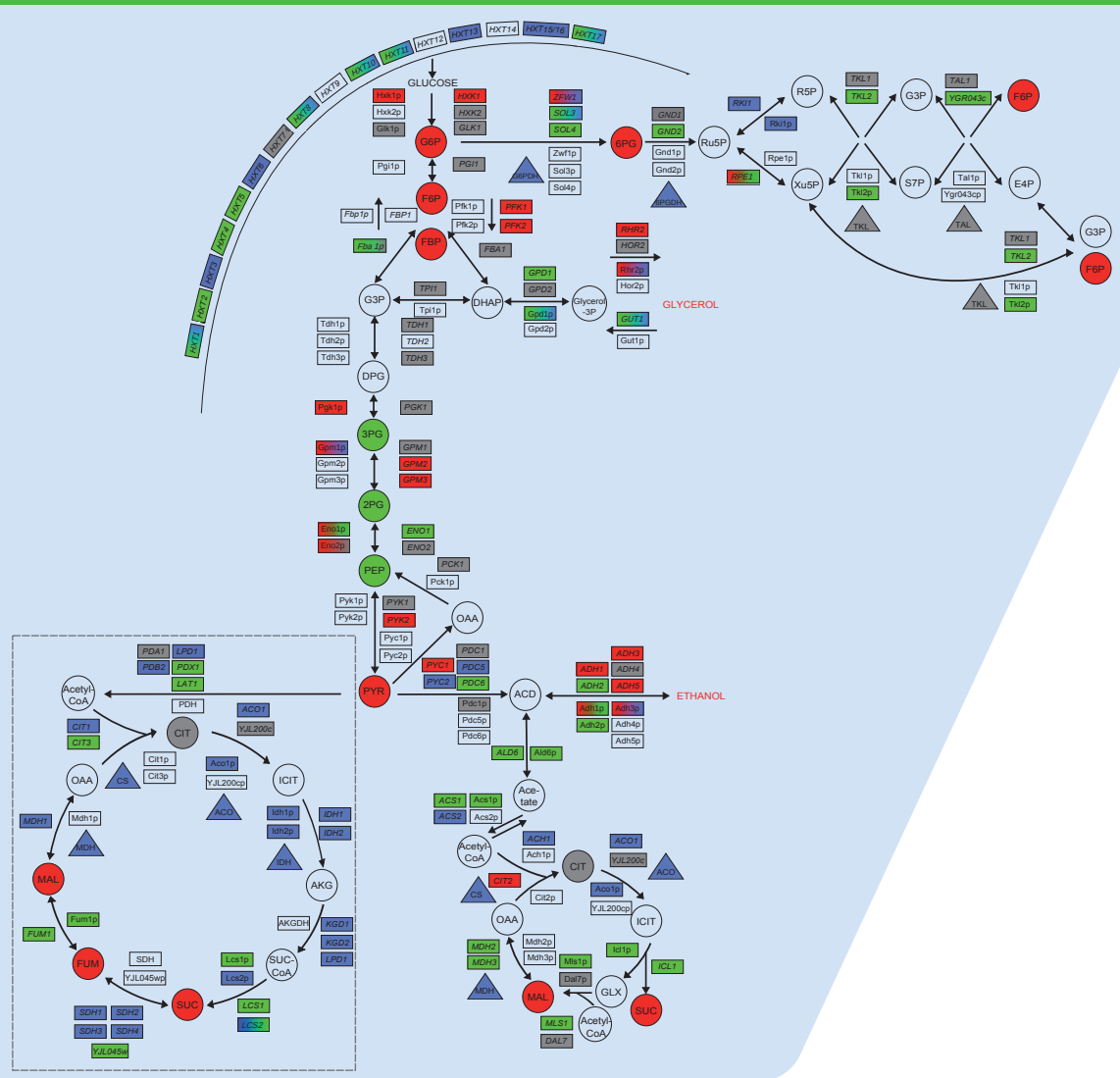

Eija Rintala

Effects of oxygen provision on the physiology of baker's yeast *Saccharomyces cerevisiae*

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Effects of oxygen provision on the physiology of baker's yeast *Saccharomyces cerevisiae*

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Keywords *Saccharomyces cerevisiae*, oxygen, transcriptome, proteome, hexose transporters, central carbon metabolism, trac, metabolites

Abstract

The availability of oxygen has a major effect on all organisms. The yeast *Saccharomyces cerevisiae* is able to adapt its metabolism for growth in different conditions of oxygen provision, and to grow even under complete lack of oxygen. Although the physiology of *S. cerevisiae* has mainly been studied under fully aerobic and anaerobic conditions, less is known of metabolism under oxygen-limited conditions and of the adaptation to changing conditions of oxygen provision. This study compared the physiology of *S. cerevisiae* in conditions of five levels of oxygen provision (0, 0.5, 1.0, 2.8 and 20.9% O_2 in feed gas) by using measurements on metabolite, transcriptome and proteome levels. On the transcriptional level, the main differences were observed between the three level groups, 0, 0.5–2.8 and 20.9% O_2 which led to fully fermentative, respiro-fermentative and fully respiratory modes of metabolism, respectively. However, proteome analysis suggested post-transcriptional regulation at the level of 0.5 O_2. The analysis of metabolite and transcript levels of central carbon metabolism also suggested post-transcriptional regulation especially in glycolysis. Further, a global upregulation of genes related to respiratory pathways was observed in the oxygen-limited conditions and the same trend was seen in the proteome analysis and in the activities of enzymes of the TCA cycle.

The responses of intracellular metabolites related to central carbon metabolism and transcriptional responses to change in oxygen availability were studied. As a response to sudden oxygen depletion, concentrations of the metabolites of central carbon metabolism responded faster than the corresponding levels of gene expression. In general, the genome-wide transcriptional responses to oxygen depletion were highly similar when two different initial conditions of oxygen provision (20.9 and 1.0% O_2) were compared. The genes related to growth and cell proliferation were transiently downregulated whereas the genes related to protein degradation and phosphate uptake were transiently upregulated. In the cultures initially receiving 1.0% O_2, a transient upregulation of genes related to fatty acid oxidation, peroxisomal biogenesis, response to oxidative stress and pentose phosphate pathway was observed.

Additionally, this work analysed the effect of oxygen on transcription of genes belonging to the hexose transporter gene family. Although the specific glucose uptake rate was highest in fully anaerobic conditions, none of the *hxt* genes showed highest expression in anaerobic conditions. However, the expression of genes encoding the moderately low affinity transporters decreased with the decreasing oxygen level. Thus it was concluded that there is a relative increase in high affinity transport in anaerobic conditions supporting the high uptake rate.

Eija Rintala. Effects of oxygen provision on the physiology of baker´s yeast *Saccharomyces cerevisiae* [Hapen vaikutus leivinhiiva *Saccharomyces cerevisiaen* aineenvaihduntaan]. Espoo 2010. VTT Publications 747. 82 s. + app. 93 s.

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Tiivistelmä

Toisin kuin useimmat aitotumalliset eliöt, leivinhiiva *Saccharomyces cerevisiae* pystyy kasvamaan erilaisissa happipitoisuuksissa, jopa täysin hapettomissa oloissa. Tätä ominaisuutta on hyödennetty laajasti erilaisissa bioprosesseissa. Jotta näistä prosesseista saataisiin mahdollisimman tehokkaita, on tärkeä tietää miten leivinhiivan aineenvaihduntaa säädellään hapen vaikutuksesta. Tässä väitöskirjatyössä tutkittiin leivinhiivan aineenvaihduntaa olosuhteissa, joissa syötetyn hapen määrä oli tarkasti määritetty. Työssä käytettiin viittä eri happipitoisuutta (0, 0.5, 1.0, 2.8 ja 20.9 % happea kasvatukseen syötetyssä kaasuseoksessa) sekä olosuhteita, joissa hapen syötttöä muutettiin äkillisesti. Työssä mitattiin solunsisäisiä ja -ulkoisia aineenvaihduntatuotteita ja geenien ilmentymistä. Hapensyötön eri tasoilla mitattiin myös proteiinien määriä ja entsyymien aktiivisuuksia.

Geenien ilmentymisen ja solunulkoisten aineenvaihduntatuotteiden perusteella näytti siltä, että leivinhiivan aineenvaihdunta on hyvin samankaltaista rajoitetun hapen olosuhteissa (0.5, 1.0 ja 2.8 O_2), mutta eroaa niissä selvästi hapettomista (0 % O_2) ja normaalin hapen olosuhteista (20.9 % O_2). Proteiinitasoja vertailtaessa kuitenkin havaittiin, että aineenvaihdunta ei ole täysin samanlaista happirajoitetuissa olosuhteissa, erityisesti 0.5 ja 1.0 % hapensyötön välillä nähtiin eroja, mikä kertoo todenäköisesti geenitason yläpuolella tapahtuvasta säätelystä.

Tässä työssä havaittiin myös, että suurin osa hengitykseen liittyistä geeneistä ilmentyi voimakkaammin happirajoitteisissa kuin normaalin hapen olosuhteissa, ja sama tulos näkyi myös kyseessä olevien proteiinien tasoissa ja sitruunahappokierron entsyymien aktiivisuuksissa. Tämä kertoo luultavasti siitä, että solu yrittää saada rajoitetun hapen mahdollisimman tehokkaasti käyttöönsä. Lisäksi havaittiin, että vaikka glukoosin sisäänottonopeus on suurin hapettomissa olosuhteissa, glukoosinkuljettajaproteiineja koodaavien geenien ilmentyminen ei ole tällöin voimakkaimmillaan. Sen sijaan hapen määrän laskiessa keskimääräisen

affiniteetin omaavia glukoosinkuljettajia koodaavien geenien tasot laskivat. Edellämainittu aiheuttaa todennäköisesti sen, että solukalvolla on hapettomissa olosuhteissa suhteellisesti enemmän proteiineja, joilla on korkea affiniteetti glukoosia kohtaan kuin hapellisissa olosuhteissa.

Lopetettaessa hapensyöttö äkillisesti kokonaan, aineenvaihdunnan muutokset näkyivät nopeammin solunsisäisten aineenvaihduntatuotteiden määrissä kuin geenien ilmentymisessä. Havaittiin. että muutokset olivat hyvin samankaltaisia riippumatta siitä kuinka paljon happea kasvatuksiin oli alunperin syötetty. Hapen loppuessa kasvuun ja solujen uudistumiseen liittyvien geenien ilmentymistasot laskivat, kun taas proteiinien hajotukseen liittyvien geenien ilmentymistasot nousivat. Lisäksi havaittiin stressivasteeseen liittyviä muutoksia.

Preface

This study was carried out at VTT Technical Research Centre of Finland in the Metabolic Engineering team. Financial support from the Academy of Finland (Centre of Excellence, Industrial Biotechnology 2000-2005; project number 214568, Centre of Excellence, White Biotechnology – Green Chemistry 2008–2013; project number 118573 and SYSBIO programme; project number 207435) and Tekes Finnish Funding Agency for Technology and Innovation (Project numbers 40135/04 and 40537/05) is gratefully acknowledged. I also thank the University of Helsinki for a grant for writing this thesis.

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I thank my mother Eila and my late father Seppo for the support and guidance during my life and my mother for taking care of Heikki whenever I needed time for this thesis. I also thank my brothers and their families for reminding me of life beyound science and my friends Elina, Heli, Heli and Piia for all the nice moments we have shared both indoors and outdoors.

My warmest thanks go to my dearest ones, Harri and Heikki. Harri for your love and Heikki for filling my days with joy and sunshine.

Espoo, October 2010

Contents

List of publications

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals (I–IV). In addition, data published in the abstract book of the 3rd European Federation of Biotechnology Conference: Physiology of Yeasts and Filamentous Fungi (PYFF3) and some unpublished data is presented.

I Wiebe Marilyn G., Rintala Eija, Tamminen Anu, Simolin Helena, Salusjärvi Laura, Toivari Mervi, Kokkonen Juha T, Kiuru Jari, Ketola Raimo A, Jouhten Paula, Huuskonen Anne, Maaheimo Hannu, Ruohonen Laura & Penttilä Merja. 2008. Central carbon metabolism of *Saccharomyces cerevisiae* in anaerobic, oxygen-limited and fully aerobic steady state conditions and following a shift to anaerobic conditions. FEMS Yeast Research 8(1): 140–154.

II Rintala Eija, Wiebe Marilyn G., Tamminen Anu, Ruohonen Laura & Penttilä Merja. 2008. Transcription of hexose transporters of *Saccharomyces cerevisiae* is affected by change in oxygen provision. BMC Microbiology 8: 53.

III Rintala Eija, Toivari Mervi, Pitkänen Juha-Pekka, Wiebe Marilyn G., Ruohonen Laura & Penttilä Merja. 2009. Low oxygen levels as a trigger for enhancement of respiratory metabolism in *Saccharomyces cerevisiae*. BMC Genomics 10: 461.

IV Rintala Eija, Jouhten Paula, Toivari Mervi, Wiebe Marilyn G., Penttilä Merja, Maaheimo Hannu & Ruohonen Laura. Transcriptional responses of *Saccharomyces cerevisiae* to change in oxygen provision. Accepted for publication in OMICS: A Journal of Integrative Biology. In press.

Abbreviations

2PG	2-phosphoglycerate
3PG	3-phosphoglycerate
6PG	6-phoshogluconate
ACD	acetaldehyde
ADP	adenosine 5-diphosphate
AEC	adenylate energy charge
AKG	alphaketoglutarate
AMP	adenosine 5-monophosphate
ANOVA	analysis of variance
ATP	adenosine 5-triphosphate
CER	carbon dioxide evolution rate
CIT	citrate
DHAP	dihydroxyacetone phosphate
DPG	1,3 bis-phosphoglycerate
E4P	erythrose 4-phosphate
FAD	flavin adenine dinucleotide (oxidised)
$FADH_2$	flavin adenine dinucleotide (reduced)
FUM	fumarate
F6P	fructose 6-phosphate
FBP	fructose 1,6-bisphoshate
G3P	glyceraldehyde 3-phosphate
G6P	glucose 6-phosphate
GDP	guanosine diphosphate
GLX	glyoxylate
GTP	guanosine 5-triphosphate

HXT	hexose transporter gene of *S. cerevisiae*
ICIT	isocitrate
MAL	malate
M6P	mannose 6-phosphate
mRNA	messenger RNA
NAD	nicotinamine adenine dinucleotide (oxidised)
NADH	nicotiamine adenine dinucleotide (reduced)
NADP	nicotinamine adenine dinucleotide phosphate (oxidised)
NADPH	nicotinamine adenine dinucleotide phosphate (reduced)
OAA	oxaloacetate
ORF	open reading frame
PEP	phosphoenolpyruvate
PPP	pentose phosphate pathway
PYR	pyruvate
OUR	oxygen uptake rate
ROS	reactive oxygen species
SUC	succinate
SUC-CoA	succinyl-CoA
S7P	sedoheptulose 7-phosphate
T6P	trehalose 6-phosphate
TCA	tricarboxylic acid
TRAC	transcript analysis with the aid of affinity capture

1. Introduction

1.1 Oxygen

Oxygen is an essential molecule for most eukaryotic organisms. In the early, prebiotic atmosphere of earth, oxygen was present only in trace amounts if at all [1, 2]. Approximately two to three billion years ago the emergence of the first photosynthetic organisms led to slow accumulation of atmospheric oxygen [3]. The concentration of oxygen in the atmosphere has varied between 10 and 35% during the last 550 million years [4] and stabilised at its present level of 21% the during last 200 million years [5].

The first eukaryotes appeared on earth at around the same time as the increase in atmospheric oxygen occurred [6–8]. The level of oxygen has been suggested to have constrained the evolution of receptor proteins, which are important in the communication across membranes and between cells and are thus crucial for eukaryotes [9]. As the oxygen level increased, the size and number of communication-related transmembrane proteins increased [9]. In addition to transmembrane proteins, sterols play a key role in the transport of materials across the cell membrane. The biosynthesis of sterols is an oxygen-dependent process facilitated by high atmospheric oxygen levels. Only a few prokaryotes are able to synthesise sterols [10].

Most known eukaryotes rely on oxygen during growth even though oxygen can also be harmful to them. Free radicals which are formed in the mitochondrial reactions can damage the cell membranes and DNA [11, 12]. There are also some eukaryotes which can survive and grow (temporarily) without oxygen [13]. The unicellular eukaryote *Saccharomyces cerevisiae* (baker's yeast) is able to grow both in the presence and absence of oxygen. However, in the absence of oxygen, sterols and unsaturated fatty acids have to be obtained from the envi-

ronment [14, 15]. The ability of growth in diverse oxygen concentrations and the ability to produce ethanol even in the presence of oxygen, has made *S. cerevisiae* an important industrial organism.

S. cerevisiae has long been used for the leavening of bread and for biomass production. *S. cerevisiae* and other *Saccharomyces* yeasts also make an important contribution in the brewing and wine-making. More recent applications of *S. cerevisiae* can be found in the production of heterologous proteins such as hepatitis b vaccine and insulin and in the production of bulk chemicals such as fuel ethanol and lactic acid [16]. In this thesis, the term yeast refers to *S. cerevisiae*.

The provision of an optimal level of oxygen is still problematic in industrial scale bioreactors. The level of oxygen influences product and by-product formation and thus the economics of the process [17, 18]. Full oxygenation in large reactors is expensive and sometimes even impossible. On the other hand, too high oxygen levels may lead to biomass growth at the expense of product formation. Anaerobic conditions lead to ethanol production, which is not favourable in cultivations for protein production and anaerobic conditions may be energetically less efficient since ATP is produced by substrate-level phosphorylation only. Furthermore, production of e.g. heterologous proteins may require high cell densities, which makes mixing more difficult and leads to temporal or local oxygen gradients inside the production vessels. These gradients cause differences in the physiology of the cells [19].

1.2 Fermentative and respiratory metabolism of *S. cerevisiae*

During fermentative and mixed respiro-fermentative growth, *S. cerevisiae* converts six-carbon sugars to two- and three-carbon components. This conversion, and subsequent use of the two- and three-carbon components (ethanol, acetate, glycerol) as carbon source, is energically less efficient than conversion of sugars directly to CO_2 by respiration. However, this strategy (make-accumulate-consume–strategy) gives yeast an advantage over many microorganisms for which ethanol is toxic [20]. In nature, *S. cerevisiae* lives on fruit surfaces and competes for resources with other yeasts, moulds and bacteria [16].

The ability of *S. cerevisiae* to consume ethanol is thought to have arisen from the duplication and differentiation of the *ADH* gene 80 million years ago [21], after the whole genome duplication which occurred 100 million years ago [22, 23]. The genome of *S. cerevisiae* contains five alcohol dehydrogenases which

are involved in the metabolism of ethanol. *ADH1*, *ADH3*, *ADH4* and *ADH5* encode alcohol dehydrogenase isoforms used during the formation of ethanol whereas the enzyme encoded by *ADH2* is used during the consumption of ethanol. At about the same time as *ADH* duplication, eight additional gene duplications occurred, six of which are involved in the conversion of glucose to ethanol [21].

In *S. cerevisiae*, fully fermentative metabolism occurs only under anaerobic conditions while fully respiratory metabolism occurs on the respiratory carbon sources (*e.g.* ethanol, glycerol, acetate, fatty acids) and at low specific growth rates on the fermentative carbon sources (*e.g.* glucose, fructose, galactose). When both oxygen and excess sugars are present, *S. cerevisiae* uses respiro-fermentative metabolism. As the sugars become used, yeast undergoes a diauxic shift during which the growth is switched from the respiro-fermentative to the respiratory mode. The respiro-fermentative growth in high concentrations of sugars in the presence of oxygen has been suggested to result either from the rate of glycolysis exceeding the rate of pyruvate dehydrogenase enzyme or from carbon catabolite repression [24, 25]. The most studied repressor is glucose, which is known to repress the genes needed in mitochondrial respiration, utilisation of alternative carbon sources and gluconeogenesis, via a complex mechanism which is not yet fully understood (for review see [24, 26]). The presence of glucose also affects mRNA turnover and protein translation rate and degradation [24]. In addition to glucose, the presence of other sugars such as fructose, maltose and galactose leads to respiro-fermentative metabolism in the presence of oxygen [24]. The yeasts that produce ethanol from sugars in the aerobic conditions are called crabtree-positive. In the study of Vemuri *et al.* [27], an alternative oxidase from *Histoplasma capsulatum* was over-expressed in *S. cerevisiae*, resulting in increased expression of several genes encoding the enzymes of the TCA cycle and decreased aerobic ethanol formation in the presence of high concentration of sugars. These results suggest that the crabtree effect is a consequence of limited capacity of the respiratory system involved in oxidation of mitochondrial NADH.

1.3 Central carbon metabolism of *S. cerevisiae*

The central carbon metabolism of *S. cerevisiae* provides precursors for biosynthesis, energy as ATP and reducing power in the form of NAD(P)H and $FADH_2$ (Figure 1). In glycolysis, glucose taken up by hexose transporters is rapidly phosphorylated and converted to pyruvate. Pyruvate can be further converted to

acetaldehyde by pyruvate decarboxylase (fermentation), to acetyl-CoA by pyruvate dehydrogenase (respiration) or to oxaloacetate by pyruvate carboxylase (gluconeogenesis). In high intracellular concentrations of pyruvate, the pyruvate decarboxylase reaction is favoured [28]. In this fermentative pathway to ethanol, NADH formed in the glycolysis and biomass formation is reoxidised. NADH can also be oxidised by respiration and through glycerol formation, the latter occurring especially under anaerobic conditions and under conditions in which respiration is repressed [29].

The control of glycolysis is still largely unknown, although it has been extensively studied [30–32]. Individual enzymes are known to be allosterically regulated [33–38] and glucose transport has been suggested to play a role in the regulation of glycolytic rate [39]. Transcriptional regulation of the genes encoding glycolytic enzymes also occurs [37, 40–42] although the flux through glycolysis is thought to be controlled mainly post-transcriptionally [43, 44].

Under conditions in which aerobic respiration takes place, pyruvate is taken into the tricarboxylic acid (TCA) cycle through Acetyl-CoA. Acetyl-CoA enters the TCA cycle in a reaction in which citrate synthase converts oxaloacetate to citrate. The TCA cycle produces NADH and $FADH_2$ for the respiratory chain and precursors for amino acid biosynthesis. The genes encoding the enzymes of the TCA cycle are subject to glucose-repression [45, 46]. However, the flux through the TCA cycle is most probably regulated by growth rate or glucose uptake rate and not by extracellular glucose concentration [47]. In addition, several genes encoding the enzymes of the TCA cycle are under positive regulation by the Hap2/3/4/5p complex which also regulates other genes related to respiration [48]. In respiratory-deficient cells, the control of *CIT1*, *ACO1*, *IDH1*, and *IDH2*, encoding for enzymes of the TCA cycle, switches to transcription factors Rgt1p, Rgt2p and Rtg3p. This switch has been suggested to ensure the synthesis of α-ketoglutarate [49]. In batch cultures with high glucose concentration and in the of oxygen, TCA cycle functions only partially (see section 1.5), providing a precursor for amino acid synthesis [50].

To enable growth on non-sugar carbon sources, gluconeogenesis is used to synthesise glucose. Gluconeogenesis is essentially the reversal of glycolysis, but as two enzymes in glycolysis catalyse irreversible reactions, these reactions are circumvented by the enzymes pyruvate carboxylase (Pyc1p, Pyc2p), phosphoenolpyruvate carboxykinase (Pck1p) and fructose-1,6-bisphosphatase (Fbp1). The genes encoding these enzymes are under transcriptional regulation [51–53]. In addition, Fbp1p is subject to glucose-induced protein degradation [54].

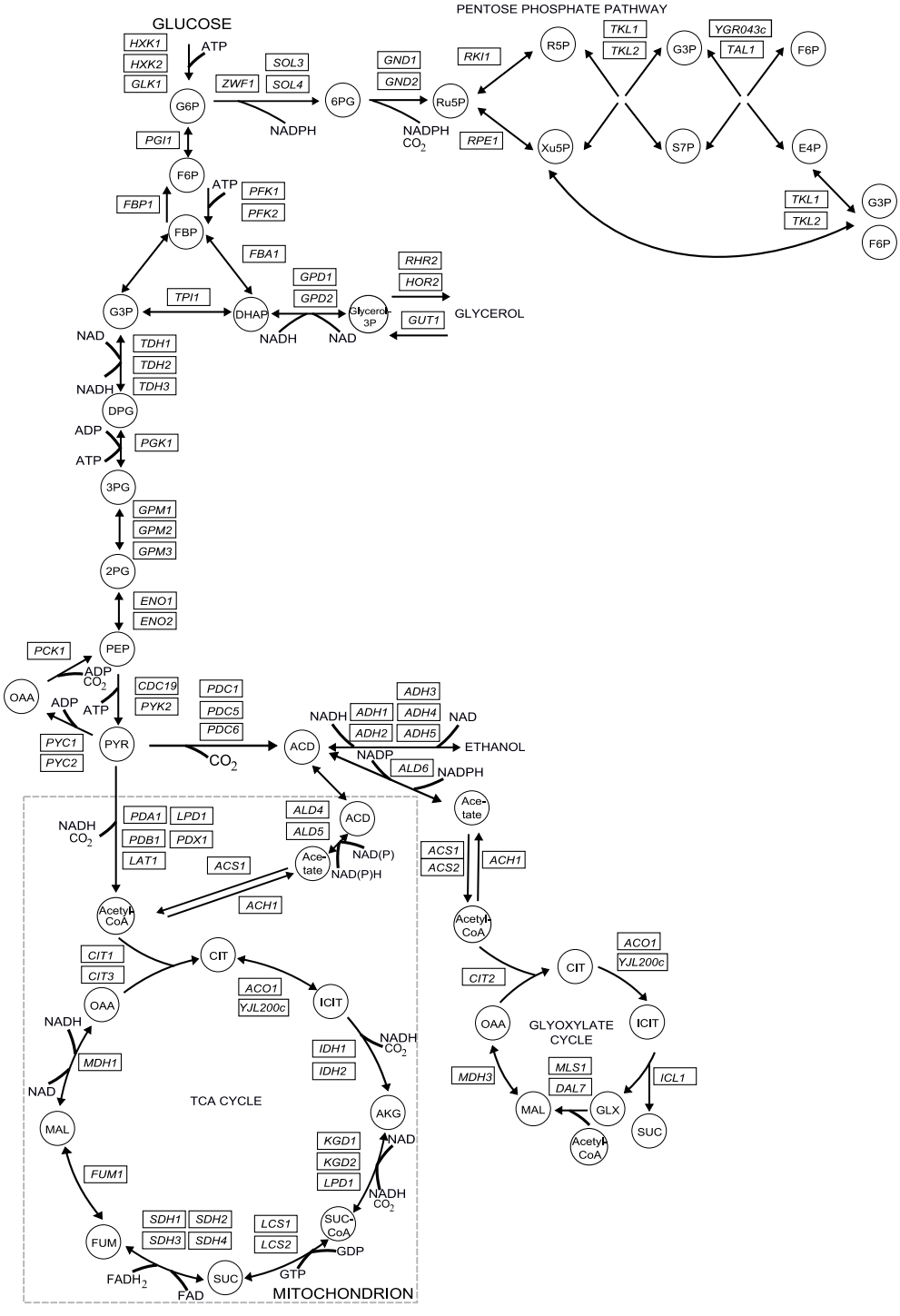

Figure 1. Genes encoding the enzymes of central carbon metabolism in *S. cerevisiae*.

In addition to gluconeogenesis, the glyoxylate cycle is required for growth on two-carbon substrates. In the glyoxylate cycle, these two-carbon substrates are converted to four-carbon compounds. Many of the reactions of the TCA cycle and the glyoxylate cycle are identical, but are catalysed by different isoenzymes. Whereas enzymes of the TCA cycle are located in mitochondria, the glyoxylate cycle occurs in cytosol and in peroxisomes [55]. The enzymes functioning only in the glyoxylate cycle are isocitrate lyase (Icl1p) and malate synthase (Mls1p, Dal7p) [56–58]. Synthesis of these enzymes is repressed in cells grown on glucose [55].

Under aerobic conditions, the reactions of the pentose phosphate pathway (PPP) produce reducing power in the form of NADPH and precursors for nucleotide and amino acid biosynthesis in the form of ribose 5-phosphate and erythrose 4-phosphate. The regulation of PPP has been thought to occur through the need for NADPH and biosynthetic precursors [59]. Especially the activity of the first enzyme of the PPP, glucose 6-phosphate dehydrogenase (Zwf1p), is largely affected by the ratio of NADP to NADPH [60, 61]. However, regulation of other enzymes of the PPP also affects the activity of the pathway and in addition PPP is important in the protection against oxidative stress and is subject to regulation by the Yap1p and Stb5p transcription factors [62, 63]. Many of the enzymes of the PPP exist as two isoforms. The physiological role of the minor isoforms is not known, but it is known that they respond similarly during diauxic shift and in response to *e.g.* histone depletion, heat shock and nitrogen depletion [46, 64, 65].

1.4 Mitochondrial respiratory chain

The respiratory chain uses electrons from NADH and $FADH_2$ to create a transmembrane proton gradient that is used to synthesise ATP by ATP synthase. In *S. cerevisiae*, the respiratory chain is present under both aerobic and anaerobic conditions, although the protein levels are lower in the absence of oxygen [66, 67]. Activity of the respiratory chain can be detected within 25–30 minutes after oxygenation of glucose-repressed anaerobic cells of *S. cerevisiae*, although at least 400 minutes are needed for full activity [68].

The respiratory chain in yeast consists of complexes II, III, IV and V whereas it lacks the complex I (Figure 2). The functions of complex I (NADH dehydrogenase) are replaced by an internal (Ndi1p) and two external (Nde1p and Nde2p) NADH dehydrogenases and a glycerol 3-phosphate dehydrogenase shuttle con-

sisting of cytosolic glycerol 3-phosphate dehydrogenase (Gpd1p) and mitochondrial FAD-linked glycerol 3-phosphate dehydrogenase (Gut2p) [69, 70].

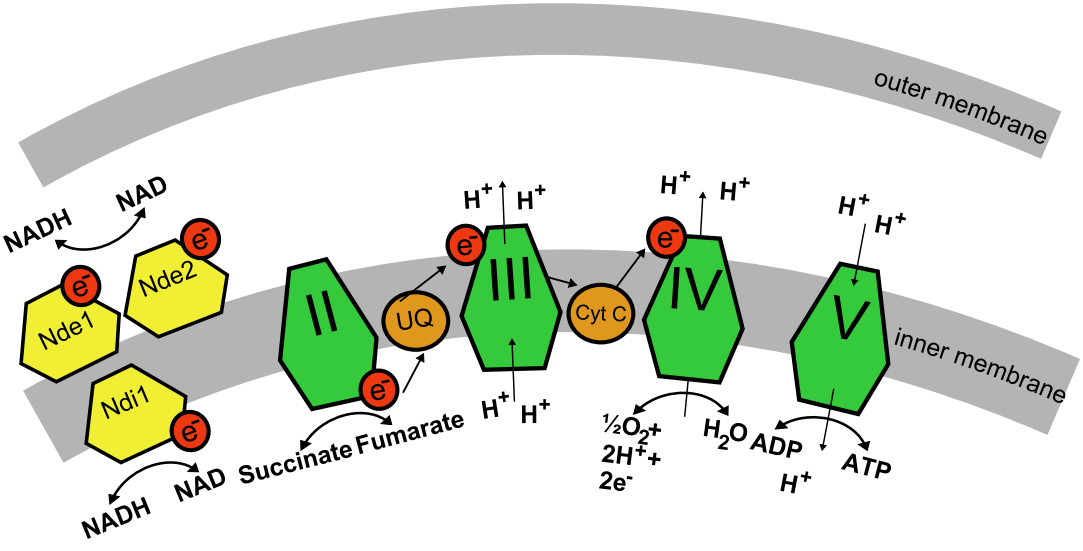

Figure 2. The mitochondrial respiratory chain of *S.cerevisiae.*

Complex II (succinate dehydrogenase) oxidises succinate to fumarate and reduces ubiquinone. Ubiquinone is oxidised by complex III (Cytochrome bc) and the electrons are transferred to cytochrome *c*. Complex IV (cytochrome *c* oxidase) oxidises cytochrome *c* by reducing oxygen to water. Complexes III and IV translocate protons across the inner mitochondrial membrane, resulting in a proton gradient. Complex V (ATP synthase) uses the proton gradient in the synthesis of ATP. Complexes III and IV can form supercomplexes and complex V can exist both as a monomer and a dimer [71]. In addition, complex II can form supercomplexes with the mitochondrial membrane-bound dehydrogenases Gut2p, Nde1p, Nde2p and Ndi1p [72].

Succinate dehydrogenase (complex II) consisting of four subunits Sdh1p, Sdh2p, Sdh3p and Sdh4p, is also a component of the TCA cycle [73]. The cytochrome bc (complex III) consists of three catalytic subunits encoded by *COB1*, *RIP1* and *CYT1* and seven additional subunits encoded by *COR1*, *QCR2* and *QCR6-10* [74]. Cytochrome *c* is encoded by two isoforms, *CYC1* and *CYC7*, of which *CYC1* is expressed under aerobic conditions and *CYC7* under hypoxic and

anaerobic conditions [75]. Cytochrome *c* oxidase (complex IV) consists of 9 subunits encoded by both mitochondrial and nuclear genomes. The three largest subunits performing catalytic functions (encoded by *COX1, COX2* and *COX3*) are mitochondrially encoded [76]. Nuclearly encoded subunits function in the assembly or stability of the holoenzyme or modulate the catalysis (*COX4, COX5a, COX5b* and *COX6-COX9*) [76]. Cox5a and Cox5b are interchangeable subunits, which affect the turnover rate of the enzyme [77]. *COX5a* is expressed under aerobic conditions, whereas *COX5B* is expressed under hypoxic and anaerobic conditions [75, 78]. Genes encoding for different subunits of cytochrome *c* oxidase exhibit different kinetics during transition from anaerobic to aerobic conditions. Some of them are fully induced rapidly, whereas others need more than two hours for full induction [75]. ATP synthase (complex IV) is encoded by *ATP6, ATP8* and *ATP9* located in the mitochondrial genome and *ATP4, ATP5, ATP7, ATP14, ATP17-12, INF1, STF1* and *STF2* which are encoded in the nuclear genome [79]. The genes encoding the subunits of respiratory chain complexes and cytochrome *c* are glucose repressed [80–82] and are regulated by oxygen concentration (see section 1.5). In addition, under glucose limitation, the amounts of cytochrome *c* oxidase, cytochrome *c* and cytochrome bc are maximal when 5% oxygen is provided in the gas stream, whereas the activity of cytochrome *c* oxidase is maximal when 10% oxygen is provided [83].

In addition to harvesting the chemical energy and storing it as ATP, mitochondria house parts of the metabolism of amino acids, lipids, heme and iron [84–87], and play an important role in apoptosis [88]. Sickmann *et al.* identified 750 different proteins in yeast mitochondria using various protein separation methods and tandem mass spectrometry [89]. Reinders *et al.* refined the mitochondrial proteome of *S. cerevisiae,* covering 851 proteins [90]. When the mitochondrial proteome of yeast was compared under fermentative (glucose) and respiratory (glycerol) conditions, the overall differences were small; only 18 proteins were found to be differentially expressed under these conditions [91]. When yeast proteomes from cells grown on glucose and lactate were compared, more proteins were detected in lactate-grown cells [92]. A study by Reinders *et al.*(2006) which identified yeast mitochondrial phosphoproteins suggested that many mitochondrial functions are regulated by reversible phosphorylation of proteins [90].

1.5 Growth under anaerobic conditions and oxygen-mediated transcriptional regulation

Under anaerobic conditions, energy is harvested by substrate-level phosphorylation in glycolysis. The conversion of one molecule of glucose yields two molecules of ATP and two molecules of NADH. This NADH can be reoxidised through conversion of pyruvate to ethanol. Residual TCA cycle activity that is maintained to provide precursors for biosynthetic reactions also produces reducing equivalents in the form of NADH and $FADH_2$. The NAD/NADH ratio is regulated by production of glycerol, particularly through the action of NADH-dependent glycerol 3-phosphate dehydrogenase. This enzyme is encoded by two genes, *GPD1* and *GPD2*, the latter of which is primarily used for redox balancing under anaerobic conditions [93]. $FADH_2$ is reoxidised by cytoplasmic fumarate reductase, encoded by *FRDS1* [94]. Flavin co-factors are exchanged between the cytosol and mitochondria by the carrier protein Flx1p [95, 96]. Under anaerobic conditions, TCA cycle operates as two branches because of low or zero oxoglutarate dehydrogenase and succinate dehydrogenase activities [97, 98]. In addition to production of biosynthetic precursors, the action of the TCA cycle also leads to excretion of organic acids under anaerobic conditions.

The biosynthesis of heme, sterols, unsaturated fatty acids and deoxyribonucleotides is generally thought to require oxygen [14, 15, 99, 100], although contradicting evidence has also been reported [66, 101]. Consequently, the plasma membrane of *S. cerevisiae* contains more saturated fatty acids and less total sterol, less ergosterol and squalene under anaerobic than under aerobic conditions [102]. In yeasts that cannot grow under anaerobic conditions, synthesis of pyrimidines requires oxygen, but in *S. cerevisiae* the enzyme dihydro-orotate dehydrogenase is cytosolic and independent on the functionality of the respiratory chain [99, 103].

Deoxyribonucleotides are synthesised from ribonucleotides by ribonucleotide reductases (RNRs) [100]. There are three classes of RNRs of which class I proteins are dependent on oxygen, class III proteins operate only in the absence oxygen and class II proteins can function both in the presence and absence of oxygen. Only class I RNR is known in *S. cerevisiae* [104, 105], but as the sequence homologies of these proteins is very low, it is possible that class II or III proteins are also present [19].

During anaerobic growth, sterols and unsaturated fatty acids must be provided in the growth medium [14, 15, 99, 100]. Unsaturated fatty acids are synthesised

from saturated fatty acids by a single oxygen-dependent acyl-CoA desaturase, encoded by *OLE1* [106] whereas in the sterol biosynthesis pathway oxygen is required in six enzymatic reactions [107] and references therein. In the absence of oxygen, the cell wall of *S. cerevisiae* is remodelled for the import of sterols and unsaturated fatty acids [19, 108, 109]. Under aerobic and anaerobic conditions, different classes of cell wall mannoproteins are used and this switch is regulated on the transcriptional level. Under anaerobic conditions, *CWP1* and *CWP2* transcription is on a lower level and transcription of *PAU, DAN* and *TIR* genes are on a higher level than under aerobic conditions [110].

The transcription factors Mox1p, Mox2p, Rox1p, Mot3p Upc2p, Ecm22p and Sut1p are known to play a role in the remodelling of cell walls and import of sterols [19, 111]. Nearly one third of anaerobically upregulated genes contain Upc2p/Ecm22p-binding sites in their promoters [108, 112]. Upc2 regulates the expression of *DAN*/*TIR* genes and the genes of sterol biosynthesis. Mox1p and Mox2p modulate the action of Upc2 in a heme-dependent way and Mot3p also regulates some of these genes [109]. In addition to Upc2p, Ecm22p regulates the genes of sterol biosynthesis. Upc2p and Ecm22p bind the same sequence and the binding is dependent on sterol concentration [113]. The target genes of Sut1p are not known, but the overexpression *SUT1* has been shown to enable uptake of sterols under aerobic conditions [114, 115].

Heme levels decline during growth under anaerobic conditions, and the concentration of heme plays an important role in the regulation of genes needed under anaerobic and strictly oxygen-limited conditions [116–118]. However, it has been reported that cells grown under anaerobic conditions contain small amounts of heme and it has thus been suggested that electron carriers other than oxygen could function during synthesis of heme [66, 101]. In addition, there are at least two types of heme pools in the cell, a protein-bound and a free pool, and it is not known how these two pools contribute to the transcriptional regulation [116]. Under aerobic conditions, a heme-activated transcription factor Hap1 activates the expression of genes encoding the respiratory chain complexes and those related to oxidative stress [118, 119]. Hap1p also induces the expression of *ROX1*, which encodes a repressor of genes needed during severe hypoxia or under anaerobic conditions [117, 120]. In addition, Hap1p acts as a repressor of genes involved in ergosterol biosynthesis in the absence of heme [121]. Another heme-activated transcription factor Hap2/3/4/5p is also involved in the activation of many genes related to respiratory metabolism in the presence of oxygen [48, 122]. However, the exact mechanisms of regulation of Hap2/3/4/5p by heme and

oxygen are unknown [123]. In addition, Hap2/3/4/5p regulates the expression of respiratory genes during glucose derepression [124].

Under strictly oxygen-restricted conditions, *S. cerevisiae* adapts the expression of certain genes to improve oxygen utilisation [118]. These genes are related to those functions that require oxygen (respiration and heme, sterol and unsaturated fatty acid biosynthesis). Some of these genes have counterparts that are used under aerobic conditions [125]. As stated above, Rox1p acts as a repressor of many of the genes needed under strictly oxygen-restricted conditions [117]. In addition, Ixr1p functions in the induction of certain genes during severe oxygen restriction [126–128]. Furthermore, induction of *OLE1* is regulated by low oxygen response element (LORE) [129] and cytochrome *c* oxidase is involved in the induction of at least *OLE1* and *CYC7* [130]. Additionally, computational evidence of Hypoxia response elements (HRE) in the yeast genome has recently been published [131]. In mammals, these elements are crucial in the regulation of gene expression under oxygen restriction [132].

In the absence of oxygen, mitochondria are present as precursor structures called promitochondria, which differ in their number, morphology and ultrastructure from the mitochondria present in the presence of oxygen [133, 134]. During growth on the respiratory carbon source glycerol, mitochondria are typically strongly branched and tubular, whereas during growth on fermentable carbon source glucose, the mitochondrial network is relatively simple [135]. Cells under anaerobicity typically contain only one promitochondrion whereas aerobic, ethanol- grown cells contain 20-30 mitochondria [68, 136, 137]. The mitochondrial structure is maintained by balanced fusion and fission [138]. In addition, it has been shown that dimerisation of ATP synthase is involved in control of the biogenesis of the inner mitochondrial membrane [139]. However, although respiration and mitochondrial morphology are linked, respiration is not required for normal mitochondrial morphology [140]. Furthermore, during transition from anaerobic to aerobic conditions, changes in the mitochondrial morphology continue for several hours after respiratory capacity has reached its maximum, indicating that one particular mitohcondrial morphology is not a prerequisite for increased respiration rate. This suggests that mitochondrial structure is formed and maintained for other functions than respiration, one of which is the mitochondrial inheritance [140]. In addition, mitochondria have been suggested to have a role in the anaerobic uptake of sterols. Deletion of certain genes encoding mitochondrial proteins leads to deficiency in the aerobic uptake of

sterols and to the formation of electron-dense mitochondrial inclusions in a mutant that otherwise would be able to transport sterols in the presence of oxygen [141].

1.6 Oxidative stress

During growth in the presence of oxygen, the mitochondrial respiratory chain produces reactive oxygen species (ROS) [11, 142]. In addition, ROS are generated when cells are exposed to heavy metals, ionising radiation or redox-cycling chemicals [143]. It has also been suggested that ROS produced by the mitochondrial respiratory chain function as signalling molecules during oxygen sensing especially under oxygen-restricted conditions [125, 144, 145]. Transiently elevated ROS levels are seen as a response to anoxia [146, 147]. In addition, transient oxidative stress as a response to anoxia is seen as increased levels of carbonylation of mitochondrial and cytosolic proteins, accumulation of 8-hydroxy-2'-deoxyguanosine in the mitochondrial and nuclear DNA, and as increased expression of *SOD1* [146].

Generally, the cells respire more when more oxygen is available and consequently many genes involved in the protection against oxidative stress are induced by oxygen [145]. Furthermore, respiring cells are more resistant to external oxidants such as H_2O_2 and superoxide anions than fermentative cells [148]. When oxygen provision exceeds 30% of the gas stream, toxic effects are observed [149]. In addition, exposure to sub-lethal concentrations of oxidants leads to an adaptive response which protects cells against subsequent exposure to higher concentrations of oxidants [150, 151]. Different oxidants confer somewhat different responses in *S. cerevisiae*, the most studied ones being H_2O_2 and menadione, which produce superoxide anions in the cells [148, 152, 153].

Cells use both enzymatic and non-enzymatic systems in the defence against oxidative stress (reviewed by [143]). In *S. cerevisiae*, glutathione, metallothioneins, thioredoxin, glutaredoxin and possibly trehalose and flavohaemoglobin act as non-enzymatic defence systems. The enzymes used in the protection against reactive oxygen species are catalases and superoxide dismutatases. In addition, as glutathione and thioredoxin reductases require NADPH, the pentose phosphate pathway plays an important role in defence against oxidative stress [154].

The transcriptional responses to oxidative stress are mediated by Yap1p, Skn7p and Msn2p/4p transcription factor,s of which Msn2p/4p is also involved in the regulation of general stress response evoked by many stress situations [64,

155–157]. As a response to oxidative stress, Yap1p relocalises from the cytoplasm to the nucleus, inducing the transcription of its target genes. [157]. Yap1p and Skn7p regulate a partially shared group of target genes. Skn7p and Yap1p are needed for induction of catalase, superoxide dismutase and of proteins of the thioredoxin system whereas only Yap1p upregulates the genes related to the glutathione system and the pentose phosphate pathway [63].

1.7 Genome-wide studies on responses to oxygen

Although major changes in the physiology of *S. cerevisiae* under anaerobic conditions are observed, only 23 genes, for most of which the function is unknown, are essential only under anaerobic conditions [158]. In addition, of 1300 genes that are essential for aerobic growth only 33 are not required for anaerobic growth. Interestingly, these genes are not regulated by oxygen [158]. Further, gene regulation under anaerobic and aerobic conditions also depends on other factors such as carbon or nitrogen source [159, 160]. Ter Linde and co-workers identified 369 genes which had highly different levels of expression in aerobic and anaerobic glucose-limited conditions [161]. The genes that had the greatest differences between the two conditions included those involved in respiration, oxygen toxicity and fatty acid oxidation, and also included many with unknown functions. Piper and co-workers also compared the aerobic and anaerobic transcriptome of *S. cerevisiae* under glucose limitation and found 877 transcripts to be differentially expressed [162]. Tai and co-workers found that only 155 of these genes responded consistently to anaerobiosis under four different macronutrient limitations [160]. These genes included those of transport, cell wall organisation, metabolism and energy and once again, 55 of them were of unknown function.

Lai and co-workers studied the transcriptome of yeast during transition from aerobic to anaerobic conditions in batch cultivations on galactose and glucose [163, 164]. On galactose, DNA replication and repair-, cell cycle-, rRNA processing and protein synthesis -related networks controlled by Fhl1p, MCB, SCB, PAC and RRPE transcription factor binding sites were transiently downregulated. At the same time, the Msn2/4p controlled networks related to import and utilisation of different carbon sources were transiently upregulated. These responses, which were not seen on glucose, are similar to the general stress response in *S.cerevisiae*. These responses were suggested to result from cessation of respiration, which is not as significant on glucose due to the already low ac-

tivity of respiration under conditions of glucose repression [163]. In accordance with this hypothesis, it was shown that treatment of galactose-grown cells with the respiratory chain inhibitor antimycin A leads to a similar transient transcriptomic response to that resulting from anoxia [165]. On both glucose and galactose, slower responses of Hap1p, Hap2/3/4/5p, Rox1p, Upc2p and Mot3p – regulated networks were observed [163, 164]. Lai et al. also studied the transcriptional response after re-oxygenation and found that this response was similar in both glucose and galactose and dominated by Yap1p-controlled networks related to oxidative stress and networks regulated by heme [164]. In a study of Kundaje by co-workers (2008), a machine learning algorithm was used to integrate information about the oxygen-related regulation in *S. cerevisiae*. The results indicated that the network of oxygen regulation is significantly different from the general stress response network [166].

On the proteome level, less is known concerning the effect of oxygen than on the transcriptional level. The proteome of *S. cerevisiae* has been compared in anaerobic and aerobic glucose-limited conditions and during growth on xylose [44, 167, 168]. Bruckmann and co-workers used 2D electrophoresis and reported differences mainly in cytoplasmic proteins involved in energy metabolism, c-compound and carbohydrate metabolism. Altogether 110 spots were identified, the levels of which differed more than twofold between aerobic and anaerobic conditions [168]. De Groot and co-workers used methods based on mass spectrometry and were able to quantify and identify 474 proteins. The results of the study of de Groot and co-workers indicated that glycolysis, amino-acyl-tRNA synthesis, purine nucleotide synthesis and amino acid biosynthesis are regulated on the post-transcriptional level [44]. The levels of proteins involved in translation and synthesis of precursor molecules for translation were on a higher level under anaerobic than aerobic conditions, even though the overall translation rate was equal under both conditions. The authors suggested that this could be due to an increased need for synthesis of glycolytic proteins, which represent a substantial percentage of cellular protein [44].

1.8 Glucose transport

Glucose and fructose are the preferred carbon and energy sources for *S. cerevisiae* [169]. Generally, Crabtree-positive yeasts such as *S. cerevisiae* utilise only facilitated-diffusion glucose-transport systems, whereas high-affinity proton-symport mechanisms with much lower Km values for glucose are common

in Crabtree-negative yeasts and are used by them under glucose-limited conditions [25, 170–172]. The facilitated-diffusion glucose-transport system of *S. cerevisiae* is encoded by 20 genes, of which 18 encode transporters (Hxt1p-Hxt17p, Gal2p) and two encode sensor proteins (Snf3p, Rgt2p) [173, 174]. The diversity of the transporters gives *S. cerevisiae* the ability to utilise efficiently different levels of glucose.

The transporters encoded by *HXT1* to *HXT4* and *HXT6* to *HXT7* are considered to be the major hexose transporters in *S. cerevisiae*. These transporters have been classified as high (Hxt6p, Hxt7p), moderately low (Hxt2p, Hxt4p) and low (Hxt1p, Hxt3p) affinity transporters. However, Hxt2p exhibits both high and low affinity transport kinetics in cells grown on low glucose concentration [175]. In addition, *HXT5* encodes a moderately low affinity transporter, the specific function of which is not known [176]. However, it is known to be regulated by growth rate, osmolarity, sporulation and glucose concentration [176, 177]. Depending on the strain background, deletion of either *HXT1-7* or of all 18 hexose transporters is needed to completely abolish growth on glucose [178, 179].

Extracellular glucose concentration sensed via the Snf3p and Rtg2p receptors affects the transcription of the major hexose transporters [180–183] (Figure 3). As a response to glucose, Snf3p and Rgt2p generate a signal that stimulates the degradation of Mth1p and Std1p via Grr1p-dependent degradation [184–186]. Mth1p and Std1p associate with Rgt1p which represses the *HXT* genes [187]. The release of repression of the *HXT* genes requires both the degradation of Mth1p and Std1p and phosphorylation of Rgt1p [180]. Rgt1p is phosphorylated by protein kinase A, which in turn is activated by the G-protein coupled receptor Gpr1p [188]. In addition, in high glucose concentrations, Rgt1p acts as an activator of the low affinity transporter Hxt1p [189]. Furthermore, the glucose repression pathway mediated by Snf1p-Mig1p acts by repressing the expression of genes encoding the moderately low affinity transporters *HXT2* and *HXT4* and genes encoding *MTH1* and *SNF3* [190].

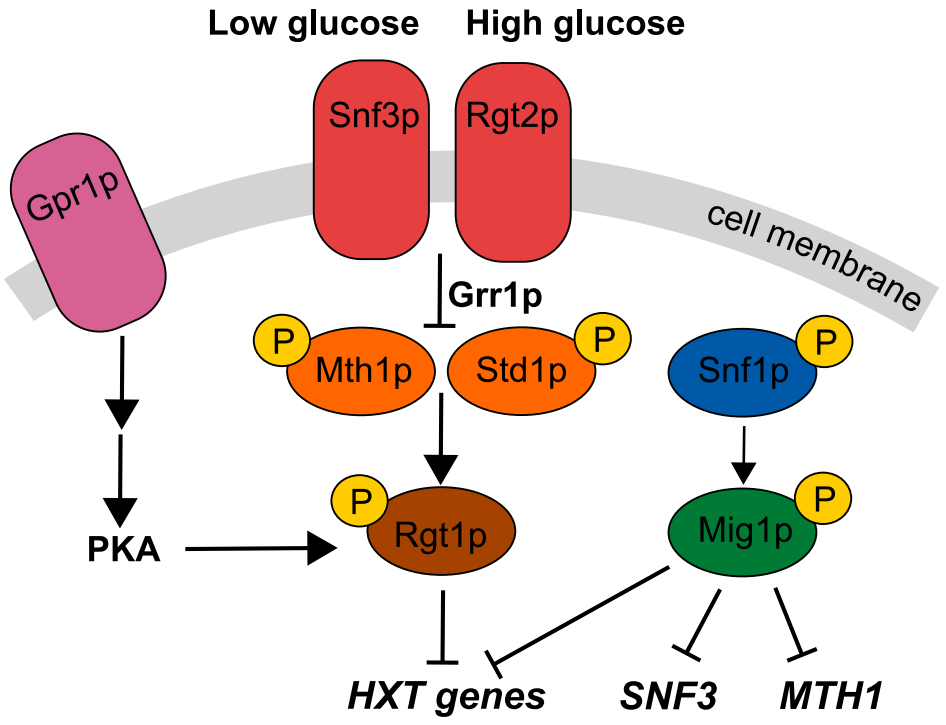

Figure 3. Regulation of genes encoding the major hexose transporters. Positive regulation is marked by a line with an arrowhead and negative regulation by a line with a bar at the end. The figure is adapted from Kim and Johnston 2006 [188].

HXT8-HXT17 encode transporters with mainly unknown functions. Although the expression of all but *HXT11* and *HXT12* is regulated by extracellular glucose concentration, the expression levels of all these genes is very low both under glucose limitation and glucose excess [183, 191]. Some of these transporters may function in the transport of other compounds than glucose. Transporters encoded by *HXT9* and *HXT11* are involved in drug resistance process [192], and Hxt9p and Hxt10p are able to transport arsenic trioxide into the cell [193]. In addition, *HXT17* contains a binding site for Mac1p transcription factor, which regulates copper-uptake genes under copper-deficient conditions [194, 195]. Further, *HXT13* and *HXT17* are induced on non-fermentable carbon sources and *HXT17* is upregulated in cells grown on medium containing galactose and raffinose at pH 7.7, but not at pH 4.7 [196].

1.9 Aims of the study

Oxygen has a major effect on cellular metabolism. The general aim of this study was to obtain further knowledge on the regulation of the metabolism of *S. cerevisiae* in regard to oxygen provision. This knowledge would greatly benefit the planning of new bioprocesses and the control of those already in use.

In the beginning of this work, most of the studies available had concentrated on comparing the fully aerobic and fully anaerobic growth of S. cerevisiae whereas less data existed on oxygen-limited conditions. Especially the genome-wide data and studies combining simultaneous measurements of different levels of metabolism were lacking. In addition, it was not known whether previous adaptation to oxygen-restricted conditions prepared the cells to sudden oxygen depletion, compared to cells grown under fully aerobic conditions.

Although glycolysis has a central role in the metabolism of S. cerevisiae, the control of this pathway is still not fully understood. One of the controlling mechanisms has been suggested to be the transport of hexoses into the cell. Oxygen greatly affects the glucose uptake rate and the flux through glycolysis. Thus this study aimed at determining out how the glucose transporters are regulated by the availability of oxygen.

The specific aims of this study were

1) to compare the physiology of *Saccharomyces cerevisiae* under conditions of different oxygen provision by using measurements of multiple levels of metabolism
2) to study the responses of transcriptome and intracellular metabolites to changes in oxygen availability
3) to analyse the effect of oxygen provision on the expression of genes belonging to the hexose transporter gene family.

2. Materials and methods

2.1 Summary of methods

Materials and methods are described in detail in the original articles I–IV. Methods are summarised in Table 1.

Table 1. Methods used in this study.

Method	**Study**
Biomass determination	I
Enzyme activity assays	III
Extracellular metabolite analysis	I
Intracellular metabolite extraction and analysis	I
Microarray analysis	III, IV
Protein identification	III
RNA extraction	III, IV
Sequencing	II
Steady state chemostat cultivations of *S.cerevisiae*	I, II, III, IV
Time-course chemostat cultivations of *S.cerevisiae*	I, II, III, IV
TRAC	I, II
2DE proteome analysis	III

2.2 Chemostat cultures

Chemostat cultures and analyses carried out in this study are summarised in Table 2.

Table 2. Chemostat cultivations carried out in studies I–IV and in unpublished studies and the analyses of these cultivations.

Cultivation	**Analyses**	**Study**
Steady state cultures receiving 0, 0.5, 1.0, 2.8 or 20.9% oxygen	Extracellular metabolite analysis Intracellular metabolite analysis Transcription analysis using TRAC Transcription analysis using microarrays Proteomics analysis using 2DE Enzyme activity measurements	I I I, II III III III
Time course analysis from aerobic (0.5, 1.0, 2.8 or 20.9% oxygen) to anaerobic conditions	Extracellular metabolite analysis Intracellular metabolite analysis Transcription analysis using TRAC Transcription analysis using microarrays	I I I, II IV
Time course analysis from anaerobic to aerobic (1.0 or 20.9% oxygen) conditions	Extracellular metabolite analysis Intracellular metabolite analysis Transcription analysis using TRAC	II, PYFF3, unpublished results

3. Results

3.1 Cultivations and physiology (I)

In order to study the effects of oxygen on *S. cerevisiae*, both steady state and time-course analyses were used. In the steady state setup, cells were grown in glucose-limited chemostats with 0, 0.5, 1.0, 2.8 or 20.9% oxygen provision in the incoming air. In the first time-course setup, oxygen feed was replaced with nitrogen in cultures which initially received 0.5, 1.0, 2.8 or 20.9% O_2. The cultures were followed until a new anaerobic steady state was achieved (cultures initially receiving 0.5, 1.0 and 20.9% O_2) or for 4 hours after anaerobicity was reached (cultures initially receiving 2.8% O_2). In the second time-course analysis, cultures initially in anaerobic steady state were given 1.0% or 20.9% O_2 and followed until a new steady state was achieved or until the cultures started to oscillate (yeast cells synchronised their cell cycle). Oscillations were observed in cultures provided with 20.9% O_2.

The biomass concentration (g L^{-1}) and the specific oxygen uptake rate (OUR) had very strong positive correlation (>0.9) with oxygen provision in the steady states receiving 0–2.8% O_2 (Table 3). In 20.9% O_2, the biomass concentration and OUR were only slightly higher compared to 2.8% O_2. The specific carbon dioxide evolution rate (CER), the specific glucose consumption rate and the specific ethanol production rate had high (>0.7) negative correlation to oxygen provision in 0–2.8% O_2. However, in 20.9% O_2 CER, specific glucose consumption rate and specific ethanol consumption rate were only slightly lower than in 2.8% O_2. Glycerol was produced only under anaerobic conditions. The main difference between 2.8% and 20.9% O_2 was that 20.9% O_2 sustained fully respiratory metabolism, as no ethanol was produced at this level of oxygen provision (I).

Table 3. Physiological parameters in glucose-limited chemostat cultivations of CEN.PK113-1A under provision of 0, 0.5, 1, 2.8 or 20.9% oxygen (I).

	0%	**0.5%**	**1.0%**	**2.8%**	**20.9%**
Oxygen solubility (μM)*	0	6	12	34	250
Biomass ($g\ L^{-1}$)	1.0 ± 0.02	2.1 ±0.02	3.0 ± 0.03	4.8 ± 0.05	5.0 ± 0.03
Yield (x/C) ($Cmol\ Cmol^{-1}$)	0.12 ± 0.03	0.27 ± 0.01	0.36 ± 0.03	0.56 ± 0.01	0.60 ± 0.01
Specific OUR [$mmol\ (g\ DW)^{-1}h^{-1}$]	0	1.2 ± 0.02	1.7 ± 0.02	2.5 ± 0.04	2.7 ± 0.04
Specific CER [$mmol\ (g\ DW)^{-1}h^{-1}$]	11.3 ± 0.30	4.6 ± 0.06	3.7 ± 0.04	3.0 ± 0.03	2.6 ± 0.03
Specific glucose consumption rate [$Cmol\ (g\ DW)^{-1}h^{-1}$]	37.1 ± 3.0	14.3 ± 1.1	11.4 ± 0.5	8.0 ± 0.3	6.6 ± 0.5
Specific ethanol production rate [$Cmol\ (g\ DW)^{-1}h^{-1}$]	16.7 ± 1.6	5.5 ± 0.5	3.2 ± 0.2	0.2 ± 0.01	0
Specific glycerol production rate [$Cmol\ (g\ DW)^{-1}h^{-1}$]	3.0 ± 0.3	ND**	ND	ND	ND

* Solubility of O_2 in pure water at 30°C

** Not determined

In the time-course experiments in which oxygen (0.5–20.9%) was replaced with nitrogen, the ethanol and glycerol concentrations started to increase within one hour of the switch (I, Figure. 3). Biomass concentration also started to decrease almost immediately, but the cells continued to grow at a rate of 0.06 h^{-1} during the washout and returned to 0.1 h^{-1} after approximately 15 hours. A new steady state was achieved in 36 hours (I).

When the initially anaerobic cultures were given 1.0 or 20.9% O_2, biomass accumulation and oxygen uptake started after two hours. Between 2 and 10 hours cultures receiving 1.0% and 20.9% O_2 had specific growth rates of 0.21 h^{-1} and 0.32 h^{-1}, respectively. After 10 hours, the growth rate of 0.1 h^{-1} was restored. In both cultures receiving 1.0 or 20.9% O_2, glycerol production stopped as soon as oxygen was present in the environment and glycerol was washed out of the culture at a rate of ~0.13 h-1. In cultures receiving 20.9% O_2, ethanol was washed out at the dilution rate for the first 2 to 3 h, and then at rates up to ~0.81 h-1 until all ethanol was removed. In cultures receiving 1.0% O_2, ethanol production con-

tinued after the shift at a slower rate than before the shift and ethanol was removed from these cultures at a rate of 0.04 h^{-1} [197], (II).

3.2 Intracellular metabolites (I)

The concentrations of metabolites of upper glycolysis (G6P, F6P, FBP) and the TCA cycle (citrate, succinate, fumarate, malate) were higher in the anaerobic than in the aerobic conditions as were the concentrations of pyruvate, 6-phosphogluconate (6PG), combined pentose phosphate pool and mannose 6-phosphate. Concentrations of the metabolites of lower glycolysis (2PG+3PG, PEP) and trehalose 6-phosphate (T6P) were lower under anaerobic than under aerobic conditions (I, Figure. 1).

When the aerobic (0.5–20.9% O_2) conditions were turned to anaerobic, the levels of metabolites started to change immediately (within 10 minutes) and mostly in the direction predicted on the basis of the steady state data. However, it took 30 hours before they had reached the new steady state level. Furthermore, the concentrations of most of the metabolites responded similarly independently of the initial oxygen concentration. Clear exceptions were T6P, which showed transient upregulation in 0.5–2.8% O_2 before the final downregulation, and 6PG, which showed transient downregulation in the 1.0% and 20.9% O_2 before the final upregulation. In addition, decrease in concentration of 6PG was observed already at 0.2 hours in the initially fully aerobic cultures, whereas in the initially oxygen-limited cultures the decrease was not seen until 3 hours (I, Figure 4 and Figure 5).

When oxygen (1.0 or 20.9%) was added to anaerobic cultures, the levels of TCA cycle intermediates and FBP decreased within 1 to 2 h, whereas the levels of metabolites of lower glycolysis increased. Generally, changes in the metabolite concentrations required more than 10 minutes. Similar changes were observed with 1.0% and with 20.9% O_2 [197].

3.3 Transcriptional analyses (I, II, III, IV)

3.3.1 Targeted analysis using TRAC (I,II)

3.3.1.1 Analysis of central carbon metabolism (I)

Transcription of 71 selected genes, mostly related to the central carbon metabolism, was measured with the TRAC method. 92% of these genes showed significant ($p<0.05$) differences in their expression between the fully aerobic (20.9% O_2) and anaerobic conditions, most of them having higher expression levels in aerobic than anaerobic conditions. Only *ADH1, COX5b, ACS1* and *PYC1* were more highly expressed under anaerobic than under aerobic steady state conditions. Expression of most of the genes related to glycolysis was on the same level in 0 to 1.0% O_2 whereas higher levels were observed in 2.8% than in lower oxygen levels. The expression of genes related to the TCA cycle showed higher levels already at 0.5% O_2 than under anaerobic conditions. Most of the genes related to the pentose phosphate pathway (PPP) showed higher expression levels in 2.8% O_2 than in the lower oxygen concentrations. In total, 50% of all the genes measured showed significant differences in expression between 2.8 and 20.9% O_2 (I, Figure1).

During the time-courses from aerobic (0.5–20.9% O_2) to anaerobic conditions, the expression levels of many genes did not change during the first hour or in some cases even during the first 8 hours. Many genes showed transient responses of both down- and upregulation. The duration of these responses was affected by the initial level of oxygen provision. Glycolytic genes generally showed no downregulation until 24 hours after the shift and some of them showed transient upregulation. The genes of the TCA cycle were downregulated already 2 to 3 hours after the shift. Most of the genes related to the PPP showed either transient of permanent downregulation, but transient upregulation was also observed. Genes related to ethanol consumption, respiration and some genes involved in acetate metabolism were consistently downregulated within 1 hour (I, Figure 6 and Figure7).

During the time-courses from anaerobic to aerobic (1.0 or 20.9% O_2) conditions, most glycolytic genes were transiently downregulated within 10 min after the shift. Most TCA cycle genes were upregulated after 2-3 hours. Provision of 1.0% O_2 had little effect on the genes of the PPP whereas GND1, ZFW1 and

TKL1 were upregulated as a response to provision of 20.9% oxygen. In general, the level of oxygen provision affected the transcriptional responses [197].

3.3.1.2 Analysis of hexose transporters (II)

The transcription of *HXT2, HXT4* and *HXT5*, encoding for moderately low affinity transporters was on a higher level in the fully aerobic (20.9% O_2) than in any of the intermediate oxygen or anaerobic conditions (II, Figure. 1). The transcription of *HXT6*, encoding for a high affinity transporter, and *HXT13* and *HXT15/16*, encoding for transporters with unknown functions, was on a higher level under the intermediate oxygen conditions compared to either the fully aerobic or anaerobic conditions. The expression of *HTX7*, encoding for a high affinity transporter with high similarity to the protein encoded by *HXT6*, reached its highest level in 2.8% and 20.9 % O_2. None of the *HXT* genes showed higher level of transcription in the anaerobic than under the fully aerobic conditions. Expression of *HXT9, HXT14* and *GAL2* was not detected under the conditions studied (II).

As a response to the change in oxygen provision (from 1.0 or 20.9% O_2 to anaerobic and vice versa), the transcription of most of the hexose transporters *HXT1* to *HXT7* changed either transiently or permanently. The permanent changes were in the direction predicted from the steady state analysis. The transient changes were affected by the level of aeration.

As a response to lack of oxygen in the cultures which were initially fully aerobic, *HXT1, HXT2, HXT4* and *HXT5* were downregulated, *HXT6* was permanently upregulated and *HXT3* and *HXT7* were transiently upregulated. While *HXT1-HXT4* and *HXT6-HXT7* responded in 0.2–1 hours, the transcription of *HXT5* remained unchanged for the first 3 hours. Lack of oxygen in the cultures which were initially oxygen-limited, led to responses of some of the *HXT* genes which were clearly different than those observed in the initially fully aerobic cultures. *HXT1, HXT4,* and *HXT7* were either transiently or permanently downregulated and *HXT3, HXT5* and *HXT6* were transiently upregulated before downregulation. In addition, *HXT2* was upregulated.

During transition from the anaerobic to the fully aerobic conditions, *HXT3, HXT6* and *HXT7* were downregulated and *HXT2* and *HXT4* were upregulated either transiently or permanently. *HXT5* was downregulated before final upregulation. The responses to oxygen provision were similar, but not exactly the same when limited oxygen was provided, compared to 20.9% oxygen provision. When limited oxygen was provided to the anaerobically grown cells, *HXT2* was per-

manently and *HXT6* transiently downregulated. *HXT4* was transiently and *HXT5* permanently upregulated.

In general, the responses of the transporter genes *HXT8* to *HXT17* were weaker than the responses of *HXT1* to *HXT7*. The highest responses were observed for *HXT13* and *HXT15/16* when the oxygen provision was changed from oxygen-limited to anaerobic and vice versa. As a response to lack of oxygen in oxygen-limited conditions, *HXT13* and *HXT15/16* were downregulated and as a response to limited oxygen under anaerobic conditions, these genes were upregulated. During the transitions between fully aerobic and anaerobic conditions the expression of *HXT13* and *HXT15/16* did not change (II, Figure 2 and Figure 3).

3.3.2 Global analysis using microarrays (III, IV)

3.3.2.1 Different levels of oxygen provision (III)

The level of oxygen provision, not only the presence and absence of oxygen, affected a significant part of the transcriptome of *S. cerevisiae*. The expression of 3435 genes had significant ($p<0.01$) differences under five steady state conditions studied (0, 0.5, 1.0, 2.8 and 20.9% O_2). However, the expression level of only a few genes correlated strictly with oxygen concentration in the feed gas (III). The main differences in the transcriptome were observed between the fully aerobic, intermediate oxygen and anaerobic conditions. Especially the levels of 0.5 and 1.0% oxygen were very similar to each other: only 10 genes were found to have significant ($p<0.01$) differences in their expression levels between these two conditions (III, Figure 1 and Table 1).

Analysis of gene expression data with fuzzy c-means clustering resulted in 22 clusters with different expression profiles (III, Figure 2). The promoters and 3' untranslated regions (3'UTRs) of the genes in these clusters were analysed for the most informative regulatory motifs. 17 transcription factor binding sites and 7 3'UTR motifs, of which some had significant co-occurrence and/or co-localisation patterns, were identified (III, Figure 3) In addition, GO categories and KEGG-pathways over-represented in these clusters were analysed (III, Table S1).

Under conditions of intermediate oxygen availability (0.5–2.8 % O_2), the genes related to oxidative phosphorylation, TCA cycle and metal ion homeostasis were more highly expressed than under either aerobic or anaerobic conditions. These genes included nearly all the genes (34 out of 37) encoding the nu-

clearly-encoded subunits of the respiratory chain complexes and all but three of the genes encoding for the main enzymes of the TCA cycle. Of the genes encoding the main enzymes of the TCA cycle, *FUM1*, *LSC1* and *LSC2* had their highest expression under fully aerobic conditions. The promoters of genes of the respiratory pathway and the TCA cycle were enriched in binding sites for Hap2/3/4/5p transcription factor and for two previously undescribed 3'UTR elements. In addition, many respiratory enzymes contain metals and accordingly, 9 out of 16 genes known to be involved in transport of iron from the extracellular medium to the cytosol had higher expression levels in 2.8% than 20.9% O_2.

In addition to the respiratory pathways, several genes related to the MAPK signalling pathway of mating and filamentous growth had their highest levels of expression under the intermediate oxygen conditions. The genes encoding transcription factors Ste12p and Tec1p, that are activated by these MAPK pathways and control the expression of genes needed in mating and filamentous growth [198, 199], also showed the same behaviour.

In contrast to the respiratory pathways, most genes related to the mitochondrial protein synthesis and import were present at higher levels under all oxygen-limited and anaerobic conditions, compared to the fully aerobic conditions. These genes were enriched for Puf3p 3'UTR motif.

Lipid metabolism was highly affected by the oxygen level provided. Genes encoding activities of fatty acid β-oxidation and genes related to peroxisomal biogenesis had their highest levels of expression under the fully aerobic conditions and similar, lower levels of expression under all three intermediate oxygen levels. In the anaerobic conditions, the expression levels of these genes were similar to or even lower than under the intermediate oxygen conditions. The genes encoding known regulatory elements of these genes, namely *PIP2* and *OAF1* [200] were also found to be similarly expressed. The genes related to sterol synthesis and uptake had either the lowest level of expression in the intermediate oxygen or were transcribed at a lower level under all oxygen-containing conditions, compared to the anaerobic conditions. In the promoters of these genes two putative transcription factor binding sites with strong positive co-occurrence were over-represented. One of these motifs corresponded to AR1 and SRE motifs which are known to function in the regulation of genes of ergosterol biosynthesis [112, 201].

Stress-related effects were also seen in the data. Binding sites of several stress-related transcription factors (Msn2p/Msn4p, Gis1p and Xbp1p) [202–204] were identified in the promoter analysis of clustering results. Binding sites for the transcription factors Msn2p, Msn4p, Gis1p were over-represented among

genes in two clusters which were enriched in genes belonging to the GO category of response to stress. In one of these clusters, the expression level of genes was on similar level in 0 to 1.0% O_2, on the lowest level in 2.8% O_2 and the highest level under fully aerobic conditions. The genes in the second cluster had their lowest levels of expression under the anaerobic conditions, similar, intermediate level of expression under the intermediate oxygen conditions and the highest level of expression under the fully aerobic conditions. In the second cluster binding sites for Ume6p and two unknown sites were also over-represented. In addition, the gene encoding Xbp1 was a member of this cluster and the binding site of Xbp1p was under-represented in the promoters of genes of this cluster. Furthermore, the binding site for Xbp1 was over-represented in two clusters, the expression profiles of which negatively correlated with the expression level of *XBP1*. The four core bases of binding site of Xbp1 were found in the promoters of approximately 70% of the genes in these clusters. Many of these genes were related to cell division and cell wall organisation.

Of the genes of the central carbon metabolism, major changes in the expression of genes of the PPP were observed. The expression of genes encoding the minor isoforms of enzymes of the PPP had their highest level of expression under the fully aerobic conditions, lower level of expression in the intermediate oxygen and lowest expression under the anaerobic conditions. The expression of genes encoding the major isoforms of the PPP enzymes was not significantly affected by oxygen concentration.

3.3.2.2 Change in oxygen provision (IV)

In order to study the dynamics of transcriptional regulation by oxygen, time-course analysis was performed. Steady state cultures, which were initially fully aerobic (20.9% O_2) or oxygen-limited (1.0% O_2), were switched to anaerobicity and followed until a new steady state was obtained. Whereas the transcriptional response to oxygen depletion was faster in the initially oxygen-limited than in the fully aerobic cultures (IV, Figure 1), the overall patterns of gene expression were very similar. 1169 genes responding to lack of oxygen showed a correlation of >0.9 in their expression profiles (IV).

The multidimensional reporter features algorithm was used to analyse the transcriptional responses in the context of the network of all known interactions between transcription factors and other regulatory proteins and genes [205]. The analysis identifies the features of which the surrounding genes have had highly

correlated expression in the time series. The regulators shared by and specific for the two initial conditions are summarised in Table 4.

Analysis of gene expression data with fuzzy c-means clustering resulted in 24 and 22 clusters with different expression profiles in the initially fully aerobic and oxygen-limited cultures, respectively (IV, Figures 2 and 3). The promoters and 3' untranslated regions (3'UTRs) of the genes in these clusters were analysed for the most informative regulatory motifs. In the initially fully aerobic cultures, 8 transcription factor binding sites and 4 3'UTR motifs were identified (IV, Figure S1). In the initially fully aerobic cultures, 14 transcription factor binding sites and 8 3'UTR motifs were identified (IV, Figure S2). In addition, GO categories and KEGG-pathways over-represented in these clusters were analysed (IV, Tables S2 and S3).

Table 4. Reporter features identified when initially fully aerobic (20.9% O_2) or oxygen-limited (1.0% O_2) cultures were switched to anaerobicity.

20.9% and 1.0% O_2	**specific for 20.9% O_2**	**specific for 1.0% O_2**
Growth BAS1, RAP1, IFH1, RSC30, ESA1, GTS1 **Protein degradation** RPT6, SNF7 **Stress** MSN2, MSN4, HSF1, HOG1 **Fatty acid β-oxidation** OAF1, PIP2 **Sterol biosynthesis** UPC2 **Carbon-source regulation** ADR1 **Vesicle trafficking** SLY1 **Methionine biosynthesis** MET1	**Growth** LYS14, AMA1 **Protein degradation** RPT4 **Stress and nutrient limitation** TPK2, RAS2, HAA1 **Metabolic kinases** SNF1, SNF4 **Glycolysis** GCR1 **Response to copper ion** CUP2 **Unknown function** RIM9	**Growth** SFP1, MBP1 **Protein degradation** RPN4 **Heme** HAP2/3/4/5, HAP1 **Methionine biosynthesis** MET4 **Arginine transport** YHC3

Both culture conditions responded to the lack of oxygen by transient downregulation of genes related to growth and cell proliferation (amino acid and purine metabolism, ribosomal biogenesis, RNA processing, biogenesis of RNA polymerases and genes related to cell cycle and DNA replication and repair). Some

of the clusters containing these genes showed more rapid responses in the oxygen-limited cultures, but mostly the gene expression patterns were similar under the two conditions studied. Under both conditions, PAC motif and binding sites of transcription factors Rap1p and Xbp1p were over-represented in the promoters of the genes belonging to these clusters. Under the fully aerobic conditions, two putative 3'UTR motifs were over-represented. One of these motifs was also identified in a shorter form under the oxygen-limited conditions. Additionally under the oxygen-limited conditions, RRPE motif, binding sites of transcription factors Bas1p and Swi4p, and 3'UTR motifs for binding of Puf4p and Puf5p were over-represented.

Specifically, the genes related to biosynthesis of the amino acid methionine and to sulphate assimilation were rapidly and transiently downregulated in both the fully aerobic and the oxygen-limited cultures. In the fully aerobic cultures, the response of these genes was over after 1 h whereas in the oxygen-limited cultures the recovery was complete only after 8 hours.

Transient downregulation was also seen in the transcription of genes related to mitochondrial translation and protein targeting to mitochondria in both culture conditions studied. In the fully aerobic cultures, the expression of these genes recovered to a higher level than in the initial steady state. Under both conditions, 3'UTR motif for binding of Puf3p was over-represented in the clusters containing these genes.

Transient upregulation of genes related to protein degradation mechanisms was observed in both the fully aerobic and the oxygen-limited cultures. In the oxygen-limited cultures, binding sites of Msn2p/Msn4p, Gis1p, Rpn4p and one putative transcription factor binding site were enriched among genes related to protein degradation. Furthermore, genes related to reserve energy metabolism (storage and degradation of trehalose and glycogen) were transiently upregulated in both cultures. In the oxygen-limited cultures, binding sites of Msn2p/Msn4p, Gis1p and Ume6p, a putative transcription factor binding site and a putative 3'UTR motif were enriched among these genes.

Under both culture conditions, genes related to fatty acid oxidation, peroxisomal biogenesis and response to oxidative stress showed downregulation towards the anaerobic steady state. In the initially oxygen-limited cultures binding site of Ume6p, one putative transcription factor binding site and two putative 3'UTR motifs were over-represented in the cluster containing these genes. Furthermore, in the initially oxygen-limited cultures, some genes related to fatty acid oxidation and peroxisomal biogenesis, together with genes related to re-

sponse to oxidative stress, genes of oxidative phosphorylation, TCA cycle and pentose phosphate pathway were transiently upregulated before the final down-regulation.

Genes related to transport of different compounds responded to lack of oxygen in both the initially fully aerobic and oxygen-limited cultures. Genes related to sterol and iron transport and cell wall biogenesis were upregulated towards steady state. In the initially oxygen-limited cultures the upregulation did not start until after 3 hours whereas in the fully aerobic cultures, a response was seen already at 0.2 hours. Under both culture conditions, genes encoding phosphate transporters were transiently upregulated as a response to lack of oxygen. Furthermore, many genes related to uptake of amino acids and other nitrogen containing metabolites responded by either transient or permanent upregulation during the adaptation to anaerobic conditions in both cultures (IV, Figure 4).

Additionally, the redox cofactor NADH was identified as a Reporter Metabolite after 24 h, when the anaerobic steady state was established, independent of the initial metabolic state, but in the initially fully aerobic cultures NADH was identified as a Reporter also in the earlier phase of adaptation, between 1 and 3 h. In the initially oxygen-limited cultures, the cofactor NADPH was identified as a Reporter Metabolite between 1 and 3 h after the switch to anaerobic conditions.

The responses of the genes related to central carbon metabolism were seen in the clustering analysis and were further studied by the Reporter metabolite analysis. If genes encoding the enzymes producing and/or consuming a metabolite have significantly differential gene expression between different time points, the metabolite is defined as a reporter by the Reporter metabolite algorithm [206]. The Reporter metabolite analysis revealed that the temporally differential expression of the genes encoding the enzymes of central carbon metabolism as a response to oxygen depletion was dependent on the initial metabolic state of the culture (IV, Figure 7). The metabolites of the pentose phosphate pathway and the upper glycolysis were identified as reporters between 0 and 0.2 hours in the initially oxygen-limited cultures whereas in the initially fully aerobic culture metabolites of the pentose phosphate pathway and glyoxylate cycle were identified as reporters between 0.2 and 1 hours. In the initially fully aerobic cultures, the metabolites of the TCA cycle and cofactor NADH responded after 1 and 3 hours. Additionally, NADH was identified as a reporter between 1 and 3 hours and between 24 and 79 hours. In the initially oxygen-limited cultures, NADPH was identified between 1 and 3 hours. In the clustering analysis, it was observed that in the initially oxygen-limited cultures, genes of NADPH regeneration and

the pentose phosphate pathway were either transiently downregulated (TAL1, TKL1, SOL3, RKI1, GND1) or showed transient upregulation before final downregulation (SOL4, GND2, TKL2), whereas in the initially fully aerobic cultures, these genes were transiently (TAL1, TKL1, RKI1, GND1, ADH6, PYC2) or permanently (SOL4, GND2, TKL2) downregulated.

3.3.3 Comparison of TRAC and microarray analyses (I, III, IV)

The gene expression levels of selected genes related to central carbon metabolism were measured with the TRAC and Affymetrix methods (I, III, IV) and the results of these analyses were compared for the steady state data (III) and for the data from initially fully aerobic time-course analysis (data not published). For the initially oxygen-limited cultivation, the comparison was not performed since separate cultivations had been performed for the TRAC and Affymetrix analysis and the sampling points were not exactly the same. In addition, the expression levels of *HXT* genes were measured with both these methods (II, III, IV), but the Affymetrix measurements were not reliable for all these genes because of high sequence homologies in the coding regions of the genes. The probes used in the TRAC analysis were manually designed so that these homologies were taken into account (II).

In the steady state data 61 of the 71 selected genes related to central carbon metabolism showed statistically significant differences in their expression levels with both the Affymetrix ($p<0.01$) (III) and the TRAC ($p<0.05$) (I) methods. Most of the genes (16) that showed >3-fold differences in their expression in the different oxygen levels also showed a high average correlation of 0.8 between the TRAC and the Affymetrix analyses. The genes (13) that showed 2- to 3-fold difference in their expression had a good average correlation of 0.6. Finally, the genes (24) that showed <2-fold difference in their expression had a low average correlation of 0.2. However, five of these genes also had a good correlation of >0.7. The genes that showed ≥2-fold differences in their expression levels and had low correlation between the TRAC and the Affymetrix data were *GPD2*, *CIT2*, *ACS1*, *HAP1*, *MAE1* and *PCK1*, the signals of the three latter genes being very close to the detection limit using TRAC (III).

In the time-course data of the cultivations in which fully aerobic conditions were turned anaerobic, 48 of 67 selected genes had significant changes in their expression levels. 41 of the 48 genes showed good correlation of >0.6 between the two methods. Of the 7 genes that showed correlation <0.6, 5 showed <2-fold

differences in their expression levels. The two showing >2-fold differences in their expression and low correlation between the TRAC and the Affymetrix data were *CIT2* and *CIT3*.

3.3.4 Comparison of transcriptional, proteomics and enzyme activity analyses in different oxygen concentrations (III)

2D gel analysis of cells cultivated in five different levels of oxygen provision resulted in a proteome dataset of 484 protein spots. Of the 484 spots, the intensities of 145 differed significantly ($p < 0.01$) when the cells were provided different levels of oxygen. In all the levels of oxygen provision studied, the Pearson's correlations between proteins identified in the 2D gels and the mRNA levels of the corresponding genes in the transcriptome were similar, with r-values between 0.41 and 0.55 (III). For a more detailed comparison, the 107 protein spots from the 2D gels and the corresponding transcripts that showed significant differences between the different oxygen levels were hierarchically clustered (III, Figure 5). The clustering analysis revealed that for many protein and transcript pairs, correlation of expression levels was high in 0, 1, 2.8 and 20.9% O_2 and low in 0.5% O_2.

Enzymes of the TCA cycle and proteins involved in respiration showed either a slight increase in quantity (1.5- to 2-fold) under the intermediate oxygen conditions (0.5–2.8% O_2) compared to the fully aerobic and the anaerobic conditions, or a strong increase (3 to 64-fold) under the fully aerobic conditions, or did not differ in the different levels of oxygen provision. Activities of the enzymes of the TCA cycle could not be measured directly, but the combined activities of all isoforms of the enzymes citrate synthase (CS), aconitase (ACO), isocitrate dehydrogenase (IDH) and malate dehydrogenase (MDH) were analysed from crude cell extracts (III, Figure 4). All these enzymes showed highest activities under the intermediate oxygen conditions and strongly correlated (correlation >0.89) with the transcriptome data for the corresponding genes of the TCA cycle (*CIT1*, *ACO1*, *IDH1,2* and *MDH1*, respectively). In the proteome analysis, only Idh2p and Aco1p were identified: Idh2p showed increase in intermediate oxygen whereas Aco1p did not change.

Of the enzymes of the pentose phosphate pathway, Rki1p (ribose 5-phosphate Ketol-Isomerase) and Tkl2p (transketolase 2) were identified in the proteome analysis (III, Table S1). These showed correlations of 0.86 and 0.78 to the corresponding gene expression levels, respectively. The enzyme activities of glucose

6-phosphate dehydrogenase (G6DPH) and 6-phosphogluconate dehydrogenase (6PGDH) and the combined activities of isoforms of transketolase (TKL) and transaldolase (TAL) were measured (III, Figure 4). The activity of G6PDH showed a correlation of 0.7 to *ZWF1*. The activity of 6PGDH showed correlations of 0.6 and 0.3 with *GND1* and *GND2*, respectively. The activities of TKL and TAL had a correlation of 0.5 with *TKL1* and *TAL1*, respectively, and no correlation to *TKL2* and ORF YGR043C, respectively.

Of the proteins involved in glucose fermentation many were found as multiple pI isoforms which differed in relative quantities in different oxygen levels. These included Adh1p (3 pI isoforms), Adh2p (3), Ald4p (2), Ald6p (2), Eno1p (6), Eno2p (4), Gpm1p (3), Fba1p (2) and Hxk1p (2).

4. Discussion

4.1 Physiological responses to oxygen

The effect of oxygen on the physiology of *S. cerevisiae* was studied in highly controlled glucose-limited chemostat cultivations. Under steady state conditions of five different levels of oxygen provision (0, 0.5, 1.0, 2.8 and 20.9%), data of extra- and intracellular metabolites, expression of genes, levels of proteins and levels of enzyme activities were obtained. The data obtained from the central carbon metabolism are summarised in Figure 4 for glucose transport and fermentation and in Figure 5 for the PPP and TCA cycle. In addition, the fluxes through central carbon metabolism have been measured under these conditions and published separately [207].

The provision of 20.9% or 2.8% O_2 led only to small differences in the biomass concentration and the specific glucose and oxygen consumption rates. A clear difference was that provision of 2.8% O_2 led to respiro-fermentative growth, whereas provision of 20.9% O_2 supported purely respiratory growth. However, only minor differences were observed in the metabolic flux distribution between 20.9% and 2.8% O_2, respiratory pathways also carrying the most of the carbon flux in 2.8 % O_2 [207]. Interestingly, whereas the flux distribution was similar in 20.9% and 2.8% O_2, the transcriptional profiles under these two culture conditions were clearly different. On the other hand, the provision of 0.5, 1.0 and 2.8 % of oxygen led only to small differences on the transcriptome level, but the measurements of flux distribution under these three conditions revealed distinct modes respiro-fermentative metabolism [207]. In addition, on the proteome level differences were observed between 0.5% and 1.0% O_2, suggesting that post-transcriptional regulation mechanisms were responsible for the different physiological modes.

After a switch from aerobic (0.5, 1.0, 2.8 or 20.9% O_2) to anaerobic conditions, considerable time (four to five generations) was needed for the cells to reach a

new steady state regardless of the initial oxygen concentration provided. Thus, the provision of a low amount of oxygen does not prepare yeast for full anaerobicity. In a previous study an even longer time (>10 generations) was required for the new transcriptional steady state to be obtained after a shift from galactose to glucose [208]. Interestingly, S. cerevisiae requires more time to adapt to new conditions than the filamentous fungus Trichoderma reesei, which needs less than one generation to achieve a new transcriptional steady state [209].

4.2 Fermentative pathways, glucose transport and reserve carbohydrate metabolism

In the steady state analysis, glycolytic genes were found to be largely unaffected (Affymetrix) or on a slightly higher level under the aerobic than the anaerobic conditions (TRAC), although the levels of glycolytic metabolites showed clear differences between the conditions of different oxygen provision. However, on the proteome level differences were seen. For many glycolytic proteins, different isoforms were observed which showed differences in their levels in different oxygen concentrations. These results are in accordance with earlier studies which have shown that the regulation of glycolysis occurs mostly on the post-transcriptional level [43, 44]. The time-course analyses also supported this observation, as the responses of the glycolytic metabolites and genes did not correlate with each other. Although glycolysis has been extensively studied, the exact mechanism of its control is not known. Most probably, the control is distributed over a number of steps [33, 210–212, 212]. One of the controlling mechanisms of glycolysis has been suggested to be transport of glucose into the cell [39, 213]. Under conditions of restricted respiration, the carbon flux through glycolysis is increased [50, 207, 214–216] and the specific glucose consumption rate is inversely related to oxygen provided to the system. Interestingly, in the current study, the expression levels of the genes encoding hexose transporters were not positively correlated with the glucose uptake rate. Instead, the expression levels of the genes encoding moderately low affinity transporters (*HXT2, HXT4* and *HXT5*) were low when the specific glucose consumption rate was high. It was thus concluded that the relative increase in the high affinity compared to low affinity transport was sufficient to allow for the higher specific glucose consumption rate.

During the adaptation to anaerobic conditions a reporter metabolites of upper glycolysis were identified only in the cultures that were initially oxygen-limited. As upper glycolysis is the entry point of the storage carbohydrates, this response

may be related to the regulation of genes associated with the storage and mobilisation of trehalose and glycogen. In fact, the concentration of T6P, an intermediate in trehalose synthesis and one of the regulators of glycolysis [217], was dependent on the initial level of oxygen provision. However, in the clustering analysis a transient upregulation of genes of reserve carbohydrate metabolism was observed in both the initially fully aerobic and in the initially oxygen-limited cultures. The simultaneous upregulation of genes encoding both the enzymes needed in the mobilisation and storage of reserve carbohydrates has previously been observed as a response to stress and has been suggested to be involved in maintaining a constant glucose concentration inside the cell [218, 219].

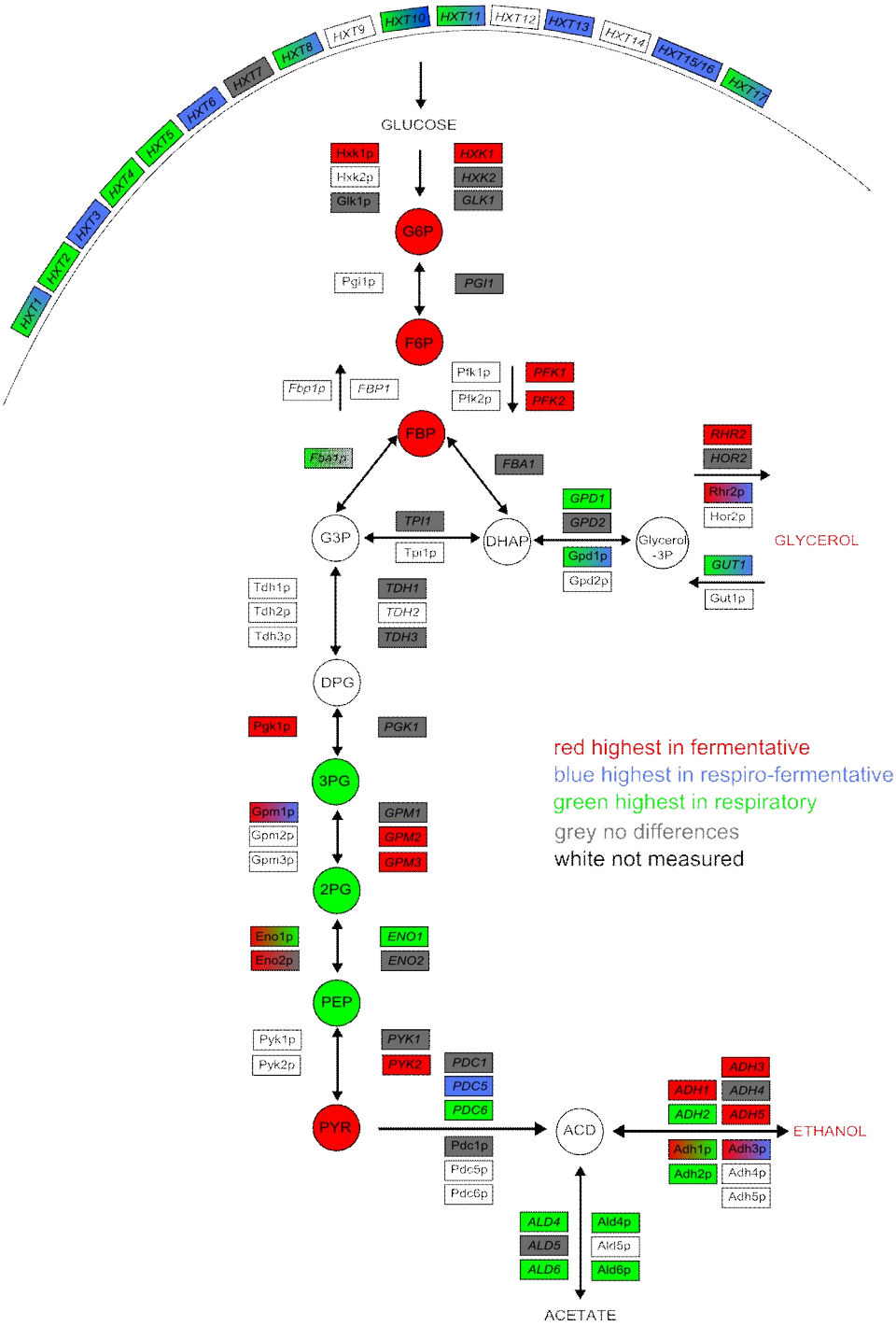

Figure 4. Relative levels of intra- and extracellular metabolites, gene expression and protein expression in the fermentative pathway. The gene expression data is derived from Affymetrix measurements except for *Hxt* genes, the data of which is derived from TRAC measurements.

4.3 The respiratory pathway and the pentose phosphate pathway

Under the steady state conditions of intermediate oxygen provision, higher levels of the genes related to the TCA cycle and respiratory pathways was observed compared to the fully aerobic or anaerobic conditions. The effect of this regulation was also seen on the proteome level as higher concentrations of some of the proteins of the TCA cycle and respiratory chain and in addition as higher activities of the enzymes of the TCA cycle. Furthermore, many genes related to transport of iron and zinc had their highest level of expression under the conditions of intermediate oxygen, reflecting the high demand of respiratory enzymes for metal ions. Of the respiratory genes Hap3/4/5p complex and two putative 3'UTR motifs were enriched. The Hap3/4/5p is suggested to play a role in the activation of respiration during growth rates above 0.08 h^{-1} to allow for higher respiratory capacity [48, 123]. The findings of the current study suggest that under the conditions of restricted oxygen, but not in the complete absence of oxygen the cells also try to enhance the respiration by upregulation of respiratory genes. Under these conditions the upregulation is not sufficient to enable fully respiratory growth. However, it is possible that the regulation is needed to sustain respiratory energy metabolism, which has been observed still to account for 25 % of ATP generation at 0.5 % O_2 [207]. Further, similar to the results of the current study, maximal amounts of cytochromes (5% O_2) and maximal activity of cytochrome *c* oxidase (10% O_2) were observed in lower oxygen provision than that which supported fully respiratory metabolism (26%) [83, 149].

In contrast to the expression of genes encoding the enzymes of the TCA cycle, the intracellular levels of the TCA cycle acids were highest under the anaerobic conditions. This in accordance with earlier studies measuring intracellular metabolites under anaerobic and aerobic conditions [220]. The extracellular concentrations of these acids are high also in anaerobic batch cultures on glucose, which is thought to be due to TCA cycle functioning as two branches under anaerobic conditions to provide biosynthetic precursors for amino acids [97, 98].

During adaptation of fully aerobic cultures to anaerobic conditions, the genes encoding the enzymes of the TCA cycle and the respiratory pathway were downregulated. However, during adaptation of the oxygen-limited cultures to the anaerobic conditions, some of these genes were transiently upregulated. Transient upregulation of the genes of oxidative phosphorylation and the TCA cycle has previously been reported during adaptation to anaerobic conditions in batch cultures on galactose, but not on glucose, suggesting that the response is linked to

termination of respiration [163, 164]. It is thus interesting that the response was not observed in the fully aerobic cultures.

The genes related to oxygen-demanding processes of fatty acid oxidation and peroxisomal biogenesis were also transiently upregulated during the adaptation of oxygen-limited cultures to anaerobic conditions. It has previously been observed that the genes related to peroxisomal activities and anaplerotic reactions are upregulated in respiratory-deficient yeast cells as a response to the loss of oxidative phosphorylation, in order to increase supplies of acetyl-CoA and OAA [221].

The PPP provides precursors and reducing power for biosynthesis, but it is also important in the protection against oxidative stress [222, 223]. In the steady state analysis, expression of the genes encoding the main isoforms of the enzymes of the PPP, and the combined activities of major and minor isoenzymes of PPP were mostly unaffected by provision of oxygen, or smaller than twofold differences were seen with exception of TAL1 in TRAC analysis. The specific flux through the oxidative part of PPP also remains constant under the conditions studied [207]. In addition, as Yap1p-regulated pathways specific to oxidative stress were not identified in either the reporter features analysis or the promoter analysis of clustered gene expression, it appears that the oxygen concentration provided was not too high for the cells even under the fully aerobic conditions. In the time-course analysis, the genes encoding the major isoforms of enzymes of the PPP were transiently downregulated during the adaptation to anaerobic conditions. This transient downregulation was possibly due to the transient decrease in the growth rate and thus decrease in the need for the biosynthetic precursors.

In contrast to major isoforms, the expression of the genes encoding the minor isoforms of the enzymes of PPP was transiently upregulated during the adaptation of oxygen-limited cultures to anaerobic conditions. Interestingly, a difference between the conditions was also observed on the metabolite level. The transient decrease in the concentration of 6PG was observed already after 0.2 hours in the initially fully aerobic cultures whereas in the initially oxygen-limited cultures the decrease was not observed until 3 hours. Further, the expression of the genes encoding the minor isoforms of the PPP enzymes was strongly affected by provision of oxygen in the steady state cultures. The physiological role of the minor isoenzymes is not known. Under the steady conditions, the expression of genes correlated to the physiological state, being highest under purely respiratory conditions, lowest in fermentative conditions and on an intermediate level under respiro-fermentative conditions. This may suggest that they are beneficial under conditions of high respiration, which hypothesis is supported by previous findings that they are induced after diauxic shift [46].

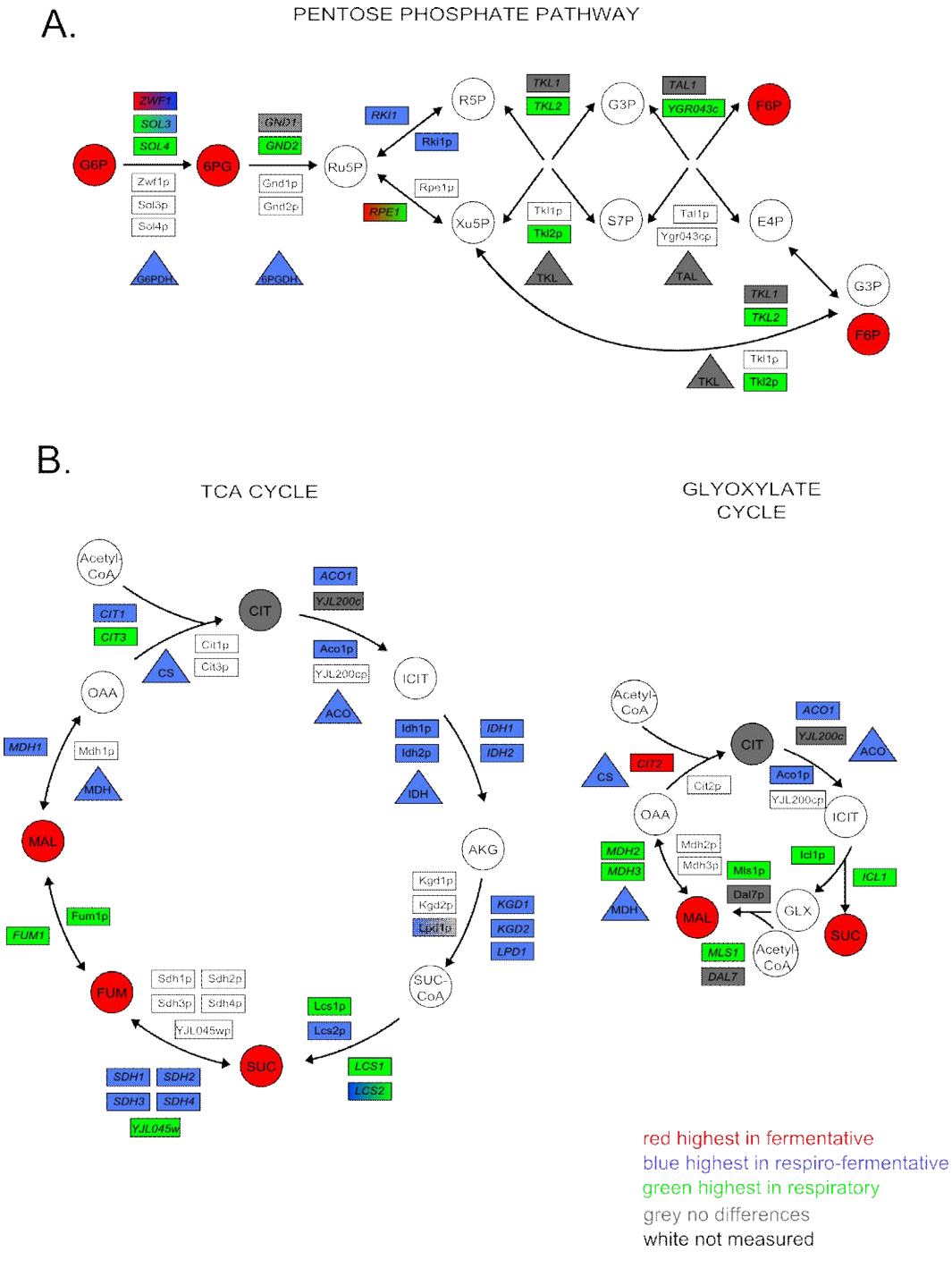

Figure 5. Relative levels of intracellular metabolites, gene expression, protein expression and enzyme activities in A. pentose phosphate pathway and B. TCA cycle and glyoxylate cycle. The gene expression data is derived from Affymetrix measurements.

4.4 Growth, protein degradation and stress

In the time-course analysis, a transient downregulation of genes related to growth and cell proliferation was observed. Especially, downregulation of both cytosolic and mitochondrial translation machineries was observed, but genes related to cell cycle, amino acid and purine biosynthesis and DNA replication and repair were also downregulated. Simultaneously to downregulation of growth-related genes, the genes encoding the protein degradation mechanisms were transiently upregulated. These changes are shared with the phenomenon of environmental stress response, a common response of certain patterns of genes to a variety of stressful situations [64, 155]. It has been suggested that at least part of this response, which also includes other processes such as reserve energy metabolism, carbohydrate metabolism and oxidative stress defence, is actually a response to changes in the growth rate [224]. Especially for the genes encoding ribosomal proteins, it has been shown that in steady state chemostat cultures, their expression is positively correlated with the specific growth-rate [225, 226]. However, it has also been suggested that under dynamic conditions their responses are regulated by the external environment rather than by the specific growth rate [227]. This is supported by the findings that the gene expression responds faster than the growth rate and also that no correlation between growth rate and ribosomal gene expression has been observed during recovery from environmental perturbations [227]. In the current study, a recovery to the original level in the expression of the ribosomal genes was also observed within 3 to 8 h whereas the specific growth rate was below 0.1 h^{-1} for approximately 15 h.

Among the genes related to growth and cell profiliferation, a set of transcription factor binding sites (PAC, RRPE, RAP1, XBP1, BAS1, SWI4) was identified which may be involved in the regulation of genes. However, the rapid (10 min) downregulation seen in particular in the expression of genes encoding the translational machineries indicates active degradation mediated by 3'UTR elements. It has been shown that in the case of heat and osmotic stress, the decay of mRNA plays an important role [228–230]. In our dataset, two putative 3'UTR motifs were identified in the fully aerobic cultures, whereas in the oxygen-limited cultures PUF4, PUF5 and a putative motif were identified. PUF4 motif is known to be involved in the decay of the mRNAs of genes related to rRNA synthesis and processing and ribosomal biogenesis [228] whereas both PUF4 and PUF5 are associated with mRNAs encoding nuclear components [231].

The genes induced in the environmental stress response are mostly regulated by Msn2p and Msn4p transcription factors [64, 155], and these were also identi-

fied in the analysis of the current data. However, a pattern of other known and putative transcription factors was also identified. Furthermore, stress-related responses mediated by Msn2p/Msn4p, Gis1p, Ume6p and Xbp1 were also observed in the steady state data. In general, the fully respiratory conditions appeared to be the most stressful for the cells.

4.5 Transport of sterols, phosphate and nitrogen-containing compounds

Under the anaerobic conditions, the cell wall and cell membrane of *S. cerevisiae* is modified to enable the uptake of substances requiring oxygen for their biosynthesis. In accordance with earlier studies [110, 112, 232], the genes of the *DAN* and *TIR* families encoding cell wall mannoproteins and the regulators of sterol biosynthesis and uptake (*UPC2, ECM22*) were on their highest level under the fully anaerobic conditions and on a lower level under all oxygen-containing conditions. The genes encoding the enzymes of ergosterol biosynthesis were also on their highest level under anaerobic conditions. Although ergosterol biosynthesis requires oxygen, upregulation of these genes has previously been observed under anaerobic and severely oxygen-restricted conditions [44, 113, 164]. It was suggested that this upregulation gives the cells an advantage in situations in which small amounts of oxygen suddenly become available [233]. In the time-course analysis, the switch to anaerobic conditions led to upregulation of the genes related to sterol transport, although in the initially oxygen-limited cultures the expression levels of these genes remained constant for the first 3 hours after the shift. A delayed response of these genes has also been observed in batch cultivations of glucose as a response to anaerobic conditions [164]. It is however unclear why the response was faster under the fully aerobic conditions.

In the time-course analysis, upregulation of amino acid transport was observed as a response to anaerobic conditions. It is interesting that at the same time the cells shut down their biosynthesis for amino acids and upregulated the genes related to the uptake of these compounds. However, it may be an energetically more feasible strategy to use externally provided resources. Lai *et al.* [163, 164] suggested that when the cells experience a sudden depletion of oxygen, at least part of this response is related to balancing the energy status.

In addition, a transient increase in the expression of genes encoding the phosphate transporters was seen in the time-course analysis. Previously, a transient increase in the intracellular phosphate and polyphosphate levels resulting from increase in transport of extracellular phosphate was observed after a shift to an-

aerobiosis [234]. Although it is unclear why this happens, it has been suggested to be related to the regulation of glycolysis.

4.6 TRAC vs. Affymetrix

In the current study, two methods for transcript analysis were used. The TRAC analysis enabled the accurate analysis of expression levels encoding the hexose transporters, the sequences of which have high similarities to each other. In addition, with TRAC we were able to analyse a selected set of genes of central carbon metabolism in a steady state setup and in six different time-course setups. Genome-wide Affymetrix analysis was then used to analyse the steady state cultures and two of the time-course setups.

In general, the data obtained from TRAC and Affymetrix analyses correlated well in situations in which >2-fold differences in the expression levels were observed. In large-scale studies comparing different methods of gene expression analysis, lower correlations have been often also been observed with smaller changes than with larger changes [235–237]. However, discrepancies were also seen in situations in which large changes in the gene expression were observed. These could result from multiple different factors, such as probe design, sample treatment and normalisation of the data.

5. Conclusions and future perspectives

In *S. cerevisiae,* provision of 0%, 0.5–2.8% and 20.9% oxygen led to fully fermentative, respiro-fermentative and fully respiratory modes of growth, respectively. On the transcriptional level, the main differences were observed between these three modes of metabolism. Especially the expression levels in 0.5 and 1.0% of oxygen provision were very similar. However, these two conditions differed on the proteome level, suggesting that post-transcriptional regulation occurred at this level of oxygen provision. In addition, proteomic analysis of glycolytic enzymes revealed oxygen-responsive isoforms, the level of which varied in the different oxygen concentrations. As the controlling mechanisms of glycolysis are still not fully understood, it would be important to study the role of these isoforms in the oxygen-mediated regulation of the pathway. One of the controlling mechanisms of glycolysis has been suggested to be transport of glucose into the cell. In this work, it was concluded that to enable the higher specific glucose uptake rate in the anaerobic and oxygen-limited than fully aerobic cells, the transcription of moderately low affinity transporters was decreased.

Under the oxygen-limited conditions, transcriptional adjustments for more efficient energy metabolism were observed. A global upregulation of genes encoding the respiratory pathways was accompanied by higher concentrations of the proteins related to respiration and higher activities of the enzymes of the TCA cycle. In addition, the genes encoding the mitochondrial translation machinery were more highly expressed in all the oxygen-limited and anaerobic than under the fully aerobic conditions, suggesting separate regulation mechanism from that of genes directly related to respiration. This also indicates an important, non-respiratory–related role for mitochondria under anaerobic conditions. Although mitochondria are known to exist in the absence of oxygen in the form differing from that of aerobic mitochondria, their function under anaerobic conditions is not known and would be an interesting subject of further studies.

There were only small differences in the transcriptional responses of cells initially in the oxygen-limited and the fully aerobic metabolic states to sudden oxygen depletion. Thus at least the levels of oxygen limitation used in this work did not prepare the cells for complete anaerobiosis. As the oxygen provision was stopped, there was transient decrease in the growth rate and in the expression of genes related to growth and cell proliferation. In addition, stress-related changes were observed and the transient upregulation of genes related to protein degradation suggested a remodeling of the metabolism for the new state.

Mass spectrometry–based methods for measurements of intracellular metabolite levels and for studies of the proteome are constantly developing. With the use of these new methods a more complete analysis of the metabolism will become possible. Especially, a larger spectrum of metabolites would enable the use of more sophisticated computational tools to combine the transcriptional and metabolite level data. In addition, although much is already known concerning the mechanisms regulating the metabolism of *S. cerevisiae* as a response to oxygen, there has been no evidence for the proteins that may directly sense the oxygen concentration in the environment. It might be that those kind of proteins do not exist at all, but as it is known that the lipid composition of the cell membrane is greatly affected by the provision of oxygen it would also be particularly interesting to study the behaviour of the membrane proteins.

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Author(s)
Eija Rintala

Title

Effects of oxygen provision on the physiology of baker´s yeast *Saccharomyces cerevisiae*

Abstract

The availability of oxygen has a major effect on all organisms. The yeast *Saccharomyces cerevisiae* is able to adapt its metabolism for growth in different conditions of oxygen provision, and to grow even under complete lack of oxygen. Although the physiology of *S. cerevisiae* has mainly been studied under fully aerobic and anaerobic conditions, less is known of metabolism under oxygen-limited conditions and of the adaptation to changing conditions of oxygen provision. This study compared the physiology of *S. cerevisiae* in conditions of five levels of oxygen provision (0, 0.5, 1.0, 2.8 and 20.9% O_2 in feed gas) by using measurements on metabolite, transcriptome and proteome levels. On the transcriptional level, the main differences were observed between the three level groups, 0, 0.5–2.8 and 20.9% O_2 which led to fully fermentative, respiro-fermentative and fully respiratory modes of metabolism, respectively. However, proteome analysis suggested post-transcriptional regulation at the level of 0.5 O_2. The analysis of metabolite and transcript levels of central carbon metabolism also suggested post-transcriptional regulation especially in glycolysis. Further, a global upregulation of genes related to respiratory pathways was observed in the oxygen-limited conditions and the same trend was seen in the proteome analysis and in the activities of enzymes of the TCA cycle.

The responses of intracellular metabolites related to central carbon metabolism and transcriptional responses to change in oxygen availability were studied. As a response to sudden oxygen depletion, concentrations of the metabolites of central carbon metabolism responded faster than the corresponding levels of gene expression. In general, the genome-wide transcriptional responses to oxygen depletion were highly similar when two different initial conditions of oxygen provision (20.9 and 1.0% O_2) were compared. The genes related to growth and cell proliferation were transiently downregulated whereas the genes related to protein degradation and phosphate uptake were transiently upregulated. In the cultures initially receiving 1.0% O_2, a transient upregulation of genes related to fatty acid oxidation, peroxisomal biogenesis, response to oxidative stress and pentose phosphate pathway was observed.

Additionally, this work analysed the effect of oxygen on transcription of genes belonging to the hexose transporter gene family. Although the specific glucose uptake rate was highest in fully anaerobic conditions, none of the hxt genes showed highest expression in anaerobic conditions. However, the expression of genes encoding the moderately low affinity transporters decreased with the decreasing oxygen level. Thus it was concluded that there is a relative increase in high affinity transport in anaerobic conditions supporting the high uptake rate.

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Tekijä(t)
Eija Rintala

Nimeke

Hapen vaikutus leivinhiiva *Saccharomyces cerevisiaen* aineenvaihduntaan

Tiivistelmä

Toisin kuin useimmat aitotumalliset eliöt, leivinhiiva *Saccharomyces cerevisiae* pystyy kasvamaan erilaisissa happipitoisuuksissa, jopa täysin hapettomissa oloissa. Tätä ominaisuutta on hyödennetty laajasti erilaisissa bioprosesseissa. Jotta näistä prosesseista saataisiin mahdollisimman tehokkaita, on tärkeä tietää, miten leivinhiivan aineenvaihduntaa säädellään hapen vaikutuksesta. Tässä väitöskirjatyössä tutkittiin leivinhiivan aineenvaihduntaa olosuhteissa, joissa syötetyn hapen määrä oli tarkasti määritetty. Työssä käytettiin viittä eri happipitoisuutta (0; 0,5; 1,0; 2,8 ja 20,9 % happea kasvatukseen syötetyssä kaasuseoksessa) sekä olosuhteita, joissa hapen syötttöä muutettiin äkillisesti. Työssä mitattiin solunsisäisiä ja -ulkoisia aineenvaihduntatuotteita ja geenien ilmentymistä. Hapensyötön eri tasoilla mitattiin myös proteiinien määriä ja entsyymien aktiivisuuksia.

Geenien ilmentymisen ja solunulkoisten aineenvaihduntatuotteiden perusteella näytti siltä, että leivinhiivan aineenvaihdunta on hyvin samankaltaista rajoitetun hapen olosuhteissa (0,5; 1,0 ja 2,8 O_2) mutta eroaa niissä selvästi hapettomista (0 % O_2) ja normaalin hapen olosuhteista (20,9 % O_2). Proteiinitasoja vertailtaessa kuitenkin havaittiin, että aineenvaihdunta ei ole täysin samanlaista happirajoitetuissa olosuhteissa. Erityisesti 0,5 ja 1,0 % hapensyötön välillä nähtiin eroja, mikä kertoo todenäköisesti geenitason yläpuolella tapahtuvasta säätelystä.

Tässä työssä havaittiin myös, että suurin osa hengitykseen liittyistä geeneistä ilmentyi voimakkaammin happirajoitteisissa kuin normaalin hapen olosuhteissa, ja sama tulos näkyi myös kyseessä olevien proteiinien tasoissa ja sitruunahappokierron entsyymien aktiivisuuksissa. Tämä kertoo luultavasti siitä, että solu yrittää saada rajoitetun hapen mahdollisimman tehokkaasti käyttöönsä. Lisäksi havaittiin, että vaikka glukoosin sisäänottonopeus on suurin hapettomissa olosuhteissa, glukoosinkuljettajaproteiineja koodaavien geenien ilmentyminen ei ole tällöin voimakkaimmillaan. Sen sijaan hapen määrän laskiessa keskimääräisen affiniteetin omaavia glukoosinkuljettajia koodaavien geenien tasot laskivat. Edellä mainittu aiheuttaa todennäköisesti sen, että solukalvolla on hapettomissa olosuhteissa suhteellisesti enemmän proteiineja, joilla on korkea affiniteetti glukoosia kohtaan, kuin hapellisissa olosuhteissa.

Lopetettaessa hapensyöttö äkillisesti kokonaan aineenvaihdunnan muutokset näkyivät nopeammin solunsisäisten aineenvaihduntatuotteiden määrissä kuin geenien ilmentymisessä. Havaittiin. että muutokset olivat hyvin samankaltaisia riippumatta siitä, kuinka paljon happea kasvatuksiin oli alun perin syötetty. Hapen loppuessa kasvuun ja solujen uudistumiseen liittyvien geenien ilmentymistasot laskivat, kun taas proteiinien hajotukseen liittyvien geenien ilmentymistasot nousivat. Lisäksi havaittiin stressivasteeseen liittyviä muutoksia.

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