions, irrespective of the nature of the growth-limiting nutrient (glucose, ammonia, sulfate or phosphate). Competitive anaerobic chemostat cultivation showed that only 5 out of the 27 mutants (*eug1Δ*, *izh2Δ*, *plb2Δ*, *ylr413wΔ* and *yor012wΔ*) had a significant disadvantage relative to a tagged reference strain. Implications of this study are that: (i) transcriptome analysis has a very limited predictive value for the contribution of individual genes to fitness under specific environmental conditions and (ii) competitive chemostat cultivation of tagged deletion strains offers an efficient approach to select relevant leads for functional analysis studies.



Introduction

While the number of completely sequenced microbial genomes continues to grow explosively, assignment of biochemical and physiological functions to the corresponding genes progresses at a much lower rate. A case in point is the extensively studied yeast *Saccharomyces cerevisiae*. Ten years after the completion of its genome sequence (Goffeau *et al.*, 1996), 21 % of its genes neither have an experimentally confirmed function nor a function that can be predicted with a high degree of confidence based on similarity with genes from other organisms (Saccharomyces Genome Database, August 28, 2006 <http://www.yeastgenome.org/cache/genomeSnapshot.html>) (Hirschman *et al.*, 2006).

Accurate determination of gene function often requires sophisticated and costly experimental techniques. It is therefore worthwhile to select priority targets for functional analysis via high-throughput methods such as for synthetic-lethality screening (Tong *et al.*, 2001; Tong *et al.*, 2004), mapping of physical interaction (Gavin *et al.*, 2002; Krogan *et al.*, 2006) or expression analysis. With respect to the latter, DNA microarrays have been extensively used to map genome-wide transcriptional responses to a multitude of environmental parameters (Boer *et al.*, 2003; Causton *et al.*, 2001; Daran-Lapujade *et al.*, 2004; Gash *et al.*, 2000). This approach yields sets of genes that show common and specific transcriptional responses to individual environmental parameters. The resulting sets of transcriptionally responsive genes often show enrichment for genes with known functions that can be directly correlated with the environmental conditions under study. Additionally, they invariably yield sets of transcripts that encode proteins with unknown function or with a known biochemical function that cannot be readily linked to the conditions studied.

It is generally assumed that, in the case of upregulated transcripts, the biochemical functions of the encoded proteins contribute to the organism's physiological adaptation to the environmental parameter under study. However, there are few published studies that systematically investigate the extent to which this concept of 'transcriptomics-inferred function' is correct and applicable for guiding functional analysis research. Two large-scale comparisons suggest that the correlation between transcript profile and fitness of deletion strains may be far from perfect (Birrell *et al.*, 2002; Giaever *et al.*, 2002; Giaever *et al.*, 2004; Winzeler *et al.*, 1999).

Saccharomyces cerevisiae is the only yeast that can rapidly grow under aerobic as well as anaerobic conditions (Visser *et al.*, 1990). This unique ability plays a major role in various industrial applications of *S. cerevisiae*, including beer fermentation, wine fermentation and large-scale production of fuel ethanol. Still, the genetic basis for rapid anaerobic yeast growth remains unknown. In a recent chemostat-based study (Tai *et al.*, 2005), we used transcriptome analysis to investigate the response of the yeast *Saccharomyces cerevisiae* to anaerobic conditions. 65 Genes (ca. 1 % of the genome) were found to be significantly upregulated under anaerobic conditions, irrespective of the nature of the growth-limiting nutrient (glucose, ammonium, phosphate or sulfate). In separate experiments with the yeast deletion library (Snoek and Steensma, 2006), 24 genes were shown to be essential for anaerobic (but not for aerobic) growth. Surprisingly, when these two sets of genes, obtained from different experimental approaches, were compared, no overlap was found.

In the present study, we investigate whether genes that are transcriptionally upregulated in anaerobic cultures of *S. cerevisiae* contribute to its fitness under anaerobic conditions. In order to be able to identify subtle effects on fitness, competitive cultivation of a reference strain and a set of null mutants, was performed in anaerobic chemostats.

Materials and methods

Strains

S. cerevisiae CEN.PK113-7D (MATa MAL2-8c SUC2) (van Dijken *et al.*, 2000) was used as the prototrophic reference strain. All knockout strains were constructed in this genetic background. Strains were constructed by using standard yeast media and genetic techniques (Burke, Dawson, and Stearns, 2000). The kanamycin resistance cassette was amplified by PCR by using specific primers and the pUG6 vector as template (Guldener *et al.*, 1996). As part of the deletion process, each gene disruption was replaced with a KanMX module and uniquely tagged with two 20mer sequences (http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.htm). The gene YGR059W was either tagged with a unique dntag sequence or an uptag sequence. The deletion of YOR012W carried along inactivation of neighbouring and overlapping ORF

YOR013W. The double mutant strain yor012W Δ /yor013W Δ will be referred as yor012W Δ in the rest of the manuscript. Strains were routinely grown at 30 °C on complete media (YPD).

Shake flask cultivation

Shake-flask cultivations were performed in 500 ml flasks containing 100 ml of medium, which were incubated at 30 °C on an orbital shaker set at 200 rpm. The composition of the synthetic medium (SM) was as follows: 20 g liter⁻¹ glucose, 5 g liter⁻¹ (NH₄)₂SO₄, 6 g liter⁻¹ KH₂PO₄, 0.5 g liter⁻¹ MgSO₄, trace elements and vitamin solutions (Verduyn *et al.*, 1990). The pH of the medium was adjusted to 5.0 and sterilized by autoclaving. Glucose was autoclaved separately. Vitamins were filter-sterilized and added to the medium. Growth of the various strains was monitored by OD measurements at 660 nm. After growing all strains to mid-exponential phase, an equivalent amount of each mutant strain, corresponding to 0.02 OD_{660nm} units, was aseptically pooled to prepare a mixed inoculum (30 ml total volume) for the competition experiments.

Chemostat cultivations

Chemostat cultivation was performed at 30 °C in 1-liter working volume laboratory fermenters (Applikon, Schiedam, The Netherlands) at stirrer speed of 800 rpm, pH 5.0, with a dilution rate (D) of 0.10 h⁻¹ as described previously (van den Berg *et al.*, 1996). The pH was kept constant, using an ADI 1030 biocontroller (Applikon, Schiedam, The Netherlands), via the automatic addition of 2 M KOH. The fermentors were flushed with pure nitrogen gas for anaerobic growth and air for aerobic growth at a flow rate of 0.5 liter min⁻¹ using a Brooks 5876 mass-flow controller (Brooks Instruments, Veenendaal, The Netherlands). The dissolved-oxygen concentration was continuously monitored with an Ingold model 34 100 3002 probe (Mettler-Toledo, Greifensee, Switzerland) and was 0 % for anaerobic growth and above 70 % for aerobic growth. To sustain anaerobiosis, the medium vessels were sparged with pure nitrogen gas and Norprene tubing was used to minimize oxygen diffusion into the fermentors. Anaerobic carbon-limited steady-state chemostat cultures of the reference strain *S. cerevisiae* ygr059w Δ ::uptag (see Results section) were grown on a synthetic medium as described previously (Verduyn *et al.*, 1992). Aerobic carbon-limited chemostat cultures contained the same medium but

with 7.5 g liter⁻¹ glucose and without the anaerobic growth factors Tween-80 and ergosterol. When steady state was achieved, the 30 ml competition mix was aseptically injected into the culture using a syringe. Samples were taken via the effluent line every 24 hours for a period of 216 hours. The samples were chilled on ice, spun down and frozen at -20°C for high-molecular-weight DNA extraction.

High-molecular-weight DNA extraction

DNA samples were purified using an adapted method described by (Burke, Dawson, and Stearns, 2000). 40 ml of cell culture broth was spun down and resuspended in 1 ml of DNA extraction buffer (2 % Triton X-100, 1 % SDS, 100 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA pH 8.0). 400 µl of the resuspended cells was added to an equal volume of phenol/ chloroform/ isoamyl alcohol (25/24/1) pH 8.0 and 0.3 g sterile glass beads. The Bio101 Fastprep (Qbiogene, CA) was used to break the cell walls with a speed setting of 4.5 for 15 s. After centrifugation, the supernatant was transferred to 500 µl phenol/chloroform/ isoamyl alcohol (25/24/1) pH 8.0 and vortexed. Supernatant was transferred to 1 ml of absolute ethanol (-20°C) for precipitation of DNA and centrifuged for 15 min (13,000 rpm) at room temperature. The DNA pellet was resuspended in 400 µl TE buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA pH 8.0), 15 µl RNase cocktail (Ambion 2286) and placed at 37 °C until fully dissolved. After centrifugation, the chromosomal DNA was re-precipitated with 5 µl 7.5 M ammonium acetate and 1 ml absolute ethanol (-20°C) and immediately centrifuged at 13,000 rpm for 15 min at room temperature. The air-dried DNA pellet was resuspended in 50 µl TE buffer. Quality of DNA was checked with 1% TAE agarose gel. DNA quantity was analysed at OD₂₆₀.

Quantitative real-time PCR

qrtPCR was run on an DNA engine Opticon I system (BioRad, Hercules CA) with the following settings: 94 °C for 2 min. 94 °C for 10 s, 55 °C for 10 s, 72 °C for 10 s and plate reading. The denaturation, annealing, elongation and reading steps were repeated for 49 cycles. A melting curve from 55 to 94 °C was performed at the end of the reaction. The reaction mixture of 20 µl consisted of 10 µl SybrGreen TAG readymix (Sigma S1816), 0.2 mM forward primer, 0.2 mM reverse primer and 50 ng DNA. The C(t) value was calculated with the Opticon

Monitor™ software version 1.08 (BioRad, Hercules CA) by setting the threshold for significant detection levels to 10-times the standard deviation over the cycle ranged from 1 to 15. Each time point was carried out in triplicate readings.

Data and statistical analysis

The C(t) values were converted to amounts of DNA concentration (X_{DNA}) via the exponential relationship of X_{DNA} and C(t): $X_{\text{DNA}} = a \cdot \exp^{-C(t)}$, where a is a variable constant for each strain due to qrtPCR efficiency. For each strain, all X_{DNA} values measured during the 216 hours competition experiment were normalized to the X_{DNA} value at $t = 0$ to eliminate bias from PCR efficiency. Fitness was calculated by taking the slope of the best-fit linear trend line. The relative reduction of the fitness of mutant strains was calculated from the biomass balance (Giaever *et al.*, 2002):

$$X_t = X_o \cdot \exp^{(\mu - D)t}$$

where t = time (h), X_t = biomass concentration at time t , X_o = initial biomass concentration, μ = growth rate (h^{-1}) and D = dilution rate (h^{-1}). Statistical analysis was done using the modified Z-score (Iglewicz and Hoaglin, 2006) to identify mutants that showed significant reduction in fitness (outliers). The modified Z-score was then subjected to a two-tailed T-distribution test with 2 degrees of freedom in accordance to the Grubbs' test (Barnett and Lewis, 1994) to calculate the significance p -values for each mutant strain. Only mutants with p -value < 0.01 were deemed significantly reduced in fitness.

Results

Selection of target genes and construction of deletion strains

A previous transcriptome analysis of *S. cerevisiae* chemostat cultures yielded 65 genes that showed a higher transcript level in anaerobic chemostat cultures than in aerobic cultures (we will refer to these genes as 'anaerobically upregulated'), irrespective of the growth-limiting macronutrient (Tai *et al.*, 2005). From these 65 genes, a set of 24 genes was selected for further analysis (Figure 1), based on the following criteria.

Firstly, the gene must have a high change in transcript level (> 3 fold). This led to the elimination of 3 genes whose transcript level varied between 2

and 3-fold. Secondly, the gene has to have an unclear or unknown function. For example, 8 of the 65 genes are related to sterol and unsaturated fatty acid metabolism. As these processes require molecular oxygen, their anaerobic upregulation is understood and we therefore eliminated these genes from the present study. Thirdly, the gene must not be a part of a family of genes with high sequence similarity. For example, 21 of the 65 anaerobically upregulated genes belong to the seripauperin family (*DAN*, *PAU* and *TIR* genes). Since multiple members of this family were present in the set, redundancy might well obscure the interpretation of the competitive cultivation experiments carried out with single deletion strains. We therefore decided to eliminate members of large gene families from this study. Fourthly, the gene should not have a previously established clear relation with anaerobic growth.

Five additional genes were selected for inclusion in the further experiments. YGR059w was selected as a physiologically neutral marker gene based on transcript data. YGR059w encodes a sporulation-specific septin that functions in cytokinesis, meiosis I, and sporulation, and was not expressed in the haploid CEN.PK113-7D strain in 20 different chemostat conditions (table 1). *URA3*, which is essential for uracil biosynthesis, was included as a negative control: in the absence of uracil, *ura3Δ* strains should not grow. Additionally *DAN1*, *UPC2* and *ANB1* were included as extensively studied, anaerobically upregulated genes. *DAN1* encodes for a cell wall mannoprotein induced during anaerobic growth, initially excluded as a member of the seripauperin (*PAU*) family (Viswanathan *et al.*, 1994). *UPC2* (Uptake control 2) encodes a sterol regulatory element binding protein involved in the regulation of sterol biosynthetic gene expression and the uptake and intracellular esterification of sterols (Wilcox *et al.*, 2002). Finally *ANB1* encodes the translation initiation factor eIF5A that displays a specific and strong anaerobic transcriptional upregulation (Wei, Kainuma, and Hershey, 1995). In total, 29 genes were further studied by mean of competitive cultivation.

Table 1: Expression data of YGR059W and ACT1 over 20 different chemostat culture conditions at a dilution rate of 0.1h⁻¹.

C-source	N-source	Aeration	limitation	YGR059W
glucose	(NH ₄) ₂ SO ₄	+ air	carbon	12.0±0.0
glucose	(NH ₄) ₂ SO ₄	+ air	Nitrogen	12.0±0.0
glucose	(NH ₄) ₂ SO ₄	+ air	Phosphorus	12.0±0.0
glucose	(NH ₄) ₂ SO ₄	+ air	sulfur	12.0±0.0
glucose	(NH ₄) ₂ SO ₄	+ 100% N ₂	carbon	14.8±3.0
glucose	(NH ₄) ₂ SO ₄	+ 100% N ₂	Nitrogen	12.0±0.0
glucose	(NH ₄) ₂ SO ₄	+ 100% N ₂	Phosphorus	16.2±4.0
glucose	(NH ₄) ₂ SO ₄	+ 100% N ₂	sulfur	12.0±0.0
ethanol	(NH ₄) ₂ SO ₄	+ air	carbon	12.0±0.0
acetate	(NH ₄) ₂ SO ₄	+ air	carbon	12.0±0.0
maltose	(NH ₄) ₂ SO ₄	+ air	carbon	12.0±0.0
galactose	(NH ₄) ₂ SO ₄	+ air	carbon	12.0±0.0
glucose	(NH ₄) ₂ SO ₄	+ 100% CO ₂	carbon	12.3±0.6
glucose	(NH ₄) ₂ SO ₄	+ 79%CO ₂ +21% O ₂	carbon	12.0±0.0
glucose	(NH ₄) ₂ SO ₄	+ 79%CO ₂ +21% O ₂	nitrogen	12.0±0.0
glucose	ASN	+ air	carbon	13.5±2.6
glucose	PRO	+ air	carbon	12.0±0.0
glucose	PHE	+ air	carbon	12.0±0.0
glucose	LEU	+ air	carbon	12.0±0.0
glucose	MET	+ air	carbon	12.0±0.0

ACT1	reference	GEO access no
2628.0±204.9	(Tai et al., 2005)	GSE1723
2265.3±106.0	(Tai et al., 2005)	GSE1723
2314.4±265.5	(Tai et al., 2005)	GSE1723
2172.1±249.4	(Tai et al., 2005)	GSE1723
3726.5±135.9	(Tai et al., 2005)	GSE1723
2286.7±362.9	(Tai et al., 2005)	GSE1723
2516.5±101.4	(Tai et al., 2005)	GSE1723
2345.9±248.9	(Tai et al., 2005)	GSE1723
3226.6±494.9	(Daran-Lapujade et al., 2004)	n/a
3233.9±375.6	(Daran-Lapujade et al., 2004)	n/a
4674.3±581.8	(Daran-Lapujade et al., 2004)	n/a
2676.4±120.3	Not published	n/a
2974.6±322.6	(Aguilera et al., 2005)	n/a
2332.6±219.6	(Aguilera et al., 2005)	n/a
2273.2±225.1	(Aguilera et al., 2005)	n/a
2418.1±122.1	Not published	n/a
2294.8±127.6	Not published	n/a
2917.2±575.3	(Vuralhan et al., 2003)	n/a
2148.8±204.2	Not published	n/a
2392.7±143.3	Not published	n/a

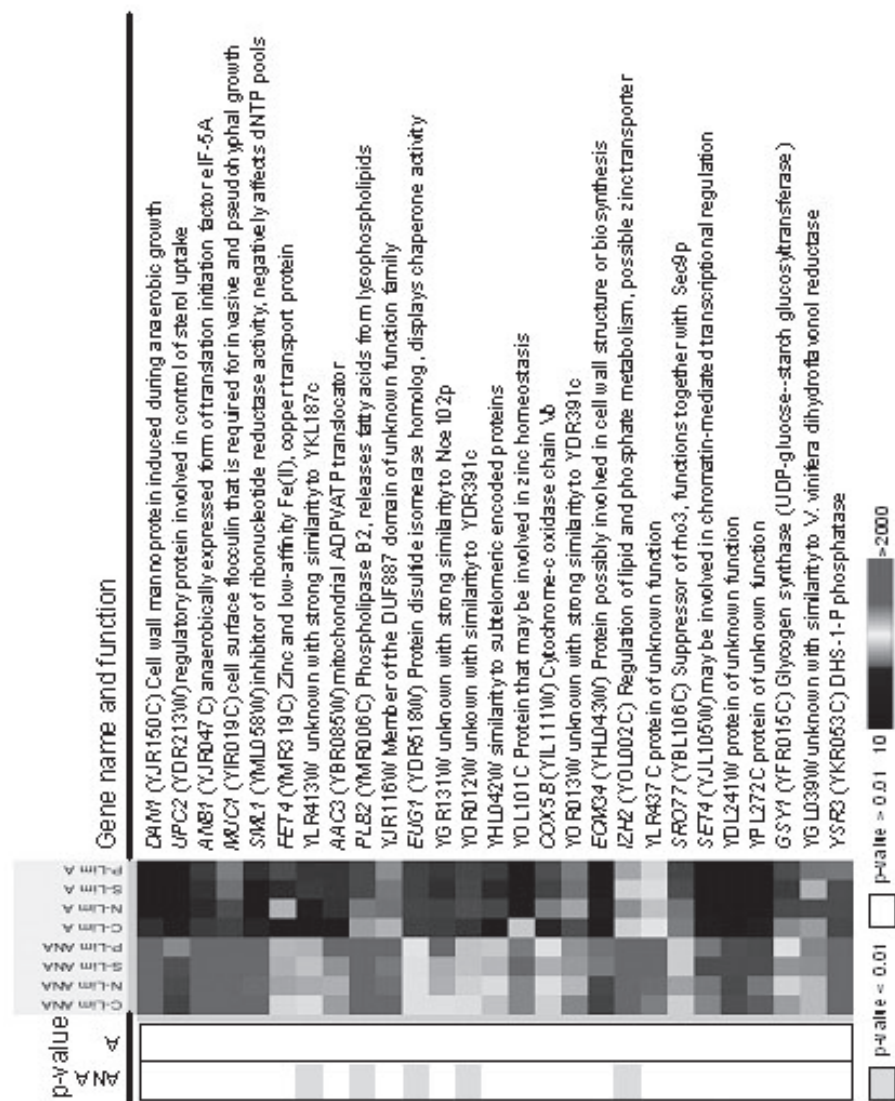
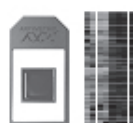
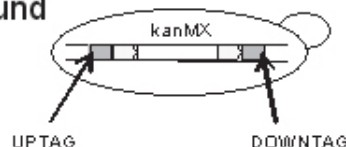


Figure 1: Genes included in the competitive cultivation experiments. Transcript intensities are depicted with low intensities in black and high intensities in red. Biochemical functions of the encoded proteins are derived from the Yeast Proteome Database (www.proteome.com). P-values represent the significance of reduced fitness of the respective mutant strain during aerobic and anaerobic growth. C (carbon), N (nitrogen), P (Phosphorus), S (sulfur), ANA (anaerobic), A (Aerobic)

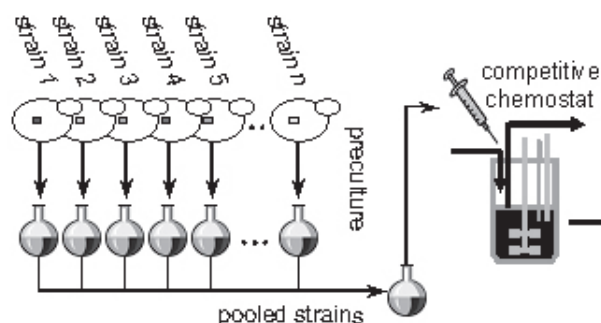
1-Selection of genes based on transcriptome data



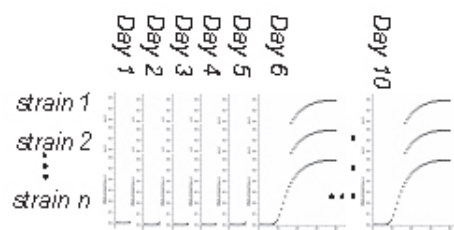
2-Strain construction in prototrophic background



3-Competitive chemostat cultivation



4-quantitative PCR



5-Fitness quantification

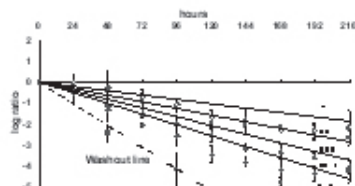


Figure 2: Experimental design.

Competitive chemostat experimental design

An outline of the experimental design is presented in Figure 2. All 29 genes were deleted from the start to stop codon in *S. cerevisiae* CEN.PK113-7D and replaced with the kanMX deletion cassette flanked by two gene-specific 20-nucleotide tag sequences (Winzeler *et al.*, 1999), see materials and methods section. The kanMX cassette has previously been shown not to confer a selective (dis)advantage during prolonged chemostat cultivation of *S. cerevisiae* (Baganz *et al.*, 1997).

In contrast to previous large-scale functional profiling studies (Giaever *et al.*, 2002; Giaever *et al.*, 2004; Winzeler *et al.*, 1999) where auxotrophic mutant collections were screened, all mutants used in this study were generated in the prototrophic CEN.PK113-7D strain (van Dijken *et al.*, 2000). The use of prototrophic strains (with the exception of the *ura3* negative control strain) eliminates the risk that results are influenced by the nutritional requirements of auxotrophic strains (Pronk, 2002).

Subsequently, steady-state chemostat cultures were grown with the neutral control mutant *ygr059wΔ* containing only the uptag (Figure 2). A second *ygr059wΔ* strain carrying a specific downtag sequence was also made and added to the mutant pool. This latter strain was used to normalize the population dynamics of the other mutants. The mix of deletion strains (see Methods section) was then injected into the steady-state chemostat culture. We prefer this approach over inclusion of the mutant pool during the start-up of the chemostat as previously reported by (Baganz *et al.*, 1997), where cultivation conditions are dynamic and the selective pressure may differ from that under steady-state conditions.

The culture was then sampled daily over a period of nine days (216 h). This time frame was chosen to reduce the impact of evolutionary adaptation, which would render a comparison of the fitness of individual tagged mutants impossible (Jansen *et al.*, 2005; Novick and Szilard, 1950) (Figure 2). After DNA isolation, samples were then analysed by quantitative real-time PCR, using the molecular tags to monitor the abundance of each mutant. After normalization to the initial sample, the abundance of the deletion strains was normalised to that of the *ygr059wΔ::downtag* reference strain included in the mutant pool.

Competitive anaerobic chemostat cultivation

During the competitive anaerobic chemostat experiments, strains that did not grow ($\mu = 0 \text{ hr}^{-1}$) were expected to disappear from the culture via washout kinetics at the dilution rate of 0.10 h^{-1} . This is depicted by the washout line in Figure 3A. Indeed, the auxotrophic *ura3 Δ* strain (negative control) closely followed this line (Figure 3A). After 96 h, the abundance of the *ura3 Δ* strain did not decrease any further (Figure 3A). This abundance was taken to reflect the threshold for detection in the experimental set-up. The $C(t)$ values measured for the reference strain *ygr059w Δ ::downtag* did not vary by more than 3.6 % in the duplicate experiments over the period of 216 h.

The anaerobic competitive cultivation experiment was performed in two independent chemostat runs. The fitness of the mutants in the anaerobically upregulated genes observed in these two runs were generally in good agreement (Figures 1 and 3). The fitness data from each strain were statistically evaluated by means of a statistical test, revealing 5 outliers ($P\text{-value} < 0.01$) from the set of 27 mutants (Figure 1). Consequently we noticed that it was not possible to make reliable statements about decreases in fitness below 20 %. While prolonging the chemostat experiment might lead to increased sensitivity, we decided against this because of the high risk of interference by evolutionary processes (Jansen *et al.*, 2005; Novick and Szilard, 1950).

None of the three anaerobic marker knockout strains *anb1 Δ* , *dan1 Δ* and *upc2 Δ* displayed a significant fitness loss compared to the control strain (*ygr059w Δ ::downtag*). While such a result could be anticipated in the case of *DAN1*, which is part of a large gene family, this result was more unexpected in the case of *ANB1* and *UPC2* that participate in central pathways as transcription and translation. It may be relevant to note that a larger variation in fitness between the two experimental runs was observed for the *upc2 Δ* strain than for the *anb1 Δ* and *dan1 Δ* strains.

Regarding the remaining 24 mutants in anaerobically upregulated genes, only five (*eug1 Δ* , *izh2 Δ* , *plb2 Δ* , *ylr413w Δ* and *yor012w Δ* ; Figure 3A) showed a significant (20 – 60 %) reduction of fitness in independent replicate experiments (Figures 3A & 3C). Of the 5 genes whose deletion resulted in a reduction of fitness under anaerobic condition, *EUG1* is the most extensively documented. *EUG1* encodes a non-essential protein disulfide isomerase. (Tachibana and Stevens, 1992). The *S. cerevisiae* genome contains four additional

protein disulfide isomerases (*PDI1*, *MPD1*, *MPD2* and *EPS1*) of which only *PDI1* is essential (Norgaard *et al.*, 2001). In addition to their catalytic role in protein folding, protein disulfide isomerases act as chaperones (Kimura *et al.*, 2005). *IZH2/PHO36* has been proposed to be involved in metabolic pathways that regulate lipid and phosphate metabolism (Karpichev, Cornivelli, and Small, 2002). Additionally, *IZH2* is part of the *ZAP1* regulon and proposed to play a role in zinc homeostasis along with *IZH1*, *IZH3* and *IZH4* (Lyons *et al.*, 2004). *PLB2* encodes a lysophospholipase B involved in phospholipid metabolism (Fyrst *et al.*, 1999;Merkel *et al.*, 1999). Two additional lysophospholipase B genes are also found in *S. cerevisiae* genome, *PLB1* (62 % similarity) (Lee *et al.*, 1994) and *PLB3* (57 % similarity) (Merkel *et al.*, 1999) The two remaining genes are very poorly characterized. Several experiments indicate that Ylr413wp is localized at the cell surface (Diehn *et al.*, 2000;Huh *et al.*, 2003) but, just like that of YOR012w, its function is totally unknown.

Aerobic reference experiments

To investigate whether the observed reduction of fitness of five mutant strains was specific for anaerobic conditions, aerobic competitive chemostat experiments were run. Over a period of five days, none of the 27 mutants displayed a significant fitness reduction when compared to the reference ygr059wΔ::downtag strain (table 3 & Figure 1).

Table 3: Aerobic growth in shake flasks and fitness reduction in aerobic competitive chemostats of the five mutants that showed significant disadvantage in anaerobic competitive chemostats. Data are presented as average ± mean deviation of results from two independent cultures for each strain. n.a. not applicable

Deletion mutant	Shake flask (hr-1)	Fitness reduction (%)
plb2Δ	0.39 ± 0.00	7.0 ± 3.7
ylr413wΔ	0.38 ± 0.02	11.6 ± 3.3
izh2Δ	0.38 ± 0.01	15.8 ± 7.4
eug1Δ	0.37 ± 0.02	8.6 ± 0.1
yor012wΔ	0.34 ± 0.00	14.8 ± 5.6
ygr059wΔ	0.40 ± 0.02	-
CEN.PK 113-7D	0.39 ± 0.00	n.a.

As an additional control, the specific growth rates of the five mutant strains that showed a reduced fitness in the anaerobic cultures was measured in (semi-)aerobic shake-flask cultures and were found not to differ significantly from those of the isogenic reference strains CEN.PK113-7D and *ygr059wΔ::downtag* (table 3). This implies that the reduction in fitness encountered in five of the mutant strains during anaerobic competitive growth was specific for anaerobiosis.

Discussion

Previous systematic comparisons of transcript levels and fitness of yeast mutants in batch cultures (Birrell *et al.*, 2002; Giaever *et al.*, 2002; Giaever *et al.*, 2004; Winzeler *et al.*, 1999) used the entire *S. cerevisiae* deletion library. The present study is the first to use transcriptome data for selecting target genes in chemostat-based competitive cultivation. We have reported a fitness profiling of knockout strains in genes that showed a significant upregulation under anaerobic conditions. Our experimental approach differs in several aspects from earlier *S. cerevisiae* (Baganz *et al.*, 1997; Baganz *et al.*, 1998; Colson, Delneri, and Oliver, 2004) and *Escherichia coli* (Chao and McBroom, 1985; Dean, Dykhuizen, and Hartl, 1988; Dean, 1989; Trobner and Piechocki, 1985) chemostat-based competition experiments: injection of a mutant pool into a steady-state culture, use of qrtPCR for quantification and the selection of strains based on transcriptome studies. This novel setup was (i) sensitive (qrtPCR versus qPCR, colony plate count or Affymetrix™ tag3 array) (ii) cost-effective (goal orientated gene deletion selection) and (iii) yielded reproducible results (immediate fitness test from steady-state conditions and prototrophic strains).

Our study has yielded five priority targets for further functional analysis of the molecular basis for anaerobic growth in *S. cerevisiae*. Further analysis will involve the use of multiple mutations to narrow down gene function in the study. The available literature provides some interesting leads. Lyons *et al.* (Lyons *et al.*, 2004) reported that *IZH2* is involved in coordinating both sterol and zinc metabolism under anoxia. The possibility that *izh2* mutants may be impaired in uptake of sterols, which are essential for anaerobic growth of *S. cerevisiae* (Andreasen and Stier, 1953), merits further research. YLR413w

encodes a protein with unknown function that has a 49 % sequence similarity to YKL187c, which is transcriptionally upregulated during growth on oleate (Kal *et al.*, 1999). It is conceivable that these genes are implicated in the uptake of essential unsaturated fatty acids, which are essential for anaerobic growth. It is relevant to note that, in the present study, oleate was provided as Tween-80 (polyoxyethylene sorbitan monooleate). Tween-80 was introduced to compensate for the inability of *S. cerevisiae* to *de novo* synthesize unsaturated fatty acids under anaerobic conditions. However, for Tween-80 to act as a source of oleate, the acyl-ester bond that links the oleate chain to the polyoxyethylene sorbitan complex must be cleaved. It is conceivable that this reaction is linked to the loss of fitness recorded for the *plb2Δ* strain. Plb2 might catalyse the hydrolysis of Tween-80 at the single fatty acid ester bond to yield oleate, as it does with lysophosphatidylcholine (Fyrst *et al.*, 1999). The incomplete functional complementation of *PLB1* and *PLB3* that were also expressed under anaerobic conditions might then reflect differences in substrate affinity and specificity of all three yeast phospholipases B as already reported (Merkel *et al.*, 2005).

EUG1 encodes a protein disulfide isomerase of the endoplasmic reticulum lumen. It has been previously suggested (Ter Linde and Steensma, 2002) that *EUG1* might be involved in glycosylation and the isomerization of disulfide bonds during the folding of anaerobically synthesized Dan/Tir cell wall proteins, but this suggestion has not yet been experimentally followed up. The reason of the fitness loss of the *yor012WΔ* strain that actually corresponds to the double mutant *yor012WΔ/yor013WΔ* remained unknown. As a consequence of the overlap between the ORFs, a more elaborated knock-out strategy should be applied to study each deletion individually and sort out which of the two genes contributes to the reduction of fitness observed.

Of 24 *S. cerevisiae* genes that showed a strong and consistent transcriptional upregulation under anaerobic conditions but were not previously implicated in anaerobic metabolism based on other experimental approaches, only five were shown to contribute to fitness under anaerobic conditions via competitive cultivation of null mutants. At first glance, it might be argued that this low 'hit rate' is due to the low dilution rate in the chemostat cultures (0.1 h^{-1} , which is 3-fold lower than the maximum specific growth rate $\mu_{\max}$ of *S. cerevisiae* CEN.PK113-7D under anaerobic conditions (Kuyper *et al.*, 2004)). This interpretation is, however, not correct, as mutations that have a

negative effect on the maximum specific growth rate will directly affect fitness because they lead to a lower affinity ($\mu_{\max}/K_s$) for the growth-limiting nutrient (where K_s is the substrate saturation constant) (Button, 1991; Monod, 1942).

Even though we sought to enrich the set of target genes by only including genes that showed a strong and consistent transcriptional upregulation under anaerobic conditions, the low 'hit rate' observed in our study was consistent with two earlier genome scale comparisons between transcript profiles and fitness where *S. cerevisiae* was exposed to DNA damaging agents (Birrell *et al.*, 2002) and grown in various stressful and growth conditions (1 M NaCl, 1.5 M Sorbitol, pH 8 and galactose) (Giaever *et al.*, 2002). Our observations show that high transcript levels cannot be interpreted as evidence for a unique physiological relevance of the encoded protein under the experimental conditions. This conclusion does not, however, imply that the observed transcriptional upregulation under anaerobic conditions is without biological significance.

Several mechanisms may explain why a transcriptional upregulation of a gene is not accompanied by a reduced fitness of the corresponding null mutant under the experimental conditions.

First, functional redundancy is an inherent problem in the analysis of (single) deletion mutants. While we have sought to reduce the impact of redundancy by eliminating members of highly related gene families from our study, several of the genes display sequence similarity with a single second yeast gene (Figure 1). For example, the role of the 'anaerobic' ATP/ADP translocase encoded by *AAC3* may well be taken over by its 'aerobic' counterparts *Aac1p* and/or *Aac2p* (Drgon *et al.*, 1992). *AAC1* is the only aerobic counter part since it is only expressed under aerobic conditions, however *AAC2/PET9* despite a higher expression in the presence of oxygen, is still expressed under anaerobic conditions (table 4) (Tai *et al.*, 2005). Similar functional complementation could occur for *UPC2* and *ANB1*, since *ECM22* and *HYP2* their respective homologue, were expressed irrespective of the oxygen regime (table 4) (Tai *et al.*, 2005).

FET4 is another anaerobic marker gene. It encodes a (FeII) low-affinity iron/zinc/copper transport system, and its expression is coregulated by iron and oxygen (Jensen and Culotta, 2002). Under aerobic conditions iron uptake is mainly achieved through the product of *FET3* that encodes a (FeII) high affinity transport system (Askwith, de Silva, and Kaplan, 1996). It is well conceivable that deletion of the gene *FET4* is compensated for by overexpression of one or

more high-affinity transport systems (Figure 4). A comparable mechanism of gene expression autoregulation has been already reported. Upon deletion of *PDC1* that encodes the major pyruvate decarboxylase, growth on glucose is rescued by overexpression of *PDC5* (Hohmann and Cederberg, 1990). Overall, in *S.cerevisiae* a quarter of those gene deletions that have no phenotype are compensated by duplicate genes (Gu *et al.*, 2003).

Table 4: Transcription intensities of genes with corresponding homologues in anaerobic (ANAe) and aerobic (Ae) chemostat cultures with limitations in carbon (C), nitrogen (N), phosphorus (P) and sulfur (S). Mean $\pm$ deviations derived from three independent chemostat experiments.

Gene name	C-Lim ANAe	N-Lim ANAe	P-Lim ANAe	S-Lim ANAe	C-Lim Ae	N-Lim Ae	P-Lim Ae	S-Lim Ae
AAC3	355 $\pm$ 148	311 $\pm$ 71	588 $\pm$ 23	387 $\pm$ 105	12 $\pm$ 0	20 $\pm$ 3	21 $\pm$ 4	22 $\pm$ 7
AAC1	60 $\pm$ 2	118 $\pm$ 15	72 $\pm$ 10	103 $\pm$ 22	529 $\pm$ 76	483 $\pm$ 67	440 $\pm$ 234	353 $\pm$ 26
AAC2/ PET9	803 $\pm$ 70	463 $\pm$ 34	396 $\pm$ 23	364 $\pm$ 21	1425 $\pm$ 122	1445 $\pm$ 47	1478 $\pm$ 145	1276 $\pm$ 98
UPC2	36 $\pm$ 25	50 $\pm$ 22	90 $\pm$ 16	66 $\pm$ 15	15 $\pm$ 3	12 $\pm$ 0	14 $\pm$ 3	12 $\pm$ 0
ECM22	182 $\pm$ 58	176 $\pm$ 30	164 $\pm$ 16	201 $\pm$ 33	138 $\pm$ 12	152 $\pm$ 21	165 $\pm$ 20	176 $\pm$ 6
ANB1	3320 $\pm$ 457	2392 $\pm$ 254	3193 $\pm$ 444	2967 $\pm$ 299	25 $\pm$ 6	16 $\pm$ 3	25 $\pm$ 4	18 $\pm$ 3
HYP2	2534 $\pm$ 625	3041 $\pm$ 384	3253 $\pm$ 505	2695 $\pm$ 170	2985 $\pm$ 1161	3547 $\pm$ 167	3572 $\pm$ 66	3699 $\pm$ 496
FET4	157 $\pm$ 41	334 $\pm$ 88	293 $\pm$ 19	316 $\pm$ 28	12 $\pm$ 0	123 $\pm$ 30	55 $\pm$ 5	17 $\pm$ 3
FET3	15 $\pm$ 4	15 $\pm$ 3	13 $\pm$ 1	46 $\pm$ 23	128 $\pm$ 43	29 $\pm$ 3	136 $\pm$ 19	110 $\pm$ 36

Second, the impact of the upregulation of a gene on fitness may be context dependent. For example, ammonia-limited growth of *S. cerevisiae* leads to a coordinated upregulation of transporters and enzymes involved in the assimilation of alternative nitrogen sources, even if these are not available in the growth medium (Boer *et al.*, 2003; Magasanik and Kaiser, 2002; ter Schure *et al.*, 1998). Similar mechanisms may underly the transcriptional upregulation under anaerobic conditions of some of the genes included in this study. For example, the oxidoreductase encoded by YGL039w may provide an excellent, energy-efficient redox sink for anaerobic growth – but only in the presence of its unknown substrate. This would also mean that assessing the contribution of transcriptionally upregulated genes would imply testing strains carrying multiple combinatorial deletion of differentially expressed transcripts.

Third, the implied teleological relationship between transcript profiles and fitness does not necessarily have to exist for all genes that show a consistent transcriptional response to a given stimulus. For example, transcriptional regulation networks may have evolved to couple transcriptional responses to environmental stimuli that tend to coincide in the natural environment. When these stimuli are separated in the laboratory or in industry, not all transcriptional responses have a direct bearing on each individual stimulus.

The present study underlines that, in *S. cerevisiae*, increased transcript levels cannot be interpreted as evidence for a contribution of the encoded protein to the cell's fitness in the immediate experimental context. A similar conclusion has been drawn based on a comparison of metabolic fluxes and transcript levels of the corresponding genes, which showed that transcript levels cannot be used as 'flux indicators' (Daran-Lapujade *et al.*, 2004). Rather than diminishing the value of transcriptome analysis, these observations underline the need for integrated 'systems' approaches to understand functions of genes and genomes.

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Chapter 4

Identification of anaerobic transcription factors

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○ Abstract

A method was designed to identify transcriptional regulating factors that are specific for the upregulation of genes under anaerobic conditions. Spt3, Spt4, Sac3 and Snf7 were found. However, transcriptional profiling of deletion strains of the corresponding genes showed that none of them specifically acted under anaerobic conditions, nor did they affect specifically 'true anaerobic genes, or genes that are essential under these conditions. The reasons for this are discussed.



Introduction

The yeast *Saccharomyces cerevisiae* is one of the few eukaryotic organisms that can grow under both aerobic and anaerobic conditions. Growth in the absence of molecular oxygen requires adaptation of the cell for at least three reasons. First, energy yield is usually much lower than under aerobic conditions, second several biosynthetic pathways require molecular oxygen and third, different molecules have to be transported in and out the cell. In *S. cerevisiae* this adaptation appears mainly on the regulatory level, in particular transcription. Only 23 of the genes are specifically essential for anaerobic growth, whereas transcription levels of circa 500 genes differ significantly when aerobic and anaerobic cultures are compared. For many of these genes it is unclear why they are essential or why their expression levels are up- or down regulated under anaerobic conditions. In this study we set out to understand more about the transcriptional regulation of genes under anaerobic conditions.

All the adaptations to an anaerobic environment have to be regulated on either transcriptional or (post-) translational levels. Several factors for transcriptional regulation of anaerobic metabolism have been proposed. One of those is Rox1p which represses hypoxic genes in the presence of oxygen (Deckert *et al.*, 1995). ROX1 is a target of Hap1p, which activates the transcription of aerobic genes when bound to heme (Zhang and Guarente, 1995; Hach, Hon, and Zhang, 1999). In another regulatory system *UPC2* and *ECM22* are implicated in a dual role in the induction of anaerobic sterol import (Crowley *et al.*, 1998; Shianna *et al.*, 2001; Ter Linde, 2003). Upon sterol depletion, Upc2 levels increase, as does the amount of Upc2p bound to promoters. Ecm22, however, shows a decrease both in the total amount of protein in the cell and in the fraction bound to promoters (Davies, Wang, and Rine, 2005). Other genes have also been implicated in anaerobic regulation either because of their effect on translational levels or because of their heme-dependency, such as *SUT1* (Ness *et al.*, 2001), *ORD1* (Lambert JR, Bilanchone VW, and Cumsky MG, 1994), and *HAP2/3/4/5* (Zitomer and Lowry, 1992). All of these genes together regulate the expression of aerobically and anaerobically specific genes in a complex way. However, the transcriptional responses to anaerobiosis of many genes are still unexplained, such as the *PAU* genes, which are genes of unknown function that are strongly and consistently upregulated under anaerobic conditions (Tai *et al.*,

2005). Also, the transcriptional changes of the cell wall proteins Dan1 and Tir1 when aerobic conditions are compared to anaerobic ones, cannot be explained by the alleviation of aerobic repression by Rox1 alone (Kitagaki H, Shimoï H, and Itoh K, 1997; Ter Linde and Steensma, 2002). It has been shown that for the *DAN/TIR* genes activation through Upc2 is necessary. Repression appears to be mediated by Rox1, Mot3, Mox1, Mox2 and the Tup1/Ssn6 complex (Abramova *et al.*, 2001). Repression of *ANB1* is not completely abolished by deletion of *ROX1*, suggesting that in this case activation is also needed (Ter Linde and Steensma, 2002). Furthermore, the anaerobically upregulated YML083C gene does carry a *ROX1* binding site in its promoter, but deletion of these bases has no effect on transcription levels (Ter Linde and Steensma, 2003). Therefore other regulatory factors must exist, which regulate transcription of genes under anaerobic conditions.

In this study we describe a method to identify genes that have a role in the regulation of mRNA levels of anaerobic specific genes. One of the genes identified in this screen was *SPT3*, encoding a component of the SAGA complex (Bhaumik and Green, 2002; Sterner *et al.*, 2004; Wu *et al.*, 2004). This complex regulates transcriptional initiation by regulating the binding of TATA-binding protein to the DNA (Sterner *et al.*, 2004). It appears to function complementary to the TFIID initiation factor. TFIID affects mostly housekeeping genes, while the SAGA complex has an effect on stress-related genes (Huisinga and Pugh, 2004). To confirm its role in anaerobic transcription regulation, transcriptome analysis was performed in a wild type strain and an isogenic *spt3* deletion mutant, both under aerobic and anaerobic conditions as described in chapter 5. The other factors that were identified as putative anaerobic upregulators were *SPT4*, Spt4 together with Spt5 forms an elongator complex (Lindstrom *et al.*, 2003; Rondon *et al.*, 2003; Yamaguchi *et al.*, 2001), *SAC3*, which is known to be involved in transcription and in mRNA export from the nucleus (Fischer *et al.*, 2002; Gallardo *et al.*, 2003; Lei *et al.*, 2003; Novick, Osmond, and Botstein, 1989), and *SNF7*, which was first isolated in a screen for sucrose non fermentable mutations and was subsequently identified as a member of the endosomal sorting complex (Babst *et al.*, 2004; Bowers *et al.*, 2004). Deletion mutants of these genes were grown in chemostat cultures and analyzed for transcriptome differences with the isogenic wild type strain under anaerobic conditions only. Finally, we compared our data with the set of genes that was consistently up- or

downregulated in the presence or absence of molecular oxygen (Tai *et al.*, 2005) for information on the anaerobic specificity of the identified factors. A detailed analysis of the $\Delta spt3$ and $\Delta snf7$ strains will be presented in chapters 5 and 6 respectively.

Materials and methods

Strains and plasmids

For the screening experiments the collection of gene-deletion mutants was used, which was created by substituting each known ORF by a KanMX-cassette (Giaever *et al.*, 2002). The collection consists of several parts. In the first one, strains are represented that contain the deletion of a gene that is aerobically essential. These strains are present as heterozygotes, with only one of the two alleles replaced. The second part contains homozygous strains with deletions in aerobically non-essential genes. These deletions are also available in *MATa* or *MAT α* haploid strains. The strain background used for this collection is BY4743 (*MATa*/ α *his3 Δ 1/his3 Δ 1 leu2 Δ 0/leu2 Δ 0 lys2 Δ 0/LYS2 MET15/met15 Δ 0 ura3 Δ 0/ura3 Δ 0*). For transcriptome analysis relevant genes were disrupted in the prototrophic strain CEN.PK 113-7D (*MATa*), giving the deletion mutants GG3098 (*MATa spt3::KanMX*), GG3099 (*MATa spt4::KanMX*), GG3200 (*MATa sac3::KanMX*) and GG3201 (*MATa snf7::KanMX*).

Plasmids were amplified in *E. coli* strain XL1blue (Bullock, Fernandez, and Short, 1987). Plasmids used are listed in table 1.

Media

Yeast cells were grown in either YPD (Difco peptone 2%, Difco yeast extract 1%, glucose 2%), when necessary provided with 150 μ g/ml G418 or 50 μ g/ml ClonNAT, or in mineral medium (Zonneveld, 1986). When required, L-lysine (30 μ g/ml), L-leucine (30 μ g/ml), L-histidine (20 μ g/ml) or uracil (30 μ g/ml) were added. For anaerobic growth, 420 μ g/ml Tween80 and 10 μ g/ml ergosterol were added to the media (Verduyn *et al.*, 1992). *E. coli* was grown in LB medium (Sambrook, Fritsch, and Maniatis, 1989). If plasmids were present ampicillin was added to 60 μ g/ml. Media were solidified by the addition of 1.5% agar (Sphero).

Table1: Plasmids used in this study

Plasmid	Properties	Reference
pRUL302	<i>ARSH4</i> , <i>CEN6</i> , <i>URA3</i> , Amp ^r , ORI C, <i>LacZ</i>	(Ter Linde and Steensma, 2003)
pRUL414	pRUL302 with ClonNat marker	This study
PDAN	pRUL414 with promoter region of <i>DAN1</i>	This study
PTIR	pRUL414 with promoter region of <i>TIR1</i>	This study
PANB	pRUL414 with promoter region of <i>ANB1</i>	This study
pRS316	<i>ARSH4</i> , <i>CEN6</i> , <i>URA3</i> , Amp ^r , ORI C, <i>LacZ</i> in MCS	(Sikorski and Hieter, 1989)
pRS316-SPT3	pRS316 with <i>SPT3</i>	This study
pRS316-SPT4	pRS316 with <i>SPT4</i>	This study
pRS316-SAC3	pRS316 with <i>SAC3</i>	This study
pRS315	<i>ARSH4</i> , <i>CEN6</i> , <i>LEU2</i> , Amp ^r , ORI C, <i>LacZ</i> in MCS	(Sikorski and Hieter, 1989)
pRS315-SNF7	pRS315 with <i>SNF7</i>	This study
pKINAT	<i>ARSH4</i> , <i>CEN6</i> , ClonNat marker, Amp ^r , ORI C, <i>KICEN2</i> , KARS	(Zeeman and Steensma, 2003)

Genetic techniques

To obtain pRUL414, the ClonNat marker from pKINat was excised using BglII and StuI, and ligated into the BglII and SmaI sites of pRUL302. Plasmids were constructed by amplifying the DNA fragment of interest using PCR (primers are listed in table 2). The promoter regions of the genes *DAN1* (-1232 to +23 from the ATG start codon), *TIR1* (-1429 to +5 from the ATG start codon) and *ANB1* (-1505 to +5 from the ATG start codon) were cloned in between the HindIII and BamHI sites of pRUL414, in frame with the *LacZ* gene, giving plasmids PDAN, PTIR and PANB respectively. The *SPT3* gene was cloned into pRS316 using the BamHI and EcoRI sites. *SPT4* and *SAC3* genes were ligated in between the BamHI and the SalI sites of pRS316. The *SNF7* gene was cloned in between the BamHI and SalI sites of pRS315. All enzymes were purchased from New England Biolabs (USA), including the T4 DNA ligase. Plasmids were amplified in *E. coli* strain XL1-blue. Transformation of individual yeast strains

with the plasmids was done using the lithium acetate method (Schiestl and Gietz, 1989). Standard genetic techniques were used according to Sambrook *et al.* (Sambrook, Fritsch, and Maniatis, 1989).

Table 2: Primers used in construction of the plasmids and deletion strains

Name primer	Restriction site	Primer
DANforward	<i>Hind</i> III	CCCAAGCTTGCAAACTTCGACCTTCTGTATC
DANframe	<i>Bam</i> HI	CGCGGATCCGCTAATACTAATTCTAGACATTACTTGG
TIRforward	<i>Hind</i> III	CCCAAGCTTCTTCATCACACAGTGTCTAGCG
TIRframe	<i>Bam</i> HI	CGCGGATCCGCCATTTTTAATTATTGTAGTACTTG
ANBforward	<i>Hind</i> III	CCCAAGCTTAATGTTACATGCGTACACGCG
ANBframe	<i>Bam</i> HI	CGCGGATCCGACATGTTTTAGTGTGTGAATGA
SPT3forward	<i>Bam</i> HI	CGCGGATCCTATACGCCGGCGGCATTTCT
SPT3reverse	<i>Eco</i> RI	CCGGAATTCAATCACTGAGTTACCCCGTTAC
SPT4forward	<i>Bam</i> HI	CGCGGATCCCGTAGTCCAATTTACGTGAAG
SPT4reverse	<i>Sal</i> I	ACGCGTCGACAATTTTCTATCCTTGGACC
SAC3forward	<i>Bam</i> HI	CGCGGATCCGTTCAAGAACAGGACCTGCTCCATCC
SAC3reverse	<i>Sal</i> I	ACGCGTCGACCCGTCTGTATCATTTCTAGCAAGGC
SNF7forward	<i>Bam</i> HI	CGCGGATCCCATTTCTAGTGATTTCGCCTC
SNF7reverse	<i>Sal</i> I	ACGCGTCGACATGCAAACGTAGACGACATCG

Transformation of the collection of deletion strains

Based on the lithium acetate transformation method of Schiestl and Gietz (1989), we developed a method for high-throughput transformation of *S. cerevisiae*. Cells were grown overnight in 96-wells flat bottom microtiter plates in 140 µl YPD G418, while shaking at 900 rpm in a Titrimax 1000 incubator (Heidolph, Germany) at 30°C. The next day 14 µl of culture were transferred to a new microtiter plate containing 140 µl of fresh YPDG418 and the cells were allowed to grow for another 4 h under the same conditions. Cells were harvested by centrifugation for 3 min at 2500 rpm in a Sigma laboratory centrifuge 4-15C, rotor 09100/09366 (Qiagen, Germany). 120 µl of the supernatant were removed

and the cells were resuspended in the remaining 34 μl of medium by shaking for 5 min at 900 rpm. Then 90 μl of a transformation mixture was added per well. The transformation mixture contained per well, 62 μl 50% PEG 3350 (w/v), 10 μl 1M LiAc containing 4% DTT (w/v), 6 μl ssDNA (2 mg/ml) and 1 μg plasmid in 12 μl H_2O . After addition of the transformation mixture shaking was continued for 5 min at 900 rpm and then the cells were incubated at 30°C without shaking. After 30 min the temperature was raised to 42°C. When this temperature was reached (after approximately 5 min), the cells were left to incubate for another 40 min before 5 μl of the cell suspension was spotted onto plates that had been dried in a 65°C incubator for 20 minutes. Plates were incubated at 30°C for 3 days. When a dominant marker was used, the cells were spun down after transformation for 3 min at 2000 rpm. 90 μl of supernatant was removed and replaced by 100 μl YPD. The cells were incubated 5 hours at 30°C while shaking at 900 rpm to recover before plating.

β -galactosidase assay

In order to accommodate the large amounts of strains we slightly modified the procedure commonly used (Ter Linde and Steensma, 2003). In brief, the transformed strains were inoculated in two duplicate microtiter plates with 70 μl of mineral medium per well and incubated 36 h at 30°C without shaking. One of the plates was incubated aerobically and the duplicate plate anaerobically by the use of Anaerocult IS sacks (Merck, Germany). After incubation, 35 μl of yeast lysis reagent Y-PER (Perbio, USA) and 35 μl of ONPG (4 mg/ml) in potassium phosphate buffer (100 mM, pH 7.0) were added. The reagents were mixed by shaking 5 min at 900 rpm and then incubated at 30°C until a clearly visible yellow color appeared, usually after circa 30 min. The reaction was halted by addition of 40 μl stop solution (1M Na_2CO_3) and mixing by shaking 5 min at 900 rpm. The time between addition of the reagents and addition of the stop solution was noted. Yellow color was measured at 420 nm in a microtiter plate reader (model 3550, Biorad, USA). Activity in arbitrary units was calculated with the use of the formula: $\text{Activity} = (1000 \times A_{420}) / (t \times V \times A_{655})$, in which t is the time (in min) between the addition of the reagent mixture and the addition of the stop solution, and V is the volume of the culture (in this case 0.07 ml). The value of A_{655} is included in the formula to compensate for the differences in amount of cells present in the wells.

Deletion of ORF's

Deletion of target genes in the strain CEN.PK 113-7D was done by amplifying the relevant parts of the deletion strains created by Giaever *et al.* (2002) with additional flanking regions of about 500 bp upstream and 200 bp downstream of the original ORF's using the primers listed in table 2. The PCR products were used to transform the wild type yeast cells. The cognate genes used for complementation of the mutants were obtained by PCR using the same primers on the wild type strain BY4743.

Chemostat fermentation and microarray experiments

Three separate glucose-limited steady state cultures for each strain in mineral medium, both aerobic and anaerobic, were obtained as described earlier (Tai *et al.*, 2005). In brief, 2-liter Applikon fermenters with a working volume of 1 liter were used at a dilution rate of 0.10 h^{-1} and a temperature of 30°C . pH was kept constant at 5 by the automatic addition of a 2 M KOH solution. Stirrer speed was set at 800 rpm. Cells were harvested in liquid nitrogen. For each of the steady state cultures RNA was isolated and used for microarray analyses using Affymetrix gene chips as described previously (Tai *et al.*, 2005). Results were analyzed using the Statistical Analysis of Microarrays tool (SAM, version 2.0) with a δ -value giving a false hit rate of 1% (Tusher, Tibshirani, and Chu, 2001). Genes were considered changed in expression level when listed as such by SAM and when the fold change was more than 2-fold.

Statistical significance, other than by SAM, was determined using a homoscedastic student's t-test assuming a 2-tailed distribution. Changes were considered significant when the P-value was smaller than 0.001.

Batch fermentation and Q-PCR

An initial working volume of 1.5 liter was used in 2-liter Applikon fermenters. Cells were grown in mineral medium with an initial glucose concentration of 2%. Temperature was maintained at 30°C . pH was kept constant at 5 by the automatic addition of a 2 M KOH solution. Stirrer speed was set at 800 rpm. Cells were harvested in liquid nitrogen when the culture was in the exponentially growing phase, when the cells had been stationary for three to four hours and at the transition between these two phases. This was monitored through the CO_2 production. These experiments were done in duplicate.

For the single batch fermenters with the strains containing the PDAN plasmid, a working volume of 1 liter was used. The ClonNAT concentration was 50 µg/ml. Cells were harvested 20 hours after inoculation, which means they were in stationary phase.

Total RNA was extracted as described previously (Chantrel *et al.*, 1998). This was treated with DNase according to the RNeasy protocol of Qiagen (Germany). cDNA was synthesized according to the M-MVT-RT protocol (Invitrogen, USA). Q-PCR reactions were done using the SYBR Green Jumpstart Taq ReadyMix (Sigma, Germany) in the Continuous Fluorescence Detector of DNA Engine Opticon (MJ Research, USA). Primers were designed close to the 3' end of the *ACT1*, *DAN1* and *LacZ* genes, such that the PCR reaction would result in a product of approximately 80 base pairs.

Results

Transformation of the collection of 4491 deletion strains (48 microtiter plates) with the plasmid PDAN using the high-throughput method yielded transformants for 95% of the strains. The remaining 222 strains were transformed individually by the traditional LiAc method (Gietz *et al.*, 1995). All of the mutants could be transformed; hence genes that are essential for the ability to be transformed were not present in this deletion collection. All transformants were grown in YPD with Tween80 and ergosterol added both aerobically and anaerobically and the activity of the *LacZ* reporter gene was assayed. Three strains did not develop a yellow color in the β -galactosidase assay test when pre-incubated anaerobically and thus appeared to be unable to activate the *DAN1* promoter under anaerobic conditions. The genes deleted in these strains were *SPT3*, *SPT4* and *SAC3* and hence these genes may be involved in activation of anaerobic genes. One strain, with a deletion of *PFK2*, was able to activate the *DAN1* promoter under aerobic conditions. Additionally, using the plasmid PTIR as a reporter plasmid, we identified *SNF7* as an additional putative anaerobic activator. To check if the observed effect was specific to the promoter used or if the effect was more general for anaerobic conditions, the β -galactosidase assay was performed on the mutants, using other reporter plasmids, i.e. PTIR and PANB for the strains lacking *SPT3*, *SPT4*, *SAC3*, and PDAN and PANB for the

strain lacking *SNF7*. Deletion of each of the four identified genes, *SPT3*, *SPT4*, *SAC3* and *SNF7*, had the same effect on the three reporter constructs, with the exception of PDAN in the *snf7* mutant. This combination turned slightly yellow in the β -galactosidase test after anaerobic growth, but much less than the wild type control (table 3). Hence, the four genes are not specific for one gene and may have a more global role in anaerobic activation. Complementation experiments, using the cognate genes on the single-copy plasmids, pRS316-SPT3, pRS316-SPT4, pRS316-SAC3 and pRS315-SNF7 respectively, confirmed that the observed effect was truly due to the deletion of these genes.

Table 3: β -galactosidase activities in wild type BY4743 and (complemented) *spt3*, *spt4*, *sac3*, *snf7* deletion strains carrying the plasmids PDAN, PTIR and PANB

strain	plasmids	Aerobic activity	Anaerobic activity
BY4743	None	0.00	0.00 (0.00)
BY4743	PDAN	0.00	0.79 (0.33)
BY4743	PTIR	0.00	0.61 (0.05)
BY4743	PANB	0.00	0.27 (0.06)
$\Delta spt3$	None	0.00	0.00 (0.00)
$\Delta spt3$	PDAN	0.00	0.00 (0.00)
$\Delta spt3$	PTIR	0.00	0.00 (0.00)
$\Delta spt3$	PANB	0.00	0.00 (0.00)
$\Delta spt3$	SPT3 + PDAN	0.00	0.46 (0.24)
$\Delta spt4$	None	0.00	0.00 (0.00)
$\Delta spt4$	PDAN	0.00	0.04 (0.02)
$\Delta spt4$	PTIR	0.00	0.06 (0.03)
$\Delta spt4$	PANB	0.00	0.03 (0.01)
$\Delta spt4$	SPT4 + PDAN	0.00	0.24 (0.02)
$\Delta sac3$	None	0.00	0.00 (0.00)
$\Delta sac3$	PDAN	0.00	0.06 (0.02)
$\Delta sac3$	PTIR	0.00	0.03 (0.04)
$\Delta sac3$	PANB	0.00	0.07 (0.01)
$\Delta sac3$	SAC3 + PDAN	0.00	0.43 (0.06)
$\Delta snf7$	None	0.00	0.00 (0.00)
$\Delta snf7$	PDAN	0.00	0.21 (0.10)
$\Delta snf7$	PTIR	0.00	0.06 (0.04)
$\Delta snf7$	PANB	0.00	0.15 (0.06)
$\Delta snf7$	SNF7 PTIR	0.00	0.20 (0.04)

In order to perform controlled chemostat cultivation experiments, strains without auxotrophic markers are preferred (Pronk, 2002). Therefore, the deletions of *SPT3*, *SPT4*, *SAC3*, and *SNF7* were transferred to the CEN.PK 113-7D background as described in the Materials and Methods section. Deletion was confirmed by PCR, by phenotypic analyses, i.e. ability to grow on YPD with G418 added, and by Southern blotting (results not shown). The deletions in the CEN.PK 113-7D background had the same effect on the expression of the *LacZ* reporter gene under control of the *DAN1*, *TIR1* and *ANB1* promoters as in the BY4743 strain. Transcriptome analyses were performed on mRNA extracted from three independent anaerobic chemostat cultures of the $\Delta spt3$, $\Delta spt4$, $\Delta sac3$, $\Delta snf7$ and the wild type strains, while for the $\Delta spt3$ and the wild type strain also aerobic chemostat cultures were grown. In chapter 5 the results of the $\Delta spt3$ data set will be discussed, while the $\Delta snf7$ data set is described in chapter 6. In this chapter only the results from the microarrays performed with the *spt4* and *sac3* deletion strains are presented.

A graphic representation of the number of genes that are up- and downregulated in the deletion strains as compared to the isogenic wild type strain under anaerobic conditions is shown in figure 1.

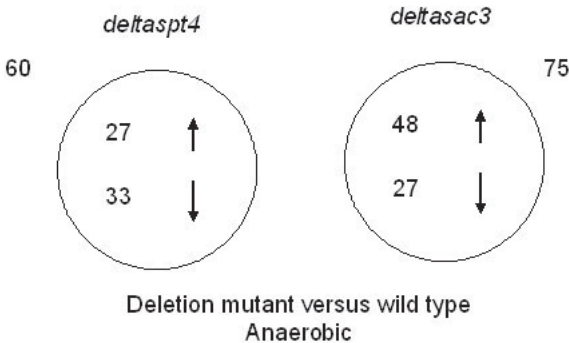


Figure 1: Number of genes that changes transcription level at least 2-fold in the deletion strains as compared to the isogenic wild type strain under anaerobic conditions.

Deletion of *SPT4* resulted in the upregulation of 27 genes and the downregulation of 33 genes, while deletion of *SAC3* caused 48 genes to be upregulated and 27 genes to be downregulated in their mRNA levels. Categorization of the genes that change transcript level more than 2-fold in one of the deletion mutants was performed with the use of the program funspec (<http://funspec.med.utoronto.ca/>),

using a cut-off value of $P < 0.001$. The results are shown in table 4. The genes that were upregulated in $\Delta spt4$ showed an overrepresentation of transporter genes. The genes upregulated in the *sac3* deletion strain had an overrepresentation of transcription factors, while the genes that were downregulated in this strain fell in the categories amino acid biosynthesis and oligopeptide transporter.

Promoter analysis and subsequent identification of motifs was performed using the RSAT (<http://rsat.scmbb.ulb.ac.be/rsat/>) and SCPD (<http://rulai.cshl.edu/SCPD/>) programs respectively. However, consensus sequences were not found.

Table 4: Results from funspec analysis on the microarray data.
The numbers behind the MIPS categories indicate
the total number of genes in that category.

Regulated in strain	MIPS category	Genes	P-value
upregulated in $\Delta spt4$	transporter (372)	<i>COR1 AST1 SIT1</i> <i>FCY21 FTR1</i> <i>BOS1 FET4</i>	7.7e-4
downregulated in $\Delta spt4$	heat shock protein (18)	<i>HSP26 HSP30</i> <i>HSP12</i>	8.5e-5
upregulated in $\Delta sac3$	perception of nutrients and nutritional adaptation (25)	<i>RPI1 PTK2 PTP2</i>	8.6e-4
	transcription factor (250)	<i>MED8 TFB3</i> <i>ASH1 GAT2</i> <i>CRZ1 BDP1</i> <i>HMS1 NDD1</i> <i>CUP9</i>	9.5e-5
downregulated in $\Delta sac3$	amino acid biosynthesis (118)	<i>LYS2 ARO4 ILV1</i> <i>TRM5 ARG1</i>	1.3e-4
	purine ribonucleotide metabolism (45)	<i>IMD1 YAR075W</i> <i>IMD2</i>	8.9e-4
	oligopeptide transporter (2)	<i>OPT1 OPT2</i>	1.8e-5

The effect for which the transcription factors were chosen, namely lowering of the transcription levels of *LacZ* under the control of the anaerobic promoters, was less clear in the transcriptome data than in the β -galactosidase assays. Most surprisingly, inspection of the mRNA levels of the *DAN1*, *TIR1* and *ANB1* genes showed that these were not changed for all three transcription factors (figure 2).

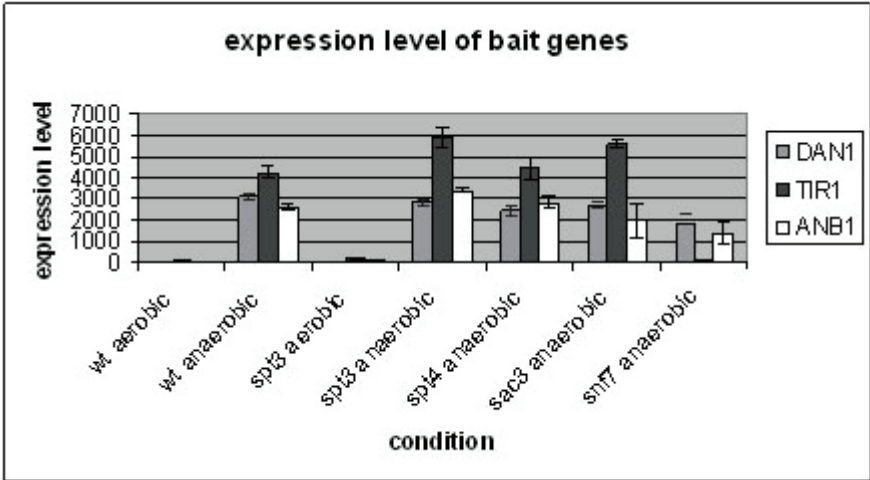


Figure 2: expression levels of bait genes in the wild type and deletion strains under anaerobic and/or aerobic conditions.

A plausible explanation could be that the effect of the deletions is context-dependent. The putative regulators identified in the screen may be active under excess-glucose conditions, which were present in the initial stages of the first screen, but not under low-glucose conditions, which were present in the glucose-limited chemostat cultures. Alternatively, there might be a link with specific growth rate or cell density, both of which were lower in the chemostat experiments than in the shake flasks. However, samples taken at different time points in batch cultures with the *spt3* deletion strain and the isogenic wild type strain all showed an expression of the endogenous *DAN1* gene comparable to that found in the chemostat cultures. This result rules out any influence of cell density, growth rate, growth phase or glucose concentration. Therefore, batch fermenters were run with the *spt3* deletion strain and the isogenic wild type, both containing the PDAN plasmid. Activity of the β -galactosidase protein was

assayed and cell lysate of wild type cells turned yellow, but that of the deletion strain did not. Q-PCR experiments on RNA extracted from these cultures showed a 50% decrease in mRNA levels for both the endogenous *DAN1* gene and the *LacZ* gene carried by the plasmid in the *spt3* deletion strain as compared to the mRNA level of the *DAN1* gene in the wild type strain.

Discussion

Identification of transcription factors

The method to identify new anaerobic transcription factors identified four genes, i.e. *SPT3*, *SPT4*, *SAC3*, *SNF7*, that were previously not correlated to the regulation of transcription levels as a response to anaerobicity. All four are known to have a link to transcription. Spt3 is a member of the yeast SAGA (Spt-Ada-Gcn5-Acetyltransferase) complex and has a role in binding TBP (TATA binding protein) (Sterner *et al.*, 2004). Spt4, together with Spt5, forms a complex that regulates RNA pol II transcript elongation (Howard, Hester, and Herman, 2003). Sac3 forms a complex with Thp1. This complex is thought to link mRNA metabolism to transcription (Gallardo *et al.*, 2003). *SNF7* is a member of a family with a vacuolar protein sorting (vps) function (Babst *et al.*, 2004) and was first identified because of its regulating effect on *SUC2* expression (Vallier and Carlson, 1991).

None of the known regulatory factors of anaerobicity were found. Deletion strains of *HAP1* and *ROX1* were not present in the library and could therefore not have been found. The strains lacking *UPC2* or *ECM22*, which encode two similar proteins that are necessary for the activation of anaerobic genes including *DAN1* (Shianna *et al.*, 2001), did not show any significant drop in β -galactosidase activity. Since the two proteins are similar in amino acid sequence, it is conceivable that they can take over each others function. *SUT1* and *SUT2*, encoding activators of sterol import (Ness *et al.*, 2001), probably pose the same kind of problem. Deletion of the genes encoding components of the Hap2/3/4/5 complex did not result in changes in β -galactosidase activity in our screen either. Since the promoter regions in the reporter plasmids were from two genes that are connected to the cell wall, i.e. *DAN1* and *TIR1* (Kitagaki H, Shimoi H, and Itoh K, 1997;Mrsa *et al.*, 1999), and from one gene that is

involved in translational initiation, i.e. *ANB1* (McCarthy, 2005), and since the Hap2/3/4/5 complex is involved in the repression of respiratory proteins (McNabb, Xing, and Guarente, 1995), it is conceivable that this complex indeed has no regulatory effect on the promoters used in this study.

It is very likely that the choice of reporter gene is responsible for the identity of the genes that were picked up. The screen done in this study appears not to be a screen for anaerobic transcription factors, but one for factors involved in the proper transcription of *LacZ*, a long and G+C rich gene. Therefore, the method in itself is not discredited, as long as a proper reporter gene is used.

Transcriptional analysis

In the *spt4* deletion strain 60 genes change transcription levels significantly, while in the *sac3* deletion strain 75 genes change. To assess whether Spt4 and Sac3 have anaerobic specificity, the genes that were changed in transcription level were compared to the genes found by Tai *et al.* (2005). They found that 65 genes were consistently upregulated under anaerobic conditions, while 90 genes were consistently downregulated. These 155 genes could be termed the 'true anaerobic genes' because they consistently change transcription levels under anaerobic conditions as compared to aerobic conditions, independently of the nutrient limitation. For the *spt4* and *sac3* deletion strains, the changes in anaerobic expression levels of the genes were compared to those in the isogenic wild type strain. While 1% of the aerobic genes (54 and 73 respectively out of 6449 (<http://www.yeastgenome.org/cache/genomeSnapshot.html>)) for $\Delta spt4$ and $\Delta sac3$ changed transcription when anaerobically grown wild type and mutants were compared, these percentages are 4% (6 of 155) for the $\Delta spt4$ strain and 1% (2 of 155) for the $\Delta sac3$ strain when the 'true anaerobic genes' are examined. However, the numbers are too small to draw conclusions with respect to the anaerobic specificity of these two factors. We also checked the genes that were experimentally found to be essential for anaerobic growth only (Snoek and Steensma, 2006) for a negative trend in fold change when the deletion strains are compared to the wild type strain. No such trend could be found. Almost all of these 23 genes show only very small fold changes and most of these are positive. This means that using this criterion, no anaerobic specificity could be observed either.

Categorization by funspec of the genes that changed expression level in the *spt4* deletion strain compared to the isogenic wild type strain, resulted in 7 genes that were categorized as 'transporter' in the upregulated group of genes and 3 genes that were categorized as heat shock proteins in the downregulated group of genes. Compared to the number of genes present in these MIPS categories, 372 and 18 respectively, and the number of genes upregulated in the $\Delta spt4$ strain in total (Causton *et al.*, 2001), indicating that many other functions are regulated by Spt4. We therefore conclude that there does not appear to be specificity for elongation by the Spt4/Spt5 complex under anaerobic conditions. The reason the *spt4* deletion strain was identified in the screen could be that *S. cerevisiae* has difficulties elongating the *LacZ* gene, as is indicated by the lack of transcription in $\Delta hpr1$, $\Delta tho1$ and $\Delta tho2$ strains (Chavez and Aguilera, 1997; Piruat and Aguilera, 1998). This is probably due to the length and G+C richness of the *LacZ* gene (Chavez *et al.*, 2001). In a transcription elongation screen using the *LacZ* gene, *SPT4* was identified as having a function in transcription elongation (Rondon *et al.*, 2003).

Among the 48 genes that are upregulated upon deletion of *SAC3* the category 'transcription factor' included 9 genes. It is therefore likely that transcriptional changes observed in the deletion strain are due to a combination of the deletion of *SAC3* itself and the changed expression levels of these nine transcription factors. Other categories that are significant in the group of genes that changed transcription level upon deletion of *SAC3* are 'perception of nutrients and nutritional adaptation', 'amino acid biosynthesis', 'purine ribonucleotide metabolism' and 'oligopeptide transporter'. These are all involved in nutrients. This could reflect the role *SAC3* has in leucine biosynthesis. The fact that the *sac3* deletion mutant gives rise to altered mRNA levels is likely due to the role of *Sac3* in linking mRNA export with transcription initiation, as was described for the *GAL* genes (Cabal *et al.*, 2006). This link appears to be dependent on the *SAGA* complex (Rodriguez-Navarro *et al.*, 2004; Cabal *et al.*, 2006).

Batch fermenters were run to examine the fact that the mRNA expression of the bait genes *DAN1*, *TIR1* and *ANB1* was not changed in respect to those of the wild type strain. The results indicate that the lack of protein level in the *spt3* deletion strain is only partly due to a decrease in mRNA level and that this decrease is dependent on the presence of the *SPT3* gene. Since the primers used in these reactions were located at the 3' end of the genes, the observed

transcripts are full-length mRNA. It could be that the decrease in transcription is due to early termination, in which case the aberrant mRNA molecules are not recognized by the primers and are probably degraded fast in the cell. This might indicate that apart from its function in transcription initiation, Spt3, and perhaps even the entire SAGA complex, has a role in transcriptional elongation as well. This explanation would also be appropriate for the *spt4* deletion strain, since this protein is part of the Spt4/Spt5 elongation complex. The effects of the *sac3* deletion could also be due to early termination of the transcripts since transcription and mRNA export and processing are linked (Cabal *et al.*, 2006; Taddei *et al.*, 2006).

In hindsight is likely that the method we developed to identify novel anaerobic transcription factors will give different results when another reporter gene is used that has less problems in elongation. Therefore, a yeast-gene, such as *CUP1* which in higher copy numbers gives resistance to higher copper ion concentrations by binding the ions, would be preferable.

Acknowledgements

We like to thank Koen de Graaff and Maurice Toirkens for the work on the fermentations, and Marinka Almering for help with the Affymetrix chips.

Chapter 5

Deletion of the SAGA component *SPT3* affects a different set of *Saccharomyces cerevisiae* genes depending on oxygen availability.

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○ Abstract

To understand more about the transcriptional regulation of genes under anaerobic conditions, the transcriptome data of a $\Delta spt3$ strain and the isogenic wild type strain were analyzed. The results of this study indicate that transcriptional activation or inhibition through Spt3 can be influenced by environmental conditions. A model is proposed in which different conditions lead to different forms of the SAGA complex. The specific composition of these SAGA-forms determines which subset of genes will be activated. In this model SAGA plays a key role in integrating the environmental conditions the cell faces to give rise to a transcriptional response that optimizes the adaptation to that specific set of conditions.



Introduction

The yeast *Saccharomyces cerevisiae* is one of the few eukaryotic organisms that can grow equally well under aerobic and anaerobic conditions. Growth in the absence of molecular oxygen requires adaptation of the cell for at least three reasons (Snoek and Steensma, 2007). First, energy yield is usually much lower than under aerobic conditions, second several biosynthetic pathways require molecular oxygen and third, different molecules have to be transported in and out the cell. In *S. cerevisiae* this adaptation appears mainly on the regulatory level, in particular transcription. Only 23 of the genes are specifically essential for anaerobic growth (Snoek and Steensma, 2006), whereas transcription levels of circa 500 genes differ significantly when aerobic and anaerobic cultures are compared (Ter Linde *et al.*, 1999). A subset of 155 of these genes changes transcription levels consistently when anaerobic and aerobic conditions are compared, irrespective of nutrient limitation (Tai *et al.*, 2005). For many of these genes it is unclear why they are essential or why their expression levels are higher or lower under anaerobic conditions.

In this study we set out to understand more about the transcriptional regulation of genes under anaerobic conditions. In our laboratory, *SPT3* was previously identified as possibly being involved with transcription under anaerobic conditions. The *SPT3* gene encodes a component of the SAGA complex (Bhaumik and Green, 2002; Sterner *et al.*, 2004; Wu *et al.*, 2004). This complex regulates transcriptional initiation by regulating the binding of TATA-binding protein to the DNA (Sterner *et al.*, 2004). It appears to function complementary to the TFIID initiation factor. TFIID affects mostly housekeeping genes, while the SAGA complex has an effect on stress-related genes (Huisinga and Pugh, 2004). We analyzed the transcriptome data of aerobically and anaerobically grown chemostat cultures of a CEN.PK 113-7D $\Delta spt3$ strain and the isogenic wild type strain. We compared these data with the transcriptome data of wild type CEN.PK 113-7D under anaerobic and aerobic conditions with different nutrient limitations described by Tai *et al.* (2005). In addition, a comparison was made with the genes that are essential under anaerobic conditions (Snoek and Steensma, 2006). These comparisons showed that Spt3 is not a specific anaerobic transcription factor, but that the effects under anaerobic conditions are indeed specific to those conditions.

Deletion of *SPT3*

Deletion of *SPT3* in the strain CEN.PK 113-7D (*Mata*) (P. Kötter, J.-W. Goethe Universität, Frankfurt, Germany) was done by amplifying the relevant part of the deletion strain created by Giaever *et al.* (2002) with additional flanking regions of 618 bp upstream and 280 bp downstream of the original ORF using the primers SPT3forward (CGCGGATCCTATACGCCGGCGGCATTTC, containing a *Bam*HI site) and SPT3reverse (CCGGAATTCAATCACTGAGTTACCCCGTTAC, containing an *Eco*R1 site). The PCR product was used to transform CEN.PK 113-7D using the LiAc method (Schiestl and Gietz, 1989), giving the deletion mutant GG3098 (*MATa spt3::KanMX*). Vent polymerase was purchased from New England Biolabs (USA). PCR was performed using an annealing temperature of 59°C. Proper deletion was confirmed by PCR, Southern blotting and phenotypic analysis (i.e. the ability to grow on YPD plates, containing 150 µg/ml G418). Media and genetic techniques were performed as described earlier (Sambrook, Fritsch, and Maniatis, 1989).

Chemostat fermentation and microarray experiments

Three separate glucose-limited steady state cultures for CEN.PK 113-7D (*Mata*) and GG3098 (*Mata spt3::KanMX*) in mineral medium, both aerobic and anaerobic, were obtained as described earlier (Tai *et al.*, 2005). In brief, 2-liter Applikon fermenters with a working volume of 1 liter were used at a dilution rate of 0.10 h⁻¹ and a temperature of 30°C. pH was kept constant at 5 by the automatic addition of a 2 M KOH solution. Stirrer speed was set at 800 rpm. Cells were harvested in liquid nitrogen. For each of the steady state cultures RNA was isolated and used for microarray analyses using Affymetrix gene chips as described previously (Tai *et al.*, 2005). Results were analyzed using the Statistical Analysis of Microarrays tool (SAM, version 2.0) with a δ -value giving a false hit rate of 1% (Tusher, Tibshirani, and Chu, 2001). Genes were considered changed in expression level when listed as such by SAM and when the fold change was more than 2-fold.

Statistical significance, other than by SAM, was determined using a homoscedastic (two-sample equal variance) student's t-test assuming a 2-tailed distribution. Changes were considered significant when the P-value was smaller than 0.001.

Results

In order to understand more about differences in transcription of genes under anaerobic conditions versus aerobic conditions, we compared microarray data from a CEN.PK 113-7D $\Delta spt3$ strain and the isogenic wild type, grown in the presence and absence of oxygen in a chemostat fermentor. The number of genes that changed transcription levels more than 2-fold when these strains and conditions were compared is depicted in figure 1. The largest number of genes with a changed expression level is observed for the *spt3* deletion strain under aerobic conditions, when compared to the wild type strain under the same conditions, 287 genes in total (figure 1A). Anaerobically, the transcriptional changes upon deletion of the *SPT3* gene are slightly less, 209 genes. The overlap between these two sets of genes is small. Only 46 genes show altered expression levels both aerobically and anaerobically.

Alternatively, transcription levels of genes under anaerobic and aerobic conditions in the same strain can be compared. Of the genes that have a fold change of at least 2 in either the wild type (711 genes) or the *spt3* deletion strain (658 genes) when anaerobic and aerobic conditions are compared, 420 genes qualitatively show the same response in these strains. 291 genes only show a significant change in the wild type strain, while 238 genes do so only in the *spt3* deletion strain (figure 1B).

Categorization of the genes that change transcript level more than 2-fold in one of the deletion mutants was performed with the use of the program funspec (<http://funspec.med.utoronto.ca/>), using a cut-off value of $P < 0.001$. The results are shown in table 1. Generally, the deletion of *SPT3* causes transcription levels of genes involved in energy and metabolism to be changed.

In order to see if there are other known transcription factors involved in these responses, promoter analysis and subsequent identification of motifs was performed using the RSAT (<http://rsat.scmbb.ulb.ac.be/rsat/>) and SCPD

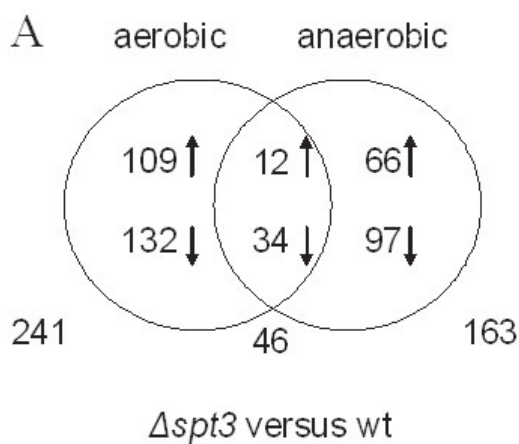
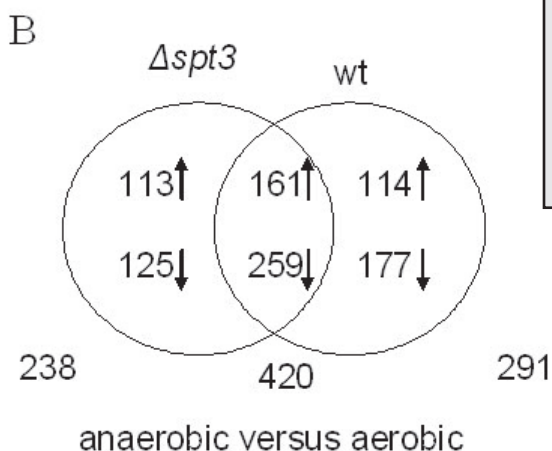


Figure 1: Number of genes that changes transcription level at least 2-fold in the deletion strain as compared to the isogenic wild type strain under anaerobic and aerobic conditions. 1A: ↑ means >2 fold higher in the mutant than in the wild type, ↓ means > 2 fold lower in the mutant than in the wild type. 1B: ↑ means > 2 fold higher in anaerobic conditions than in aerobic conditions, ↓ means > 2 fold lower in anaerobic conditions than in aerobic conditions



(<http://rulai.cshl.edu/SCPD/>) programs respectively. Genes that aerobically have a higher transcription level in the $\Delta spt3$ strain as compared to the wild type strain under aerobic conditions showed an overrepresentation of a Rap1 binding site (GCACCC, $E = 7.8e-4$). Rap1 activates transcription of ribosomal proteins (Lieb *et al.*, 2001). For genes that have a lower transcription level in this strain compared to the wild type, both under anaerobic conditions, a motif for this factor (ACCCCT, $E = 6.3 e-4$) was also found. Additionally these genes showed an overrepresentation of a motif for Mcm1-binding (GCGGCA, $E = 2.4 e-4$). Mcm1 is involved in cell-type-specific transcription and in the pheromone

response (Elble and Tye, 1991;Lydall, Ammerer, and Nasmyth, 1991). The transcription levels of *RAP1* and *MCM1* do not change when *SPT3* is deleted nor is any interaction known of Rap1 and Mcm1 with the SAGA complex.

Table 1: Results from funspec analysis on the microarray data

Regulated in strain*	Compared* conditions	MIPS category	P-value
higher transcriptional level in wild type	anaerobic vs aerobic	metabolism	4.6e-5
higher transcriptional level in $\Delta spt3$	anaerobic vs aerobic	extracellular proteins	7.7e-7
		pheromone response	4.1e-4
lower transcriptional level in $\Delta spt3$	anaerobic vs aerobic	energy	1.6e-8
		transport	3.6e-5
		tricarboxylic pathway	1.1e-4
		amino acid biosynthesis	5.1e-4
higher transcriptional level in $\Delta spt3$	aerobic vs aerobic	amino acid metabolism	1.1e-5
lower transcriptional level in $\Delta spt3$	aerobic vs aerobic	energy	4.0e-9
		extracellular proteins	8.4e-9
		metabolism	7.2e-8
		C-compound and carbohydrate metabolism	9.6e-8
		fermentation	9.8e-4
		endopeptidase inhibitor	5.6e-4
lower transcriptional level in $\Delta spt3$	anaerobic vs anaerobic	metabolism	2.1e-5
		plasma membrane	3.3e-6
		energy	4.6e-8
		cell rescue, defence and virulence	1.1e-6
		superoxide dismutase	3.6e-4

* When condition states ‘anaerobic vs aerobic’, the regulation is within one strain.

When the condition states ‘aerobic vs aerobic’ or ‘anaerobic vs anaerobic’, the regulation is of the deletion strain as compared to the isogenic wild type strain. Only significant classes (P<0.001) are shown.

Discussion

When the wild type strain and the *spt3* null mutant are compared, more changes in transcription levels were observed under aerobic conditions than under anaerobic conditions. Changes in aerobic and anaerobic transcriptional levels indicate that under these conditions 287 and 209 genes respectively (about 4% and 3% of the entire genome) are influenced by *SPT3*, both with lower and higher transcription levels. These numbers are lower than what is known about *SPT3* as a member of SAGA. Under aerobic conditions regulation of about 10 % of the yeast genes are influenced by yeast SAGA (Lee *et al.*, 2000) which activates transcription by facilitating the binding of TATA-binding protein (TBP) to the upstream activating sequence (UAS) (Larschan and Winston, 2001). This then leads to the recruitment of the RNA Polymerase II complex. Several of the components of SAGA are also present in the transcriptional activating complex TFIID (Lee *et al.*, 2000). There appears to be a differentiation of function between these complexes in which the TFIID complex regulates the housekeeping genes and in which SAGA regulates the stress-related response (Huisinga and Pugh, 2004). If this is true, categorization of the genes that have a lower transcription level upon deletion of *SPT3* should reveal some genes involved in stress response. This was indeed the case when the strains from anaerobic conditions were compared. When cells were grown with oxygen present these changes were not observed, probably due to the fact that the cells were not stressed under these conditions.

To test whether the response of the genes under anaerobic conditions can be coupled to earlier anaerobic responses of these genes, we compared our data to the 155 genes that consistently have a higher or lower transcriptional level under anaerobic conditions as compared to aerobic conditions, regardless of the nutrient limitation (Tai *et al.*, 2005). These genes could be termed 'true anaerobic genes'. When the aerobic/anaerobic fold changes of the wild type strain and the *spt3* deletion mutant are compared, 32 (21%) out of the 155 'true anaerobic genes' are significantly affected ($P < 0.001$) in their transcription levels by deletion of *SPT3*. This is more than three times as much as the percentage of 'aerobic' genes that we found to be influenced by *Spt3*, i.e 6% (418 out of 6449). The number of 'aerobic' genes was calculated by subtracting the number of 'true anaerobic genes' from the total number of ORF's (<http://www.yeastgenome.org/cache/genomeSnapshot.html>).

We also checked the genes that were experimentally found to be essential for anaerobic growth only (Snoek and Steensma, 2006) for a negative trend in fold change when the deletion strains are compared to the wild type strain. No such trend could be found. Almost all of these 23 genes show only very small fold changes and most of these are positive. The results of these two comparisons indicate that *SPT3* is not a specific anaerobic transcription factor, because it does not influence anaerobic genes only, nor does it only influence genes under anaerobic conditions. However, the transcriptomic effects under anaerobic conditions of deletion of the *spt3* gene are specific to those conditions in that a greater number of ‘true anaerobic genes’ was influenced than would be expected from chance.

Although no significant difference is observed in the number of genes with a significantly different transcription level upon deletion of *SPT3* when compared to the isogenic wild type strain under either aerobic or anaerobic conditions, a difference in identity of these genes is apparent. Of the 287 aerobically and 209 anaerobically changed genes, only 46 change in the same direction in both conditions. Of these 46 genes, 12 had a higher transcriptional level and 34 had a lower one. Thus, two distinct responses to the deletion of *SPT3* occur, one aerobically and one anaerobically. This indicates that *SPT3* does have an anaerobically specific effect.

Of the 241 (287-46) genes that are only aerobically changed between the *spt3* deletion strain and the isogenic wild type strain the genes that have a higher transcriptional level show an overrepresentation of genes involved in amino acid metabolism. The genes with lower transcriptional levels fell into several categories, among which energy, metabolism and fermentation. Of the 163 (209-46) genes that changed transcription level only under anaerobic conditions the genes that had a lower transcriptional level could be classified into groups. In addition to energy and metabolism, plasma membrane and cell rescue, defense and virulence were found. These data show that the deletion of *SPT3* generally affects genes that are involved in energy and metabolism, but that the identities of the specific genes that are affected under aerobic conditions differ from those that are regulated under anaerobic conditions. These results may indicate that transcriptional activation or inhibition through Spt3 can be influenced by the environment. We propose a model (figure 2) in

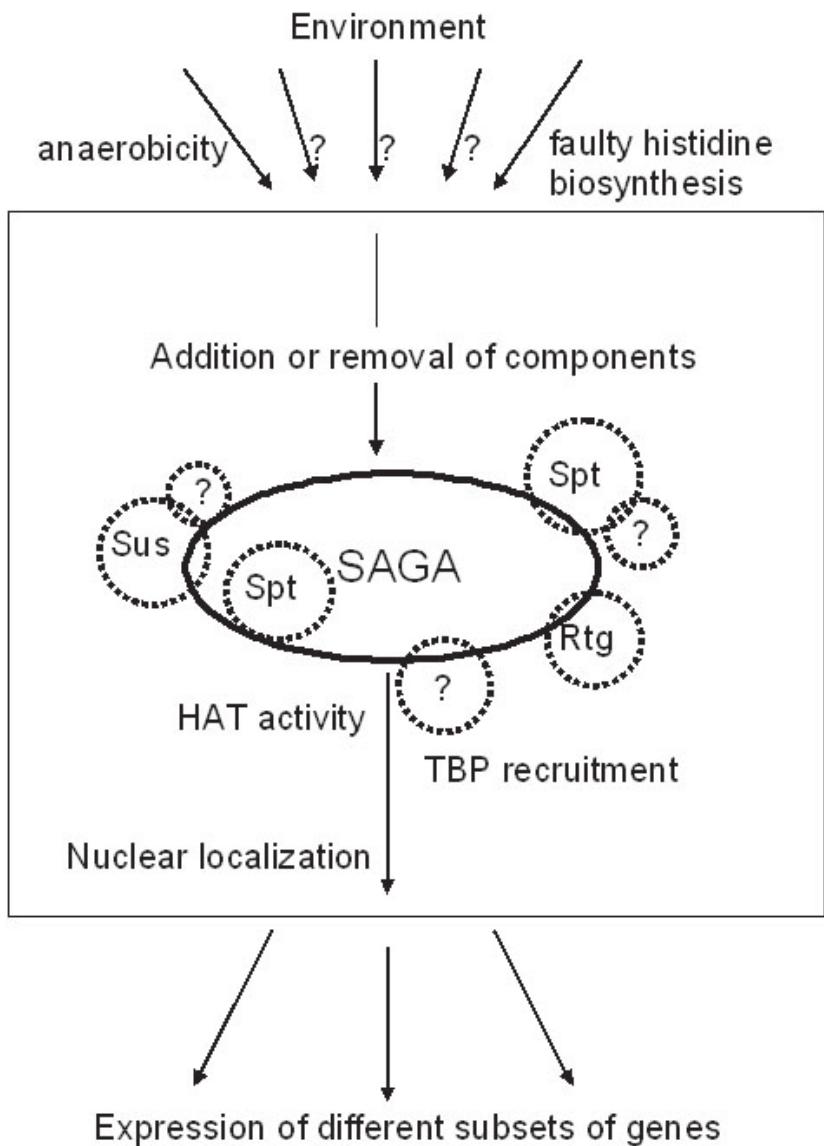


Figure 2: Model in which SAGA integrates the different environmental conditions to reach an output of expressed genes that reaches optimal adaptation to those conditions. Dashed circles represent components that are present in some of the SAGA forms, but not in others (such as Spt8), or that are present in different forms themselves (such as Spt7).

which different environmental conditions cause an adjustment of the basal transcriptional machinery, comprising among others SAGA. Such a response was observed during a transition from fermentative to respiratory growth (Roberts and Hudson, 2006). This will lead to different forms of SAGA, each missing or having certain components. An example of this is the 3D structure of SAGA which confirms that Spt8 associates dynamically with SAGA as a regulatory subunit (Timmers and Tora, 2005). Another example is SLIK, which is a transcriptionally active complex that resembles SAGA. It has the additional component Rtg2, lacks the traditional SAGA component Spt8 and has an altered form of Spt7 (Pray-Grant *et al.*, 2002). These different forms of SAGA might target different subsets of genes. Not all SAGA-dependent promoters need all components of SAGA for it to bind the promoter. Some need the HAT activity of the Gcn5 component; others need the Spt8 component (Bhaumik and Green, 2002). In this way the regulation of transcription could be the result of altered accessibility of the chromatin or of the recruitment of TBP. In addition, forms of SAGA might exist with or without the Sus1 protein (Rodriguez-Navarro *et al.*, 2004). This protein is not only a member of SAGA, but also of the nuclear pore-associated mRNA export machinery. Recent studies have shown that gene expression levels can be determined by nuclear position (Taddei *et al.*, 2006). Forms of SAGA that contain Sus1 could associate the transcription initiation complex with the nuclear pore, thus facilitating mRNA export or processing and thereby giving rise to higher expression levels (Cabal *et al.*, 2006).

In short, different conditions lead to different forms of SAGA. The specific composition of these SAGA-forms determines which subset of genes will be activated under those conditions. In this model SAGA plays a key role in integrating the environmental conditions the cell faces to give rise to a transcriptional response that optimizes the adaptation to that specific set of conditions. Analysis of the composition of SAGA under different conditions will give further understanding of this model.

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Chapter 6

SNF7 participates in the transcriptional response to oxygen availability of *Saccharomyces cerevisiae* genes encoding cell wall and plasma membrane proteins.

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○ Abstract

In a screen for genes encoding anaerobic transcription factors we previously identified the *Saccharomyces cerevisiae* *SNF7* gene. To investigate its role in anaerobic metabolism we compared microarray data of the wild type and *snf7* deletion strain, which were grown to steady states in anaerobic chemostat cultures. We found that a subset of 21 genes that are regulated transcriptionally upon anaerobiosis, and a subset of 52 genes that encode cell wall and cell membrane related proteins, such as *TIR1*, *DAN1* and *ANB1* are affected by the deletion of *SNF7*. These subsets overlap partly. It thus appears that the role of *SNF7* is not limited to anaerobic conditions and that the *SNF7* gene is needed when a change in the transcription of genes that encode cell wall and plasma membrane proteins is required for proper adaptation to environmental conditions, of which anaerobicity is one.

Introduction

The yeast *Saccharomyces cerevisiae* is one of the few eukaryotic organisms that can grow equally well under aerobic and anaerobic conditions. Transcription levels of circa 500 genes differ significantly when aerobic and anaerobic cultures are compared. Several factors for transcriptional regulation of anaerobic metabolism have been proposed. One of those is the upregulation of aerobic genes by a homodimer of Hap1p, bound to heme (Zhang and Guarente, 1995), and the derepression by Hap1p when heme is absent (Hach, Hon, and Zhang, 1999). *ROX1* is one of the targets of Hap1p and Rox1p represses hypoxic genes in the presence of oxygen (Deckert *et al.*, 1995). In another regulatory system *UPC2* and *ECM22* are implicated in a dual role in the induction of anaerobic sterol import (Crowley *et al.*, 1998; Shianna *et al.*, 2001; Ter Linde, 2003). Other genes have also been implicated in anaerobic regulation either because of their effect on translational levels or because of their heme-dependency, such as *SUT1* (Ness *et al.*, 2001), *ORD1* (Lambert JR, Bilanchone VW, and Cumsy MG, 1994), and *HAP2/3/4/5* (Zitomer and Lowry, 1992). All of these genes together regulate the response to oxygen availability in a complex way. However, the transcriptional responses to anaerobiosis of many genes are still unexplained. In a screen for anaerobic transcription factors performed previously in our laboratory (unpublished data) we found that the presence of the *SNF7* gene was required for the expression of a fusion of the *LacZ* gene with the promoter regions of the *DAN1*, *TIR1* or *ANB1* genes. These genes are upregulated under anaerobic conditions (Tai *et al.*, 2005). β -galactosidase activity was completely abolished in the *snf7* mutant with either the *TIR1* or the *ANB1* promoter driving the *LacZ* gene. Only the strain which contained the *LacZ* driven by the *DAN1* promoter yielded some expression in the *snf7* deletion mutant, but much less than the 4754 strains of the deletion collection that did not show any significant decrease in expression.

SNF7 was first identified in *S. cerevisiae* because of its regulating effect on *SUC2* expression. A *snf7* mutant does not derepress *SUC2* in response to glucose limitation, nor is it able to grow on raffinose in the presence of antimycin A (Vallier and Carlson, 1991). The mutants also grow slowly on sucrose under anaerobic conditions and show a mild growth defect on glucose at 37°C. The *snf7* mutant behaves differently from the other *snf* mutations with respect to a

double deletion with *ssn6*, which encodes a global repressor protein affecting a variety of differently regulated genes (Keleher *et al.*, 1992). The *snf7 ssn6* double mutant is severely temperature sensitive. A *snf7* null mutant has only a partial defect in invertase expression which indicates that the Snf7 protein contributes to derepression of *SUC2*, but is not absolutely required. Overall, it appears likely that the major function of *SNF7* in the cell is unrelated to invertase expression or glucose repression (Tu, Vallier, and Carlson, 1993).

SNF7 appears to be the ancestor of two other genes that encode coiled-coil proteins with similar functions, namely *MOS10* and *VPS20* (Kranz, Kinner, and Kölling, 2004). Mutations in these genes and other *VPS* genes give an E class *vps* phenotype, which is characterized by the presence of a prevacuolar organelle adjacent to the vacuole (Raymond *et al.*, 1992) and defects in the Multi Vesicular Body (MVB) sorting pathway. The MVB sorting pathway plays a critical role in the decision between recycling and degradation of membrane proteins. Monoubiquitination of both biosynthetic and endocytic cargo serves as a signal for sorting of these proteins into the MVB pathway. Protein sorting into multivesicular endosomes may also depend on lipids such as phosphatidylinositol 3-phosphate (PI3P).

Four of the class E Vps proteins (Vps2, Vps20, Vps24 and Snf7) are recruited in their monomeric form from the cytoplasm to an endosomal compartment where they oligomerize into the membrane-associated complex called ESCRT-III (Bowers *et al.*, 2004). This complex is a very large protein complex and contains numerous copies of each subunit, in a defined stoichiometry. It consists of two subcomplexes, one composed of Vps2 and Vps24 and the other of Vps20 and Snf7 (Babst *et al.*, 2004). Both Vps2 and Vps24 must be present for proper association with Vps20-Snf7 during ESCRT-III assembly. Formation of Vps20-Snf7 specifically affects the proteolytic clipping site of the vacuolar enzyme carboxypeptidase S (CPS), a cargo of the MVB pathway, by reducing the accessibility of the membrane-bound precursor (pro-CPS) clipping site to luminal proteases, but it does not affect all endosomal cargoes. The Vps20-Snf7 subcomplex is required for the membrane association of ESCRT-III. Snf7 has the ability to associate with endosomes, even in the absence of Vps20. Therefore, one possibility is that the highly charged Snf7 protein binds to membranes through interactions with charged lipid head groups. Possibly by transient interactions between Vps20-Snf7 and specific MVB cargo, there is a direct role of the Vps20-

Snf7 complex in the sorting of MVB cargo (Babst *et al.*, 2004). Dissociation of ESCRT-III from the membrane is Vps4-dependent. Vps4 binds to ESCRT-III via coiled-coil interactions with the Vps2-Vps24 subcomplex or via Snf7. This leads to oligomerization of the Vps4-dimers and subsequently to the hydrolysis of the Vps4-bound ATP. This results in a conformational change which leads to the disassembly of the class E *vps*-complex (Babst *et al.*, 1998). Raiborg *et al.* (2003) propose a model in which ESCRT-I and II pass ubiquitinated proteins along to ESCRT-III, which then functions to promote inwards vesicle scission (Raiborg, Rusten, and Stenmark, 2003).

Many of the mammalian class E *vps*-proteins have additional roles in the cell nucleus, which provides a link between protein degradation and transcription (Bowers *et al.*, 2004). In yeast several examples can be found where the endosomal sorting machinery, and Snf7 in particular have an effect on transcription. For instance, deletion of *SNF7* resulted in a transcriptional pattern similar to that found under nitrogen starvation. The *snf7* deletion strain was also sensitive to ambient stress conditions such as salinity, temperature and the presence of tunicamycin or CaCl_2 . This implicates a role for Snf7 in the response and adaptation to ambient conditions (Unno *et al.*, 2005).

Finally, Snf7 is also implicated in the RIM101 pathway, which is involved in adaptation of cells to external alkaline pH. Snf7 can interact with proteins via their Bro1-domain. A model has been proposed in which Snf7 can bind both the Bro1 protein and the Rim20 protein. The affinity of Snf7 for both proteins might be different at acidic and alkaline pH, due to a difference in the structural nature of the endosomes or of the ESCRT complexes at different pH. Only binding of the Rim20 protein will result in the recruitment of Rim13 and Rim101, promoting the cleavage and activation of the transcription factor Rim101 (Boysen and Mitchell, 2006; Wolfe and Shields, 1997).

In this study we further examined the role of *SNF7* in transcription under anaerobic conditions by transcriptome analysis of a $\Delta snf7$ strain and the isogenic wild type strain, both cultivated in anaerobic chemostats. The two data sets were compared to each other. Additionally, the list of genes that were differentially expressed in the *snf7* deletion strain as compared to the wild type was compared to the 155 genes that Tai *et al.* (2005) showed to have a consistent transcriptional response to oxygen availability.



Materials and Methods

Strains, plasmids and media

The parental *Saccharomyces cerevisiae* strains used in this study are CEN. PK 113-7D (*MATa*) (P. Kötter, J.-W. Goethe Universität, Frankfurt, Germany), or BY4743 (*MATa/Δ his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0 /ura3Δ0*) (Euroscarf, Frankfurt, Germany). The CEN.PK 113-7D $\Delta snf7$ strain was created by inserting the deletion cassette of the BY4743 $\Delta snf7$ strain, including the 20 bp tags into the CEN.PK 113-7D wild type, giving rise to strain GG3201 (*MATa snf7::KanMX*). The plasmids used are listed in table 1.

Yeast cells were grown in either YPD (Difco peptone 2%, Difco yeast extract 1%, glucose 2%), when necessary provided with 150 $\mu\text{g/ml}$ G418, or in mineral medium (Zonneveld, 1986). When required, L-lysine (30 $\mu\text{g/ml}$), L-leucine (30 $\mu\text{g/ml}$), L-histidine (20 $\mu\text{g/ml}$) or uracil (30 $\mu\text{g/ml}$) were added. For anaerobic growth, 420 $\mu\text{g/ml}$ Tween80 and 10 $\mu\text{g/ml}$ ergosterol were added to the media (Verduyn *et al.*, 1992). *E. coli* was grown in LB medium (Sambrook, Fritsch, and Maniatis, 1989). If plasmids were present ampicillin was added to 60 $\mu\text{g/ml}$. Media were solidified by the addition of 1.5% agar (Sphero).

Table1: Plasmids used in this study

Plasmid	Properties	Reference
pRUL302	<i>ARSH4</i> , <i>CEN6</i> , <i>URA3</i> , <i>Amp^r</i> , <i>ORI C</i> , <i>LacZ</i>	(Ter Linde, 2003)
pRUL414	pRUL302 with ClonNat marker	This study
PDAN	pRUL414 with promoter region of <i>DAN1</i>	This study
PTIR	pRUL414 with promoter region of <i>TIR1</i>	This study
PANB	pRUL414 with promoter region of <i>ANB1</i>	This study
pRS315	<i>ARSH4</i> , <i>CEN6</i> , <i>LEU2</i> , <i>Amp^r</i> , <i>ORI C</i> , <i>LacZ</i> in MCS	(Sikorski and Hieter, 1989)
pRS315-SNF7	pRS315 with <i>SNF7</i>	This study
pKINAT	<i>ARSH4</i> , <i>CEN6</i> , ClonNat marker, <i>Amp^r</i> , <i>ORI C</i> , <i>KICEN2</i> , <i>KARS</i>	(Steensma and Ter Linde, 2001)

Genetic techniques

To obtain pRUL414, the ClonNat marker from pKINat (Steensma and Ter Linde, 2001) was excised using BglII and StuI, and ligated into the BglII and SmaI sites of pRUL302. Promoter plasmids were constructed by amplifying the DNA fragment of interest using PCR (primers are listed in table 2). The promoter regions of the genes *DAN1* (-1232 to +23 from the ATG start codon), *TIR1* (-1429 to +5 from the ATG start codon) and *ANB1* (-1505 to +5 from the ATG start codon) were cloned in between the HindIII and BamHI sites of pRUL414, in front of the *LacZ* gene, giving plasmids PDAN, PTIR and PANB respectively. The *SNF7* (-504 bp to +1149 from start codon) gene with its own promoter and terminator was cloned in between the BamHI and SalI sites of pRS315. All enzymes were purchased from New England Biolabs (USA), including the T4 DNA ligase. Plasmids were amplified in *E. coli* strain XL1-blue. Transformation of individual yeast strains with the plasmids was done using the lithium acetate method (Schiestl and Gietz, 1989).

Table 2: Primers used in construction of the plasmids

Name primer	Restriction site	Primer
DANforward	<i>HindIII</i>	CCCAAGCTTGCAAACTTTCGACCTTCTGTATC
DANframe	<i>BamHI</i>	CGCGGATCCGCTAATATACTAATTCTAGACATTACTTGG
TIRforward	<i>HindIII</i>	CCCAAGCTTCTTCATCACACAGTGTCTAGCG
TIRframe	<i>BamHI</i>	CGCGGATCCGCCATTTTAAATTATTGTAGTACTTG
ANBforward	<i>HindIII</i>	CCCAAGCTTAATGTTACATGCGTACACGCCG
ANBframe	<i>BamHI</i>	CGCGGATCCGACATGTTTGTAGTGTGTAATGA
SNF7forward	<i>BamHI</i>	CGCGGATCCCCATTCTAGTGATTTCGCCTC
SNF7reverse	<i>SalI</i>	ACGCGTCGACATGCAAACGTAGACGACATCG

Chemostat fermentation and microarray experiments

Three separate anaerobic glucose-limited steady state cultures for each strain in synthetic medium, were grown as described previously (Tai *et al.*, 2005). The CEN.PK 113-7D strain background was used and the cells were grown in mineral medium. In brief, 2-liter Applikon fermenters with a working volume of 1 liter were used at a dilution rate of 0.10 h⁻¹ and a temperature of 30°C. pH was kept constant at 5.0 by the automatic addition of a 2 M KOH

solution. Stirrer speed was set at 800 rpm. Oxygen and carbon dioxide present in the off-gas were measured offline with a NGA 2000 Rosemount gas analyzer. Culture supernatants and media were analyzed by high performance liquid chromatography fitted with an AMINEX HPX-87H ion exchange column using 5 mM H₂SO₄ as the mobile phase. Culture dry weights were determined via filtration as described before (Postma *et al.*, 1989). Cells were harvested in liquid nitrogen. For each of the steady state cultures RNA was isolated and used for microarray analyses using Affymetrix gene chips as described previously (Tai *et al.*, 2005). Results were analyzed using the Statistical Analysis of Microarrays tool (SAM, version 2.0) with a δ -value giving a false hit rate of 1% (Tusher, Tibshirani, and Chu, 2001). Genes were considered changed in expression level when listed as such by SAM and when the fold change was more than 2-fold.

Phenotypic analysis

Calcofluor white (CFW), sodiumdodecylsulfate (SDS) and sorbitol sensitivity assays were done by growing the cells overnight in mineral medium with the supplements needed by the strains. After 18 h the cells were diluted to an OD₆₆₀ of circa 0.2. Four ten-fold dilutions were made and 2 μ l of each dilution was spotted onto an YPD plate with tween80 and ergosterol and the supplement to be tested. CFW was added to 0, 50, 100, 150, 200 and 500 μ g/ml. SDS was added to 0, 0.01, 0.02 and 0.04 %. Sorbitol was added to 0 and 1.5 M. One of the plates was incubated aerobically and the duplicate plate anaerobically by the use of Anaerocult IS sacks (Merck, Germany) for 72 h at 30°C.

For the heat shock assay the cells were grown overnight at 30°C in shake flasks in mineral medium with the appropriate supplements. The cultures were diluted 50 times and again incubated in shake flasks overnight at 30°C. These 48 h of growth were either under aerobic or anaerobic conditions. Anaerobic cultures were incubated in a Bactron Anaerobic Environmental Chamber (Sheldon Manufacturing, Inc., U.S.A.). The next morning the cells were diluted to an OD₆₆₀ of approximately 0.0002. These dilutions were exposed to 30°C, 37°C, 43°C and 46°C for 90 minutes in a T gradient thermoblock (Biometra, Germany). After this time 40 μ l was plated onto YPD plates and colonies were counted after two days aerobically at 30°C. Survival was calculated as a percentage with respect to the cells that were incubated at 30°C.

Identification of overrepresented motifs in the upstream regulatory sequences was performed using the RSAT (<http://rsat.scmbb.ulb.ac.be/rsat/>) and SCPD (<http://rulai.cshl.edu/SCPD/>) programs respectively.

Results

The initial screening in which the *snf7* deletion mutant was picked up was done with the BY4743 background, as described in chapter 4. Briefly, it was found that deletion of *snf7* resulted in the loss of expression of the *LacZ* gene under control of the *TIR1* and *ANB1* promoters. Also a reduction in expression was observed for the *LacZ* gene under control of the *DAN1* promoter. The deletion of *snf7* in the CEN.PK 113-7D background gave the same results. These results suggest that *SNF7* may play a role in the upregulation of genes under anaerobic conditions. In order to test this hypothesis, microarray experiments were performed with the CEN.PK 113-7D $\Delta snf7$ strain compared to the isogenic wild type strain, both under anaerobic conditions.

The physiology of the CEN.PK 113-7D wild type and the $\Delta snf7$ mutant in the chemostat cultures were comparable. Biomass yield of the wild type was $0.09 \text{ g.g}^{-1} \pm 0.0$, while that of the deletion strain was $0.10 \text{ g.g}^{-1} \pm 0.2$. The rates of CO_2 and ethanol production were $10.3 \text{ mmol.h}^{-1}.\text{g}^{-1} \pm 0.4$ and $9.6 \text{ mmol.h}^{-1}.\text{g}^{-1} \pm 0.1$ respectively for the wild type and $9.3 \text{ mmol.h}^{-1}.\text{g}^{-1} \pm 0.2$ and $7.6 \text{ mmol.h}^{-1}.\text{g}^{-1} \pm 0.1$ respectively for the deletion strain.

456 genes were not expressed in any of the experiments, i.e. the values were below 12 in all individual chemostat cultivations. Of the genes that were expressed, 5713 were expressed at the same level in the wild type and the *snf7* deletion strain. A total of 214 genes were differentially expressed in the two strains. The control genes *ACT1* and *PDA1* had transcript levels that were 1.15 and 1.05 fold higher respectively in the *snf7* deletion strain as compared to the wild type. These values are sufficiently close to one to assume no change in transcription levels of these kinds of genes. The values of the transcript levels of the *TIR1*, *DAN1*, and *ANB1* genes in the microarray chips are listed in table 3. These numbers confirm the results obtained by the β -galactosidase assays (table 4).

Analysis of the microarray data from the $\Delta snf7$ chemostat cultures compared to the isogenic wild type strain with the statistical program SAM yielded 99 genes that had significantly higher transcript levels in a *snf7* deletion

Table 3: Transcript expression values as measured by the Affymetrix chips for the *TIR1*, *DAN1* and *ANB1* genes in the $\Delta snf7$ strain under anaerobic conditions and the isogenic wild type strain under aerobic and anaerobic conditions. The detection limit of the β -galactosidase assay is around 1500. Values that will give a yellow color are indicated by grey boxes.

	Wild type Aerobic		Wild type Anaerobic		$\Delta snf7$ Anaerobic	
	average	standard deviation	average	standard deviation	average	standard deviation
<i>TIR1</i>	100	21	4249	292	172	9
<i>DAN1</i>	21	12	3125	140	1886	462
<i>ANB1</i>	22	3	2628	159	1383	529

Table 4: β -galactosidase activity of the BY4743 wild type and $\Delta snf7$ strains containing the PTIR, PDAN or PANB plasmid. Samples that gave a yellow color are indicated by grey boxes.

strain	plasmid	anaerobic activity
BY4743	PTIR	0.38
BY4743	PDAN	0.37
BY4743	PANB	0.16
BY4743 $\Delta snf7$	PTIR	0.00
BY4743 $\Delta snf7$	PDAN	0.18
BY4743 $\Delta snf7$	PANB	0.06

strain, while 115 genes had significantly lower transcript levels. These genes are listed in tables 5a and b. In total 3.4% of all genes are influenced in their transcriptional regulation by this deletion. Tai *et al.* (2005) found that 65 genes are consistently upregulated under anaerobic conditions, regardless of the nutrient limitation, while 90 were consistently downregulated. In this list genes influenced in transcription level by *SNF7* are enriched, as 21 (13.5%) of these 155 genes changed more than 2-fold upon deletion of *snf7*. Although not all of the 155 genes that were found by Tai *et al.* (2005) were influenced by *Snf7*, it is clear from the data in table 6 that the absence of the *SNF7* gene has a significant impact on this group of genes. Apart from an anaerobically activating effect for which *Snf7* was originally identified in our screen, the protein appears to have an anaerobically inhibiting effect.

**Table 5a: Fold changes of genes upregulated anaerobically
upon deletion of *SNF7***

Gene name	systematic name	wt an vs ae	snf7 an vs wt an	Gene name	systematic name	wt an vs ae	snf7 an vs wt an
<i>PAU7</i>	YAR020C	-2.2	5.9	<i>PXL1</i>	YKR090W	1.4	2.2
	YBL095W	-4	2.4		YLL025W	1.4	3
	YBL109W	1	2.6	<i>AQY2</i>	YLL052C	-10.6	4.5
	YBR056W	-3.7	8.9		YLL053C	-11	5.6
	YBR161W	1.9	2	<i>DAN2</i>	YLR037C	1.5	5.9
<i>SMP1</i>	YBR182C	1	5.5	<i>BOS1</i>	YLR078C	-1.2	2.6
<i>DAN3</i>	YBR301W	2.1	4	<i>ICT1</i>	YLR099C	1	2.1
	YCL044C	1.1	2.3	<i>AHP1</i>	YLR109W	-2	2.6
<i>SYP1</i>	YCR030C	-1.3	2.1		YLR202C	-1.1	2.6
<i>ABP1</i>	YCR088W	1.3	3	<i>VRP1</i>	YLR337C	-1.6	4.8
<i>UBC9</i>	YDL064W	1.8	2.4		YLR414C	-1.5	2.2
<i>VCX1</i>	YDL128W	-2.4	2.7		YML072C	-1.2	2.1
<i>ENT1</i>	YDL161W	-1.5	2		YMR087W	-2	2.4
	YDR391C	-1.4	2	<i>DDR48</i>	YMR173W	-2.1	4.7
<i>PAU2</i>	YEL049W	-1.7	2.8	<i>DDR48</i>	YMR173W	-2.1	3.8
	YEL059W	1.3	4.4		YMR181C	-1.4	2.1
<i>PRB1</i>	YEL060C	-1.2	2.3	<i>RSN1</i>	YMR266W	-1.2	3
<i>HVG1</i>	YER039C	-5.8	3.4		YMR304C	-1.6	6.5
<i>UBP5</i>	YER144C	-1.6	2.8	<i>SCW10</i>	YMR305C	-1.3	4.2
<i>PAU5</i>	YFL020C	1.5	8.4		YMR323W	1.5	2.8
<i>HAC1</i>	YFL031W	1.1	2.3		YNL045W	-1.4	2.1
<i>HAC1</i>	YFL031W	2	2.2	<i>ASI2</i>	YNL159C	-1.1	2.3
	YGL046W	1.1	5.1		YNL208W	-1.5	2
<i>CUP2</i>	YGL166W	-1.5	2.4	<i>RAP1</i>	YNL216W	-1.5	2.5
<i>GSC2</i>	YGR032W	-1.7	2.9	<i>KRE1</i>	YNL322C	1.8	2.1
<i>SCM4</i>	YGR049W	-1.7	2.3		YNR018W	-1	2
<i>CLB1</i>	YGR108W	-2.8	2.5		YOL053C	-5.9	2.5
<i>THI4</i>	YGR144W	-2.4	3.2	<i>PKH2</i>	YOL100W	-1.7	2.1
<i>DUR3</i>	YHL016C	-3.4	2.1		YOL155C	-10.7	3.6
	YHR097C	-1.5	2.3		YOL155C	-6.6	2.7
	YHR209W	-3	2	<i>TIR2</i>	YOR010C	8.6	4.3
	YIL108W	1.3	2.1		YOR084W	-2.1	2.4
<i>PRM5</i>	YIL117C	1.1	2.3	<i>SMP3</i>	YOR149C	-1.2	2.2

<i>RPI1</i>	YIL119C	-2.5	3.3	<i>RAX1</i>	YOR301W	2.2	2.1
	YIL135C	1.4	2.3	<i>KRH2</i>	YOR371C	-1.4	2
<i>RRD1</i>	YIL153W	-1.1	2.1	<i>NDD1</i>	YOR372C	-1.1	2.4
<i>IMP2</i>	YIL154C	2.6	2.6		YOR389W	-1.4	8.5
	YIR014W	-2	2.1		YOR389W	-1.4	7.2
<i>GTT1</i>	YIR038C	-4.9	2.2	<i>CAM1</i>	YPL048W	1.3	3.4
	YIR041W	-1.2	2.5	<i>LGE1</i>	YPL055C	1.3	2.2
	YJL084C	-3.2	2.6		YPL141C	1.1	2.8
<i>PRM10</i>	YJL108C	-2.3	3	<i>SVS1</i>	YPL163C	-1.2	4.1
<i>NCA3</i>	YJL116C	-6.5	3.2	<i>APM1</i>	YPL259C	1.2	2.2
<i>PTK2</i>	YJR059W	-1.3	2.6		YPL272C	1.6	3.5
	YJR061W	-1.9	5.3		YPL277C	-1.9	9.1
<i>PGU1</i>	YJR153W	1.2	10.5	<i>HAL1</i>	YPR005C	-1.1	3.4
	YKL097W	-2.8	2.7	<i>ISR1</i>	YPR106W	1	2.3
	YKL200C	-2.1	3.8	<i>PIN3</i>	YPR154W	1.4	2.5
<i>PRY2</i>	YKR013W	-1.1	3.6	<i>APG13</i>	YPR185W	-1	2
<i>KTR2</i>	YKR061W	-1.5	3.5				

Table 5b: Genes downregulated anaerobically upon deletion of *SNF7*

Gene name	systematic name	wt an vs ae	snf7 an vs wt an	Gene name	systematic name	wt an vs ae	snf7 an vs wt an
	YAL018C	2.2	-2	<i>OPT1</i>	YJL212C	2.1	-2.1
<i>POP5</i>	YAL033W	1.2	-2.2		YJL213W	8.7	-4.3
	YAL045C	-1.2	-2.1	<i>CYC1</i>	YJR048W	-5.1	-2.3
<i>ECM1</i>	YAL059W	-1.3	-2.1		YJR070C	1.7	-2
	YAR069C	3	-3.2	<i>BAT2</i>	YJR148W	1.5	-3.4
<i>IMD1</i>	YAR073W	4.7	-2.3	<i>MAE1</i>	YKL029C	6.3	-2.8
<i>SKT5</i>	YBL061C	2.6	-2.2		YKL051W	-2	-2.2
<i>GAL10</i>	YBR019C	1.8	-2.5	<i>CWP1</i>	YKL096W	-1.4	-5.6
<i>YRO2</i>	YBR054W	-6.4	-3.6		YKR012C	-1.7	-2.3
<i>TAT1</i>	YBR069C	2.1	-2.1		YKR040C	1.6	-2
<i>AAC3</i>	YBR085W	29.7	-4.9	<i>PCK1</i>	YKR097W	1.1	-2.7
<i>ICS2</i>	YBR157C	-7.6	-3.5	<i>SNF7</i>	YLR025W	-1.3	-14
<i>ARO4</i>	YBR249C	1.3	-2.9		YLR089C	1.6	-2
<i>SUL1</i>	YBR294W	-1.1	-5.3		YLR126C	1.1	-2.1
	YBR300C	4.1	-2.8	<i>ZRT2</i>	YLR130C	1.2	-2.1
	YCL049C	2.2	-2.7	<i>VPS63</i>	YLR261C	-1.2	-2
<i>SSK22</i>	YCR073C	2.5	-2.4	<i>VPS63</i>	YLR261C	-1.3	-2
<i>KNH1</i>	YDL049C	-2.1	-2.1	<i>CDA1</i>	YLR307W	2.1	-2.1
<i>SAS10</i>	YDL153C	1.2	-2.2	<i>ILV5</i>	YLR355C	1.5	-3.3
	YDL177C	1.7	-2		YLR364W	-1.2	-2.2
<i>LYS20</i>	YDL182W	1.2	-2.1	<i>CTR3</i>	YLR411W	-10	-6
	YDL187C	2.1	-2.1		YLR460C	8.2	-2.4
	YDL241W	5.3	-5.3		YML018C	1.3	-2.2
<i>BAP3</i>	YDR046C	12.8	-4.3	<i>SML1</i>	YML058W	24.7	-5.3
<i>SHU2</i>	YDR078C	1.4	-2		YML087C	-1.6	-2.9
	YDR133C	-1.6	-3	<i>ATR1</i>	YML116W	1.3	-3.9
<i>PHM6</i>	YDR281C	4.5	-3.4	<i>PHO84</i>	YML123C	1.5	-4.4
<i>TRP4</i>	YDR354W	1.2	-2.3		YMR009W	-8.4	-2
	YDR384C	-5.8	-2.5	<i>SNO1</i>	YMR095C	-1.1	-5.1
<i>HPT1</i>	YDR399W	1.3	-2	<i>SNZ1</i>	YMR096W	1.1	-5.2
<i>GNP1</i>	YDR508C	5.4	-2.9	<i>CIN4</i>	YMR138W	-1.5	-2.1
<i>UTR2</i>	YEL040W	2.1	-9.2	<i>SSO2</i>	YMR183C	1	-2
<i>MNN1</i>	YER001W	1.4	-2.2		YMR279C	4.2	-2.8
<i>TIR1</i>	YER011W	42.4	-24.7	<i>MSU1</i>	YMR287C	1.7	-2.1

<i>PET117</i>	YER058W	-1.1	-2.4	<i>FET4</i>	YMR319C	13.9	-3.3
<i>ALD5</i>	YER073W	1.4	-2.4	<i>IDH1</i>	YNL037C	-1	-2
<i>IES5</i>	YER092W	-1	-3		YNL058C	1.8	-2.1
	YFR032C	2.1	-2.1	<i>AQR1</i>	YNL065W	9.7	-4
<i>MTO1</i>	YGL236C	1.7	-2.2		YNL337W	3.1	-2.1
<i>HXK2</i>	YGL253W	1.6	-2.4		YOL014W	2.2	-2.2
<i>ZRT1</i>	YGL255W	1.5	-2.7	<i>ARG1</i>	YOL058W	1.7	-2.6
<i>YPS5</i>	YGL259W	2.5	-3.1	<i>SMF1</i>	YOL122C	-1.4	-2.8
	YGR058W	1	-2.2	<i>ENB1</i>	YOL158C	1.1	-14.5
	YGR272C	1.5	-2.4	<i>TIR4</i>	YOR009W	21.2	-2.3
	YHR029C	1	-2		YOR287C	1.4	-2.1
	YHR126C	-2	-4.4	<i>MCH5</i>	YOR306C	1.7	-2.2
<i>CTF8</i>	YHR191C	-1.2	-2.2		YOR315W	2.6	-3
	YIL064W	-1.1	-2.1	<i>FIT2</i>	YOR382W	1.4	-2.4
<i>RSM25</i>	YIL093C	1.5	-2.1	<i>SMA1</i>	YPL027W	4.2	-2.3
<i>NIT1</i>	YIL164C	1.3	-2.4	<i>PDR12</i>	YPL058C	-1.7	-9.1
	YIL165C	1.5	-2.1	<i>COX11</i>	YPL132W	1.2	-2
<i>MUC1</i>	YIR019C	32.2	-12.5	<i>MF(ALPHA)1</i>	YPL187W	1.8	-3
<i>YPS6</i>	YIR039C	1.8	-4	<i>DIP5</i>	YPL265W	32.6	-12.6
<i>YHC3</i>	YJL059W	-1.6	-2.3	<i>FCY1</i>	YPR062W	1	-2.9
<i>ARG3</i>	YJL088W	1.7	-2.5	<i>ASN1</i>	YPR145W	4	-2.7
	YJL184W	-1.2	-2.1	<i>OPT2</i>	YPR194C	13.9	-13.9
<i>ELO1</i>	YJL196C	1.1	-3.5	<i>SGE1</i>	YPR198W	-1.9	-4.1
<i>NUC1</i>	YJL208C	1.4	-2.1				

Table 6: Genes that show a consistent transcriptional response to oxygen availability (Tai *et al.*, 2005) and that are influenced by Snf7.

	higher transcriptional level in $\Delta snf7$ than in wild type	lower transcriptional level in $\Delta snf7$ than in wild type
higher transcriptional level anaerobically than aerobically (in wild type)	DAN2 PAU5 YPL272C DAN3 TIR2	YBR300C YDL241W FET4 TIR4 SML1 AAC3 MUC1 TIR1
lower transcriptional level anaerobically than aerobically (in wild type)	YBL095W YOL155C AQY2 (YLL052C YLL053C)	YML087C PDR12 CYC1 YMR009W

During the analysis of the data it became clear that genes encoding cell wall and plasma membrane proteins are statistically overrepresented among the genes that exhibit a higher transcript level in the *snf7* deletion strain than in the isogenic reference strain. Of the 214 genes changed in transcription level, 46 encode for proteins that are located in one of these cellular components as mentioned at the SGD website (<http://www.yeastgenome.org/>). Of the 21 genes that showed a consistent transcriptional response to oxygen availability and that are influenced by *SNF7* 10 encode cell wall or plasma membrane proteins (table 7a). These enrichments were statistically significant according to the funspec program (<http://funspec.med.utoronto.ca/>) using a cut-off value of $P < 0.001$. Additionally the functional categories transport facilitation ($P=1.4e-07$) and metabolism ($P=8.7e-05$) were significant in the genes that showed decreased transcription levels in the *snf7* deletion strain as compared to the wild type strain. Additionally, six genes that are differentially transcribed, either higher or lower, are involved in the organization of the cell wall (table 7b).

Analysis of the promoter regions of the genes that were affected in their transcription levels by the absence of *SNF7* showed that the sequence CTTGGC was overrepresented in the upstream region of 48 of the 99 genes that showed an increased transcript level in the *snf7* mutant ($E = 8.4\text{e-}05$). This sequence has recently been identified as a binding site for Rim101 (Macisaac *et al.*, 2006). In the upstream region of 36 of the 115 genes that had lower transcript levels in the $\Delta snf7$ strain, including *TIR1*, the sequence AGGGTC, an Nrg1 binding site (Macisaac *et al.*, 2006), was overrepresented ($E = 2.4\text{e-}3$). We also looked for overrepresented upstream motifs in the small set of genes that encode proteins which are localized in the cell wall or the plasma membrane and that were affected by the *snf7* deletion, but could not find any.

According to published data, a $\Delta snf7$ strain is defective in sporulation and raffinose utilization, and mildly temperature sensitive at 37°C (Tu, Vallier, and Carlson, 1993). Furthermore it is reported to exhibit sensitivity when grown in 1 M NaCl or in 10 μM nystatin (Giaever *et al.*, 2002). It also exhibits a growth defect on a non-fermentable, respiratory carbon source (Steinmetz *et al.*, 2002) and has a decreased glycogen accumulation (Wilson, Wang, and Roach, 2002). The mutant was also reported sensitive to ambient stress conditions such as salinity stress, exposure to CaCl_2 , and it is sensitive to high temperature and tunicamycin (Unno *et al.*, 2005). To test whether the changes in transcript levels of genes that encode cell wall and plasma membrane proteins observed under anaerobic conditions led to a weakened outer structure when molecular oxygen is absent as compared to conditions in which it is present, we performed several assays in the BY4743 background. Spot assays on rich medium indicated that the $\Delta snf7$ strain grows slightly less under anaerobic conditions than under aerobic conditions (figure 1). The $\Delta snf7$ strain is not sensitive to Calcofluor White (CFW), a compound that binds to chitin, or to 1.5 M sorbitol, neither aerobically, or anaerobically. However, strong sensitivity is found for SDS (figure 2), but this sensitivity was independent of the presence of oxygen. Similarly, we found that the mutant died more rapidly than the wild type at 43°C (figure 3), but oxygen had no effect on this temperature sensitivity either.

Table 7a: Genes influenced by *SNF7* of which the proteins are located in the cell wall or the plasma membrane. Genes marked with * are also identified by Tai *et al.* (2005).

Gene name	systematic name	Primary functional category	biological process	cellular component
UP				
<i>DAN3*</i>	YBR301W		biological_process unknown	cell wall
	YEL059W	Unclassified proteins		integral to membrane
<i>DUR3</i>	YHL016C	Regulation of/interaction with cellular environment	urea transport	plasma membrane
<i>PRM5</i>	YIL117C	Unclassified proteins	conjugation with cellular fusion	integral to membrane
<i>PRM10</i>	YJL108C	Unclassified proteins	conjugation with cellular fusion	integral to membrane
<i>CWP2</i>	YKL097W		cell wall organization and biogenesis	cell wall
	YKL200C		merged into YKL201C	membrane
<i>AQY2*</i>	YLL052C	Transport facilitation	water transport	plasma membrane
<i>DAN2*</i>	YLR037C		biological_process unknown	cell wall
<i>ASI2</i>	YNL159C	Unclassified proteins	ubiquitin-dependent protein catabolism	integral to membrane
<i>KRE1</i>	YNL322C	metabolism	cell wall organization and biogenesis	cell wall
	YOL155C	Unclassified proteins	cell wall organization and biogenesis	cell wall
*	YOL155C	Unclassified proteins	cell wall organization and biogenesis	cell wall
<i>TIR2*</i>	YOR010C	Cell rescue, defence and virulence	response to stress	cell wall

<i>RAX1</i>	YOR301W		bud site selection	integral to membrane
<i>SVS1</i>	YPL163C	Cell rescue, defence and virulence	response to chemical substance	cell wall
	YPL277C	Unclassified proteins	biological_process unknown	membrane fraction
DOWN				
<i>TAT1</i>	YBR069C	Transport facilitation	amino acid transport	plasma membrane
<i>SUL1</i>	YBR294W	Regulation of/interaction with cellular environment	sulfate transport	plasma membrane
	YCL049C	Unclassified proteins	biological_process unknown	membrane fraction
<i>KNH1</i>	YDL049C	metabolism	beta-1,6 glucan biosynthesis	cell wall
<i>BAP3</i>	YDR046C	metabolism	amino acid transport	plasma membrane
	YDR384C	metabolism	transport	plasma membrane
<i>GNP1</i>	YDR508C	metabolism	amino acid transport	plasma membrane
<i>UTR2</i>	YEL040W	Control of cellular organization	cell wall organization and biogenesis	cell wall
<i>TIR1*</i>	YER011W	Cell rescue, defence and virulence	response to stress	cell wall
<i>ZRT1</i>	YGL255W	Transport facilitation	high-affinity zinc ion transport	integral to plasma membrane
<i>MUC1*</i>	YIR019C	metabolism	pseudohyphal growth	plasma membrane
<i>YPS6</i>	YIR039C	Protein fate	biological_process unknown	cell wall

<i>ELO1</i>	YJL196C	metabolism	fatty acid elongation, unsaturated fatty acid	membrane
	YKL051W	Unclassified proteins	actin cytoskeleton organization and biogenesis	plasma membrane
<i>CWP1</i>	YKL096W		cell wall organization and biogenesis	cell wall
<i>ZRT2</i>	YLR130C	Regulation of/interaction with cellular environment	low-affinity zinc ion transport	plasma membrane
<i>CTR3</i>	YLR411W	Regulation of/interaction with cellular environment	copper ion import	integral to plasma membrane
<i>ATR1</i>	YML116W	Cell rescue, defence and virulence	multidrug transport	plasma membrane
<i>PHO84</i>	YML123C	metabolism	phosphate transport	integral to plasma membrane
<i>FET4*</i>	YMR319C	Regulation of/interaction with cellular environment	intracellular copper ion transport	integral to plasma membrane
<i>AQR1</i>	YNL065W	Transport facilitation	drug transport	plasma membrane
<i>SMF1</i>	YOL122C	Protein fate	manganese ion transport	plasma membrane
<i>ENB1</i>	YOL158C	Transport facilitation	ferric-enterobactin transport	integral to membrane
<i>TIR4*</i>	YOR009W	Cell rescue, defence and virulence	biological_process unknown	cell wall
<i>MCH5</i>	YOR306C	Transport facilitation	transport	membrane
<i>FIT2</i>	YOR382W	Unclassified proteins	siderochrome transport	cell wall

<i>PDR12*</i>	YPL058C	Transport facilitation	transport	plasma membrane
<i>DIP5</i>	YPL265W	metabolism	amino acid transport	plasma membrane
<i>OPT2</i>	YPR194C	Transport facilitation	oligopeptide transport	integral to plasma membrane
<i>SGE1</i>	YPR198W	Cell rescue, defence and virulence	response to drug	integral to plasma membrane

Table 7b: Genes of which the proteins are involved in cell wall organization, but not located in the cell wall themselves that are up- or downregulated upon deletion of *SNF7*.

Gene name	systematic name	Functional description
UP		
<i>GSC2</i>	YGR032W	cell wall synthesis and maintenance, 1,3-beta-glucan synthase activity
<i>RPI1</i>	YIL119C	affects the mRNA levels of several cell wall metabolism genes
<i>KTR2</i>	YKR061W	involved in N-linked glycosylation of cell wall mannoproteins
<i>ICT1</i>	YLR099C	null mutation leads to an increase in sensitivity to Calcofluor white
<i>SCW10</i>	YMR305C	cell wall maintenance
	YOL155C	cell wall organization and biogenesis
DOWN		
<i>SKT5</i>	YBL061C	post-translational regulator of chitin synthase III activity

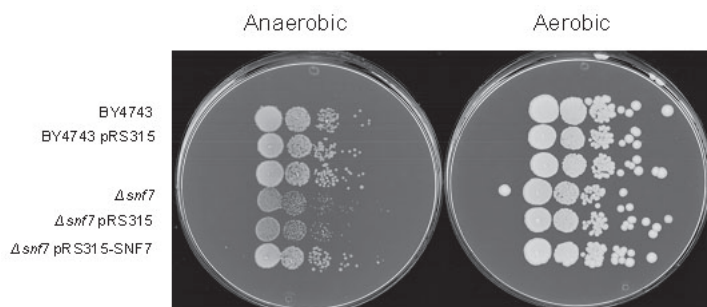


Figure 1: Growth of the $\Delta snf7$ strain and the isogenic wild type strain on YPD supplemented with Tween 80 and ergosterol, both anaerobically and aerobically. pRS315 is a single copy plasmid.

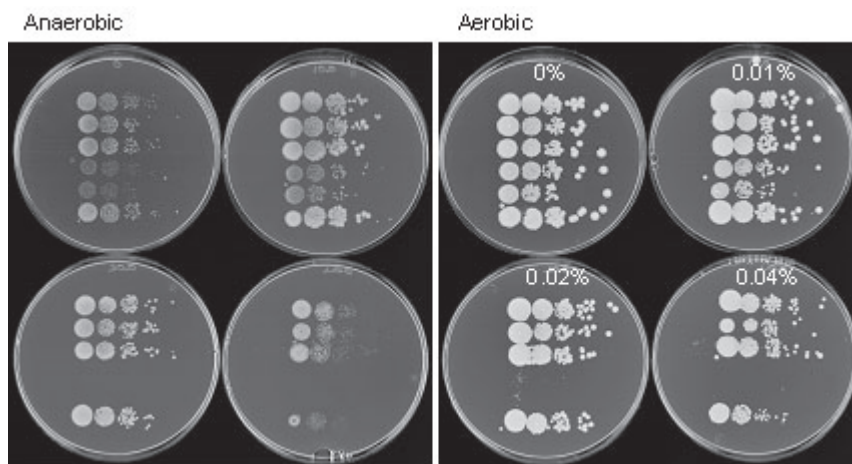


Figure 2: SDS-sensitivity of the $\Delta snf7$ strain (BY4743 background). From top to bottom the spotted strains are: wild type, wild type containing the pRS315 plasmid, wild type containing the pRS315 plasmid with the *SNF7* gene, $\Delta snf7$, $\Delta snf7$ containing the pRS315 plasmid, $\Delta snf7$ containing the pRS315 plasmid with the *SNF7* gene.

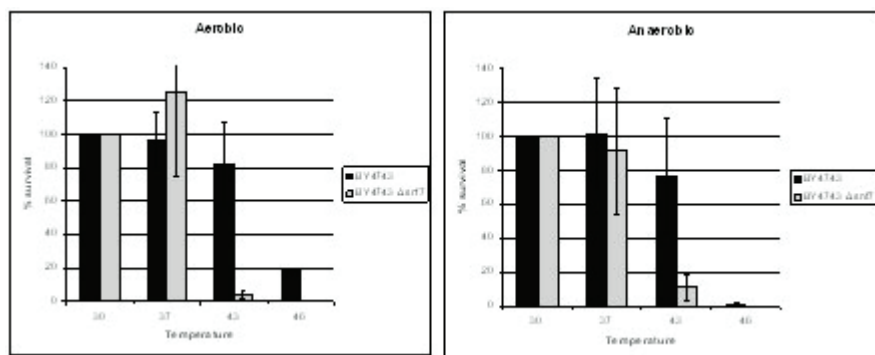


Figure 3: Survival of the *Δsnf7* strain and the isogenic wild type, preincubated aerobically or anaerobically, after 90 min at 30°C, 37°C, 43°C, and 46°C.

Discussion

In order to investigate the possible role of *SNF7* in transcriptional upregulation of genes under anaerobic conditions, chemostat fermentations were run in triplicate of the *snf7* deletion strain and the isogenic wild type strain. Since the physiological characteristics, such as biomass yield and the rate of carbon dioxide production, of these two strains in the fermentations were the same, and since the transcriptional levels of the control genes *ACT1* and *PDA1* do not differ as well, the observed effects can be ascribed to the deletion of *SNF7*.

Under anaerobic conditions the deletion of *SNF7* affects the transcription of a subset of 21 of the 155 genes that Tai *et al.* (2005) found to have a consistent transcriptomic response to oxygen availability. It also affects genes that are not known to have such a response. Thus, even though the effect of *Snf7* is not specific to anaerobically responsive genes, the absence of *SNF7* does have a significant impact on the genes that respond consistently to oxygen availability.

A significant subset of the genes that were affected by the *snf7* deletion, encode proteins that are related to the cell wall and the plasma membrane. In *S. cerevisiae*, oxygen availability has a profound effect on the expression of genes that encode cell wall and plasma membrane proteins. The changes in the cell

wall are slow to occur and take several generations for completion (Lai *et al.*, 2005). Generally, transcript levels of *CWP1* and *CWP2* decrease, while those of the seripauperin family genes, such as the *DAN*, *TIR* and *PAU* genes, increase (Klis *et al.*, 2002). These changes are quite drastic and suggest a complete switch from one set of GPI cell wall proteins to another. It is not known whether or how this change facilitates the import of supplements and if perhaps it has some additional functions. The lipid composition of the membrane under anaerobic conditions is different from that of cells grown under aerobic conditions. Anaerobically, the plasma membrane contains more saturated fatty acids, less total sterol, less ergosterol and less squalene (Nurminen, Konttinen, and Suomalainen, 1975).

46 of the 214 genes that are differentially expressed in the $\Delta snf7$ strain as compared to the wild type strain encode proteins that localize to the cell wall or the plasma membrane. Additionally, six genes were identified that code for proteins that are associated with synthesis and maintenance of these structures. Some of the genes that have a lower transcription level upon deletion of *SNF7* under anaerobic conditions, such as *TIR1* and *TIR4* are members of gene families. Other members of these families, such as *TIR2* and *DAN2* are upregulated and those could represent a compensatory response to take over the function of the downregulated genes. In line with this, we did not observe altered morphology in electron microscopy scanning images (data not shown).

Deletion of *SNF7* has a number of phenotypic consequences. Most of those, such as sensitivity to nystatin and to elevated temperature (Giaever *et al.*, 2002) can be related to a defective cell wall, and some to an altered plasma membrane, such as the sensitivity to SDS. SDS is a detergent that affects membrane stability and indirectly also cell wall construction. It can thus be used to detect cell wall defects that result in increased accessibility of SDS to the plasma membrane (de Groot *et al.*, 2001). The defect in sporulation (Giaever *et al.*, 2002) is probably related to an altered cell wall in combination with the downregulation of the *CDA1* gene (table 5b), which is required for the proper formation of the ascospore wall (Petrik, Kappeli, and Fiechter, 1983) and the downregulation of *SMA1* (table 5b), which is required for spore membrane assembly (Rabitsch *et al.*, 2001). We confirmed that a homozygous $\Delta snf7/\Delta snf7$ diploid in the CEN.PK 113-7D background does not sporulate, either aerobically or anaerobically (data not shown). Other examples of phenotypic

responses to deletion of *SNF7* are problems in the the adaptation to external alkaline pH (Boysen and Mitchell, 2006; Wolfe and Shields, 1997) and in the adaptation to high temperature (Hammond *et al.*, 2006). The latter is not known to be *SNF7*-dependent. However, a *snf7* deletion strain was reported to exhibit subtle temperature sensitivity at 37°C (Tu, Vallier, and Carlson, 1993). The strain was reported to be able to grow at 37°C as a culture, but could not form single colonies. We performed an experiment in which we measured survival after heat shock. Our experiments show that a temperature sensitive phenotype could indeed be seen at 43°C.

Even though the data above suggest cell wall integrity might be compromised in this mutant, no upregulation of Rlm1p-controlled cell wall genes and STRE-controlled genes was observed in the $\Delta snf7$ strain. Neither were the ribosomal genes and rRNA genes downregulated. These trends were previously seen in response to Calcofluor White (CFW) and zymolyase treatment (Marinissen *et al.*, 2006). This result may reflect the lack of effect the deletion of *SNF7* has on CFW sensitivity. Another known trait of mutants having a compromised cell wall structure is induced expression of *CWP1*, but not *SSR1*, which encodes a cell wall glycoprotein (Ram *et al.*, 1998). Changes in *SSR1* transcript levels were not observed, but *CWP1* was downregulated instead of upregulated in this mutant (table 5b). However, an increase in *CWP2* transcript levels was observed (table 5a). An increase in the chitin content of the cell wall has also been proposed to be part of a set of reactions induced in response to cell wall perturbation (de Deken, 1966; Ram *et al.*, 1998). The only chitin related gene differentially expressed in our dataset is *SKT5*, which is a posttranslationally active activator of the Chs3 chitin synthase. However, *SKT5* is downregulated in the $\Delta snf7$ mutant as compared to the wild type strain (table 5b). This again may reflect the insensitivity of the *snf7* deletion mutant to CFW, since this compound binds to chitin and interferes with the polymerization of this cell wall component (de Groot *et al.*, 2001). It thus appears that the *snf7* deletion mutant does not fit the classical traits of a cell wall stressed mutant.

An interdependence of growth temperature, intracellular glycerol levels, the structure of the cell wall and the MAP kinase signaling pathway has been proposed in the hyperosmotic stress response of *S. cerevisiae* (Zeeman *et al.*, 1998). The concentration of intracellular glycerol may trigger the activity of the PKC MAP kinase pathway. Increased glycerol levels may thus lead to an

increased resistance to high osmolarity (An *et al.*, 2006). It has also been shown that CFW treatment induces accumulation of glycerol (Bolotin-Fukuhara, Casaregola, and Aigle, 2006). Since the $\Delta snf7$ strain is reported to have decreased levels of glycogen accumulation (Wilson, Wang, and Roach, 2002), it may also be sensitive to high osmolarity. This was not the case, as we did not find a difference in growth on rich medium with 1.5M sorbitol between a $\Delta snf7$ and a wild type strain.

In the group of genes that had a higher transcriptional level in the $snf7$ deletion strain, the Rim101 binding site was overrepresented. No change in expression was observed for the *RIM101* gene itself in the $\Delta snf7$ strain. The *RIM101* gene is required *in vivo* for the activation of alkaline induced genes, such as ion pumps (Lamb *et al.*, 2001) and for the repression of several other genes, such as *NRG1* and *RIM8* (Lamb and Mitchell, 2003). Both functions appear to be dependent on the processing of Rim101 by Rim13. A model has been proposed in which Snf7 is required for the proper cleavage of the inactive form of Rim101 by Rim13 to produce the active form. If Snf7 is required and sufficient for proper functioning of Rim101, then the genes that show higher or lower transcriptional levels in a $\Delta rim101$ strain should show the same response in our data set. This is only true for a subset of the genes. Twelve out of the 34 genes that were identified to be affected by a deletion of the *RIM101* gene are also differentially expressed in the $\Delta snf7$ strain as compared to the wild type (Lamb and Mitchell, 2003). The group of genes that showed a higher transcriptional level in a $\Delta snf7$ strain as compared to the wild type and that had a Rim101 binding site also included genes that were not identified as direct Rim101 targets, when grown in rich YPD medium (Lamb and Mitchell, 2003). A possible explanation is that under the conditions used in our experiments, i.e. steady state chemostat cultures in mineral medium, other genes are targeted by Rim101. Interestingly, one of the genes that is repressed by active Rim101 is *NRG1* which is a repressor. Transcriptional levels of this gene changed 2 fold upon deletion of *SNF7*, although this value was not deemed significant due to a high standard deviation. Still, the data could indicate that repression by Rim101 is compromised in the $snf7$ deletion strain. A binding site for Nrg1 was found to be overrepresented in the genes that had a lower transcriptional level in the $snf7$ deletion strain than in the wild type. The observed response of these genes could therefore be due to an indirect effect via Nrg1. This connection between

SNF7 and *NRG1* could also account for the initial identification of Snf7 as a derepressor of *SUC2* in response to glucose limitation.

In general it appears that the role of *SNF7* is not limited to anaerobic conditions, since the phenotypes observed are not anaerobically specific, except for the slower growth under these conditions. Also the role that Snf7 plays in the response to an alkaline environment, through Rim101, was determined under aerobic conditions. Thus I hypothesize that the *SNF7* gene is needed when a change in the transcription of genes that encode for cell wall and plasma membrane proteins is required for proper adaptation to environmental conditions, of which anaerobicity is one.

The question remains whether the general cell wall and plasma remodeling role brought about by Snf7 is independent of the ESCRT-III complex, as is its function in the RIM101 pathway, or that the miss sorting of proteins through a malfunctioning ESCRT system creates a feedback to transcriptional control. However, none of the other members of the ESCRT protein sorting system were picked up in the initial screening, and none of them showed any transcriptional response to the deletion of *SNF7*. Moreover, the transcription level of *CPS1*, encoding the model cargo of the ESCRT complexes, was not affected. Furthermore, overrepresentation was found of a Rim101 and an Nrg1 binding site in the genes with a higher and lower transcriptional level than in the wild type respectively. Therefore it is likely that the role of *SNF7* in the transcriptional response to anaerobic conditions is independent of its role in the ESCRT-III complex and the MVB sorting pathway.

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Nederlandse samenvatting

Saccharomyces cerevisiae is één van de weinige gistsoorten die even snel kan groeien onder condities waarbij geen moleculaire zuurstof aanwezig is (anaërobe condities) als waarbij dat wel het geval is (aërobe condities) (Visser *et al.*, 1990). Deze eigenschap heeft ervoor gezorgd dat het de meest gebruikte gist in de industrie is. Anaërobe incubatie van *S. cerevisiae* speelt een grote rol in de productie van alcoholische drank en van brood.

Dit proefschrift heeft als doel meer inzicht te verkrijgen in de factoren die van belang zijn voor de eigenschap anaëroob te kunnen groeien en de regulatie daarvan. Inzichten in de processen die belangrijk zijn voor anaërobe groei en in de mechanismen welke deze reguleren zijn niet alleen wetenschappelijk interessant, maar kunnen ook bijdragen aan oplossingen voor problemen die de industrie tegenkomt bij de anaërobe incubatie van organismen.

Hoofdstuk 1 is een uiteenzetting over de aanpassingen die nodig zijn voor een organisme om anaëroob te kunnen groeien. Om te beginnen moet de mogelijkheid bestaan om energie te winnen zonder dat hierbij gebruik gemaakt wordt van de ademhalingsketen. Gist doet dit door fermentatie. De energieopbrengst uit één molecuul glucose is hierbij veel lager dan wanneer gebruik gemaakt kan worden van moleculaire zuurstof als uiteindelijke elektronenacceptor, slechts 2 in plaats van 16 moleculen ATP. Verder wordt een aantal biosynthetische routes beschreven waar een anaërobe tegenhanger voor moet zijn, zoals de productie van haemeiwitten, de productie van ergosterol en onverzadigde vetzuren en de *de novo* productie van NAD⁺. De oplossing van *S. cerevisiae* voor het omzeilen van zuurstofgebruik voor de productie van ergosterol en onverzadigde vetzuren is de import ervan. Voor dit proces, en waarschijnlijk ook voor andere doeleinden, worden de celwand en plasmamembraan drastisch veranderd. Uiteindelijk moet de cel ook nog in staat zijn om al deze verschillen tussen een aëroob en een anaëroob metabolisme te reguleren. Het lijkt erop dat dit in *S. cerevisiae* voornamelijk op transcriptioneel niveau gebeurt.

In hoofdstuk 2 is een gist 'deletiebibliotheek' (Giaever *et al.*, 2002) gebruikt om in *S. cerevisiae* genen te vinden die essentieel zijn voor de groei zonder zuurstof. In deze bibliotheek is in iedere stam steeds één gen gedeleteerd. Het

bleek dat de meeste van de ongeveer 1100 genen die aëroob essentieel zijn een net zo belangrijke rol hebben wanneer er geen moleculaire zuurstof is. Slechts 33 genen zijn helemaal niet nodig en 32 zijn belangrijk voor optimale groei in rijk medium (YPD) met Tween 80, als bron van onverzadigde vetzuren, en ergosterol. Veel van de circa 1040 genen die essentieel zijn voor zowel aërobe als anaërobe groei coderen voor mitochondriële eiwitten. Dit resultaat bevestigt dat ook onder anaërobe condities mitochondriën nog functies te vervullen hebben (Plattner and Schatz, 1969).

Het aantal genen dat alleen essentieel is onder anaërobe condities is ook klein. Van de 23 genen die nodig bleken te zijn voor groei zonder zuurstof, waren twee al bekend uit de literatuur, namelijk *NPT1* en *ARV1*. *NPT1* is nodig voor de anaërobe productie van NAD⁺ (Panozzo *et al.*, 2002) en *ARV1* is betrokken bij import en distributie van sterolen (Tinkelenberg *et al.*, 2000). De andere 21 genen hebben verscheidene functies, waaronder enkele die coderen voor transcriptie (gerelateerde) factoren. Omdat deze factoren, of de complexen waar ze in voorkomen, ook aëroob aanwezig zijn, is het niet duidelijk waarom deze genen essentieel zijn voor anaërobe groei. Uitzondering is *GUP1* dat codeert voor een glycerol transporteiwit. Mogelijk veroorzaakt de combinatie van lage ATP opbrengst tijdens fermentatie en een verlaagde eiwitsynthese een soort synthetische letaliteit. Verder bleek geen correlatie te bestaan tussen de transcriptieniveaus van de aëroob- en anaëroob essentiële genen en de aan- of afwezigheid van zuurstof.

Onder anaërobe omstandigheden is ook synthetische letaliteit vastgesteld van genen. Een voorbeeld daarvan zijn de *AUS1* en *PDR11* sterol import genen (Wilcox *et al.*, 2002). Deletie van elk gen apart heeft weinig effect, maar deletie van beide genen is letaal. Zulke genen werden in ons experiment niet geïdentificeerd, maar zijn uiteraard van groot belang voor het vermogen van organismen om te kunnen groeien in de afwezigheid van moleculaire zuurstof. Daarom zijn in de vergelijking met het *Kluyveromyces lactis* genoom behalve de genen die essentieel bevonden zijn onder anaërobe condities, ook de genen betrokken waarvan uit de literatuur bekend is dat ze een rol spelen. Deze vergelijking zou informatie kunnen verschaffen over de vraag waarom *K. lactis* niet anaëroob kan groeien, terwijl dit organisme wel kan fermenteren. Deze vergelijking leverde 20 genen op die anaëroob actief zijn in *S. cerevisiae* maar geen homoloog hebben in *K. lactis*. Het feit dat al deze genen wel aanwezig zijn in de gist *Saccharomyces kluyveri*,

die ook anaëroob kan groeien (Chantrel *et al.*, 1998), ondersteunt de conclusie dat afwezigheid van deze genen wel eens bepalend zou kunnen zijn voor het onvermogen van *K. lactis* om te groeien zonder zuurstof.

Vier van deze 20 genen, namelijk *ARV1*, *DAN1*, *AUS1* en *PDR11* zijn betrokken bij sterol import. Bovendien mist *K. lactis* ook drie genen die coderen voor transcriptiefactoren die betrokken zijn bij sterol opname, namelijk *SUT1*, *SUT2* en *UPC2*. Het importeren van sterolen is essentieel onder anaërobe omstandigheden omdat voor de synthese van deze stoffen moleculaire zuurstof nodig is. Op grond van deze data stel ik als hypothese dat *K. lactis* geen sterolen kan opnemen. Dit importdefect zou dan een reden zijn waarom *K. lactis* niet anaëroob kan groeien. Omdat er nog 13 andere anaëroob essentiële genen niet aanwezig zijn in het *K. lactis* genoom, is het onwaarschijnlijk dat de afwezigheid van sterolimport de enige oorzaak is.

In hoofdstuk 3 wordt onderzoek gerapporteerd dat dieper ingaat op de functionele genomische analyse met transcriptoomdata als uitgangspunt. Daarvoor is gekeken naar de relatieve 'fitness' onder anaërobe condities van stammen met een deletie in een gen dat in een eerdere studie consistent een hoger transcriptieniveau had onder anaërobe condities in vergelijking met aërobe condities. Relatieve 'fitness' is een maat voor het vermogen van een stam om zich te handhaven in aanwezigheid van één of meer andere stammen. De 27 geselecteerde stammen zijn samen in een chemostaatcultuur gebracht en vervolgens is onderzocht welke na het bereiken van de steady state minder frequent aanwezig waren. Van de 27 op deze manier onderzochte genen bleek de deletie van slechts 5 (19%) een effect op relatieve 'fitness' te hebben. Dit resultaat impliceert dat een hoog transcriptieniveau onder bepaalde condities niet als bewijs genomen kan worden voor een unieke fysiologische relevantie van het gecodeerde eiwit onder die condities. Dit is in overeenstemming met de resultaten in hoofdstuk 2 en die van Giaever *et al.* (Giaever *et al.*, 2002) en van Birrell *et al.* (Birrell *et al.*, 2002), die door vergelijking van al bestaande transcriptoomdata met competitieve batchcultures ook een slechte correlatie tussen deze twee aantonden.

Het gebrek aan effect van veel van de deleties kan verklaard worden door co- of crossregulatie van transcriptionele effecten. Ook kan de functie van het gedeleteerde gen overgenomen worden door andere, homologe genen.

Echter, de genen van welke de deletie wel effect had, namelijk *EUG1*, *IZH2*, *YLR413W*, *YOR012W* and *PLB2* hebben ook homologen in het *S. cerevisiae* genoom. Deze homologen kunnen dus niet, of maar gedeeltelijk de functie van de gedeleteerde genen overnemen.

De belangrijkste conclusie in dit hoofdstuk is dat het transcriptieniveau van een gen onder bepaalde condities niet altijd een functionele relatie tussen dat gen en de gebruikte condities reflecteert. Een soortgelijke conclusie is in hoofdstuk 2 getrokken, waar bleek dat de genen die essentieel zijn voor anaërobe groei van *S. cerevisiae* geen verhoogde transcriptieniveaus vertonen onder die conditie. Dit zou specifiek kunnen zijn voor anaërobe groei en een evolutionaire aanpassing van *S. cerevisiae* aan deze omstandigheid kunnen zijn. Waarschijnlijker is echter dat dit een meer algemeen fenomeen is omdat in experimenten waarin andere condities gebruikt zijn, zoals blootstelling aan DNA-beschadigende agentia, eenzelfde gebrek aan correlatie is gevonden.

Groei zonder moleculaire zuurstof vereist aanpassingen van de cel om ten minste drie redenen. Om te beginnen is de energie opbrengst meestal veel lager dan onder aërobe condities. Verder vereist de biosynthese van verschillende metabolieten moleculaire zuurstof. Bovendien moet de cel onder deze omstandigheden andere verbindingen naar binnen en naar buiten transporteren. In *S. cerevisiae* gebeurt deze aanpassing voornamelijk door transcriptie-regulatie. Slechts 23 van de genen zijn specifiek essentieel voor anaërobe groei, terwijl de transcriptieniveaus van ongeveer 500 genen significant veranderen wanneer anaërobe en aërobe condities worden vergeleken. Het is voor veel van deze genen onduidelijk waarom ze essentieel zijn of waarom hun transcriptieniveaus veranderen. Het doel van de experimenten beschreven in hoofdstuk 4 is om meer te weten te komen over de transcriptionele regulatie van genen onder anaërobe condities.

Systematisch onderzoek naar factoren die anaërobe genen activeren, waarbij gebruik is gemaakt van het *E.coli* reporter gen *lacZ* onder de controle van een anaëroob specifieke promotor, leverde vier genen op, namelijk *SPT3*, *SPT4*, *SAC3* en *SNF7*, die niet eerder in verband waren gebracht met dergelijke regulatie. Microarray analyses op mRNA uit chemostaatcultures van stammen met deleties in deze genen zijn daarop uitgevoerd. *SPT3* maakt deel uit van het SAGA complex, dat de transcriptie van zo'n 10 % van het genoom beïnvloedt.

SPT4 codeert voor een eiwit dat onderdeel is van het spt4/spt5 elongatie complex. Sac3 is betrokken bij de export van mRNA uit de celkern. SNF7 is een onderdeel van het ESCRT-III complex, dat het intracellulaire transport van onder andere transmembraaneiwitten regelt.

De deletie van *SPT4* en *SAC3* leidde niet tot veranderingen die “anaeroob-specifiek” waren. Functionele categorisatie van de genen die op transcriptioneel niveau veranderden in de *spt4* deletie stam ten opzichte van de isogene wildtype stam liet zien dat er onder anaërobe condities geen voorkeur is voor de elongatie van genen die tot een bepaalde functionele groep behoren door het Spt4/Spt5 ‘elongator complex’. De reden dat de *spt4* mutant naar voren kwam zou kunnen zijn dat de spt4 mutant moeite heeft met de transcriptie van het *E.coli lacZ* gen. Er is namelijk bekend dat dit gen door het hoge G+C gehalte (56% tegen 39% voor het *S.cerevisiae* genoom) slecht wordt getranscribeerd in gist, met name in $\Delta spt4$, $\Delta hpr1$, $\Delta tho1$ of $\Delta tho2$ mutanten (Chavez and Aguilera, 1997; Piruat and Aguilera, 1998) (Rondon *et al.*, 2003).

Inde groep genen die transcriptioneel beïnvloed werd door de deletie van *SAC3*, was de categorie ‘transcriptie factor’ significant oververtegenwoordigd. Waarschijnlijk zijn de waargenomen transcriptionele veranderingen in deze stam dus het gevolg van een combinatie van de deletie van het *SAC3* gen zelf en de veranderde mRNA niveaus van deze andere transcriptiefactoren. De veranderde transcriptieniveaus kunnen een gevolg zijn van een verband tussen mRNA export en transcriptieinitiatie, zoals dat beschreven is voor de *GAL* genen (Cabal *et al.*, 2006). Deze connectie lijkt afhankelijk te zijn van het bij transcriptie betrokken SAGA complex (Rodriguez-Navarro *et al.*, 2004; Cabal *et al.*, 2006).

Met het gebruik van het *lacZ* gen als reporter zijn geen anaëroob specifieke transcriptiefactoren gevonden. Het lijkt er dus op dat Upc2, samen met Ecm22, de enige transcriptie factoren zijn die een sterk effect heeft op de *DAN1*, *TIR1* en *ANB1* genen. Het is mogelijk dat activatie door Upc2 gemodificeerd kan worden door de activiteit van andere eiwitten, zoals de vier die we in deze studie hebben gevonden. Deze modifierende factoren zijn waarschijnlijk niet anaeroob-specifiek. Algemener kan gehypothetiseerd worden dat elke set condities een verschillende set transcriptioneel werkende eiwitten activeert. De combinatie van deze eiwitten zal dan de precieze transcriptieniveaus van de betrokken genen geven.

Hoofdstuk 5 gaat over de experimenten met de *spt3* deletie stam. *SPT3* is één van de genen die in hoofdstuk 4 beschreven zijn. Spt3 maakt deel uit van het SAGA complex. Dit complex heeft voornamelijk invloed op de transcriptie van genen die geactiveerd worden onder stresscondities. De transcriptoomdata verkregen door de *spt3* deletiestam te vergelijken met de isogene wildtype stam, zowel onder aërobe als anaërobe condities, wijzen erop dat transcriptionele inhibitie of activatie door Spt3 beïnvloed wordt door omgevingsfactoren. De set genen waarvan het mRNA niveau veranderde door deletie van *SPT3* was onder aërobe condities duidelijk een andere dan die onder anaërobe condities veranderde. Een model wordt gepostuleerd waarin verschillende condities leiden tot verschillende vormen van SAGA. De specifieke samenstelling van deze vormen van SAGA bepaalt welke subset van genen onder die omstandigheden geactiveerd wordt. In dit model speelt SAGA een sleutelrol in het integreren van omgevingscondities die de cel tegenkomt, om tot een transcriptionele reactie te komen die de aanpassing aan die condities optimaliseert.

Een ander gen dat, in de experimenten beschreven in hoofdstuk 4, is geïdentificeerd is *SNF7*. In hoofdstuk 6 wordt de rol van *SNF7* bij regulatie onder anaërobe condities verder onderzocht. De transcriptoomdata laten zien dat *SNF7* bijdraagt aan de regulatie onder anaërobe condities van genen die coderen voor eiwitten in de celwand en het plasmamembraan. Niet alleen coderen 46 van de 214 genen waarvan het transcriptieniveau verandert in een $\Delta snf7$ stam, voor eiwitten van de celwand en het plasmamembraan, maar ook zijn 6 genen gevonden die coderen voor eiwitten die betrokken zijn bij de synthese en het onderhoud van deze structuren. Sommige van deze genen die een lager transcriptieniveau hebben in een $\Delta snf7$ stam, zoals *TIR1* en *TIR4*, zijn onderdeel van genfamilies. Andere leden, zoals *TIR2* en *DAN2*, hebben juist een hoger transcriptieniveau in deze stam en zouden de functie van de genen met een lager transcriptieniveau kunnen overnemen.

Deletie van *SNF7* heeft een aantal fenotypische consequenties. De meeste van deze, zoals een gevoeligheid voor nyastatin (Giaever *et al.*, 2002), kunnen worden gerelateerd aan een defecte celwand. Sommige fenotypes zijn het gevolg van een veranderd plasmamembraan, zoals gevoeligheid voor sodium dodecylsulfaat (SDS). SDS is een detergent dat effect heeft op de membraanstabiliteit en indirect ook op de opbouw van de celwand. Daarom

kan het gebruikt worden om celwand defecten op te sporen die leiden tot een grotere toegankelijkheid van het plasmamembraan. Het defect in sporulatie (Giaever *et al.*, 2002) is waarschijnlijk gerelateerd aan een veranderde celwand in combinatie met het lagere transcriptieniveau van het *CDA1* gen, dat nodig is voor de vorming van de ascosporewand (Christodoulidou, Bouriotis, and Thireos, 1996), en het *SMA1* gen, dat verantwoordelijk is voor de opbouw van het membraan van de sporen (Rabitsch *et al.*, 2001). Andere fenotypische gevolgen van de deletie van *SNF7* zijn een veranderde aanpassing aan alkalische pH (Boysen and Mitchell, 2006; Wolfe and Shields, 1997) en aan verhoogde temperatuur (Hammond *et al.*, 2006). Dit laatste is niet bekend als *SNF7*-afhankelijk, maar een *snf7* deletiemutant heeft wel een subtiel groeidefect bij 37°C (Tu, Vallier, and Carlson, 1993). Onze experimenten tonen een sterke gevoeligheid aan bij 43°C.

Analyse van de promotergebieden van de genen die in de $\Delta snf7$ een hoger transcriptieniveau hebben, wees uit dat 46 van deze 99 genen de bindingsplaats voor de transcriptiefactor Rim101 in dit gebied heeft. Het is daarom waarschijnlijk dat deze genen gereguleerd worden via Rim101, dat in wildtype cellen geactiveerd wordt door een Snf7-afhankelijke modificatie. Een andere transcriptiefactor die misschien een rol speelt is Nrg1.

Over het algemeen is de rol van *SNF7* niet gelimiteerd tot anaërobe condities, aangezien de geobserveerde fenotypes niet anaëroob-specifiek zijn, op de langzamere groei onder deze condities na. Ook de rol die Snf7 speelt in de reactie op een alkalische omgeving is aëroob vastgesteld. Het *SNF7* gen lijkt nodig te zijn als een verandering in transcriptie van genen die coderen voor celwand- en plasmamembraanewitten vereist is voor een goede aanpassing aan omgevingsfactoren. Anaërobiciteit is één van die condities.

Met de experimenten beschreven in dit proefschrift is aangetoond dat behalve metabolische veranderingen, aanpassing van zowel de celwand als het plasmamembraan belangrijk zijn voor anaërobe groei. Bovendien lijkt een verband te bestaan tussen deze aanpassing en de import van sterolen en vetzuren, welke essentieel zijn als geen moleculaire zuurstof aanwezig is. Het belang van deze aanpassingen wordt duidelijk wanneer een genomische vergelijking gemaakt wordt tussen de obligaat aërobe *K. lactis* en de facultatief anaërobe *S. cerevisiae*. Deze vergelijking laat duidelijk zien dat de

stam die niet onder anaërobe omstandigheden kan groeien geen genen heeft die coderen voor een sterolimport systeem. Anaërobe sterolopname wordt waarschijnlijk mogelijk gemaakt door een veranderde membraanfluiditeit en een meer poreuze celwandstructuur, gecombineerd met actieve opname door transporteiwitten, zoals Dan1, Pdr11, Aus1 en Arv1. Het is waarschijnlijk dat de celwand en het plasmamembraan verbonden zijn in hun activiteit om de opname van de benodigde hoeveelheid sterol en onverzadigde vetzuren te waarborgen. De aanpassing van de celwand en het plasmamembraan aan anaërobe condities is een ingrijpend en complex gereguleerd proces, zoals blijkt uit de transcriptoomdata. Onze experimenten laten zien dat Snf7, in ieder geval gedeeltelijk, verantwoordelijk is voor deze veranderingen. Snf7 is een algemene aanpassingsfactor die de transcriptieniveaus van genen die coderen voor celwand- en plasmamembraaneiwitten reguleert in reactie op verschillende omgevingscondities.

Curriculum Vitae

Ishtar Snoek werd op 25 oktober 1976 geboren te Leiderdorp. Na zes jaar op het stedelijk gymnasium te Leiden te hebben gependeed, is zij in 1995 begonnen met de studie psychologie aan de universiteit van Leiden. Een jaar later besloot zij daar scheikunde bij te gaan doen. In 2000 werd de studie psychologie afgerond met het doctoraal examen met als richting biologische psychologie en in 2001 de studie scheikunde met als richting biochemie. Direct daarna kon zij aan de slag als promovenda bij het Instituut Biologie van de Universiteit Leiden o.l.v. dr. Yde Steensma in de werkgroep gistgenetica , onderdeel van de sectie moleculaire ontwikkelingsgenetica (prof.dr.P.J.J.Hooykaas). Zij verricht nu onderzoek als post-doc aan de industrieel belangrijke schimmel *Penicillium chrysogenum* aan de Technische Universiteit te Delft.

Stellingen

1 Het onvermogen van *Kluyveromyces lactis* om onder anaerobe condities te groeien is, in grote mate, een gevolg van de afwezigheid van een sterol-import systeem in deze soort.

Bolotin-Fukuhara *et al.* (2006) *Genome evolution: lessons from Génolevure*. Pp 165-196

Dit proefschrift

2 In *Saccharomyces cerevisiae* kunnen hoge transcriptie niveaus van een gen bij bepaalde omstandigheden niet als bewijs gezien worden van een bijdrage van dat gen aan de 'fitness' van de cel onder die specifieke experimentele condities.

Birell *et al.* (2002) *Proc. Natl. Acad. Sci. USA* **99** : 8778-8783

Giaever *et al.* (2002) *Nature* **418**: 387-391

Dit proefschrift

3 Om tot een optimale aanpassing aan omgevingsfactoren te komen dienen verschillende signalen geïntegreerd te worden. In *Saccharomyces cerevisiae* vervult het SAGA complex hierin een sleutelrol.

Roberts *et al.* (2006) *Mol. Gen. Genomics* **276** : 170-186

Pray-Grant *et al.* (2002) *Mol. Cell Biol.* **22**: 8774-8786

Dit proefschrift

4 Het *Saccharomyces cerevisiae* *SNF7* gen is nodig wanneer een verandering in de transcriptie van genen die coderen voor eiwitten van de celwand en het plasmamembraan vereist is voor de volledige aanpassing van de cel aan omgevingscondities, zoals aan anaërobiciteit.

Unno *et al.* (2005) *FEMS yeast res.* **5**: 801-812

Dit proefschrift

5 Anaeroob omschrijft een omgeving of conditie waarin geen gasvormig zuurstof aanwezig is. Dit betekent dat de meeste 'anaerobe experimenten' waarschijnlijk niet onder anaerobe ,maar onder hypoxische condities uitgevoerd zijn.

Henderson's dictionary of biology 13th ed, 2005, eds. E. Lawrence, Prentice Hall, Harlow, England

6 De dynamica van biologische systemen wordt bepaald door afstemming van de regulatie op de niveaus van transcriptie en translatie met post-transcriptionele en post-translationele processen. Deze subtiele onderlinge afstemming is een belangrijke, maar tot nog toe ondergewaardeerde factor om rekening mee te houden bij het genetisch modificeren van organismen.

Jensen *et al.* (2006) *Nature* **443**: 594-598

7 Het voorstel om de larven van *Drosophila* te gebruiken als *in vivo* test voor genotype-specifieke drugs voor het vergroten van stralingstherapie is twijfelachtig op ethische gronden, aangezien elke vorm van leven met gelijk respect behandeld moet worden.

Jaklevic *et al.* (2006) *Genetics* **174**: 1963-1972

8 Bij het gebruik van uit bacteriën geïsoleerde recombinante eiwitten dient het effect van de aanwezigheid van kleine hoeveelheden onzuiverheden niet onderschat te worden.

Montague *et al.* (1997) *J. Biol. Chem.* **272**: 6677-6684

Manteca *et al.* (2004) *J. Bacteriol.* **186**: 6325-6326

9 In tegenstelling tot wat Manteca *et al.* beweren is het verhoogde niveau van antioxiderende eiwitten welke zij waarnemen in *Streptomyces coelicolor* bij geprogrammeerde celdood waarschijnlijk niet het gevolg van oxidatieve stress veroorzaakt door deze geprogrammeerde cel dood.

Manteca *et al.* (2006) *Proteomics* **6**: 6008-6022

10 *Penicillium chrysogenum* is goed voor de gezondheid van de mens niet alleen omdat dit organisme het antibioticum penicilline kan maken, maar ook omdat het de schadelijke Cr(IV) ionen uit

afvalwater kan reduceren tot minder schadelijke Cr(III) ionen.

Pazouki *et al.* (2006) *Bioresour. Technol.* **97**:111-117

11 Het IDIC principe (oneindige diversiteit in oneindige combinaties) is een basale wetenschappelijke waarheid die elke wetenschapper in zijn achterhoofd zou moeten houden bij het doen van onderzoek.

Star Trek, the original series: 'Is there in truth no beauty?'

12 Het is wonderlijk dat een muur door Israël gebouwd om de Palestijnen buiten te houden door de wereld hevig wordt afgewezen, maar dat een muur door de Amerikanen gebouwd om de Mexicanen buiten te houden, acceptabel is.

13 Het veranderende klimaat zal de recreationele potentie van hobby-duiken zeker verhogen.

14 Tijdens een promotieonderzoek is het soms moeilijk geloof te hechten aan de woorden "Elk nadeel heb z'n voordeel".

Hendrik Johannes Cruijff