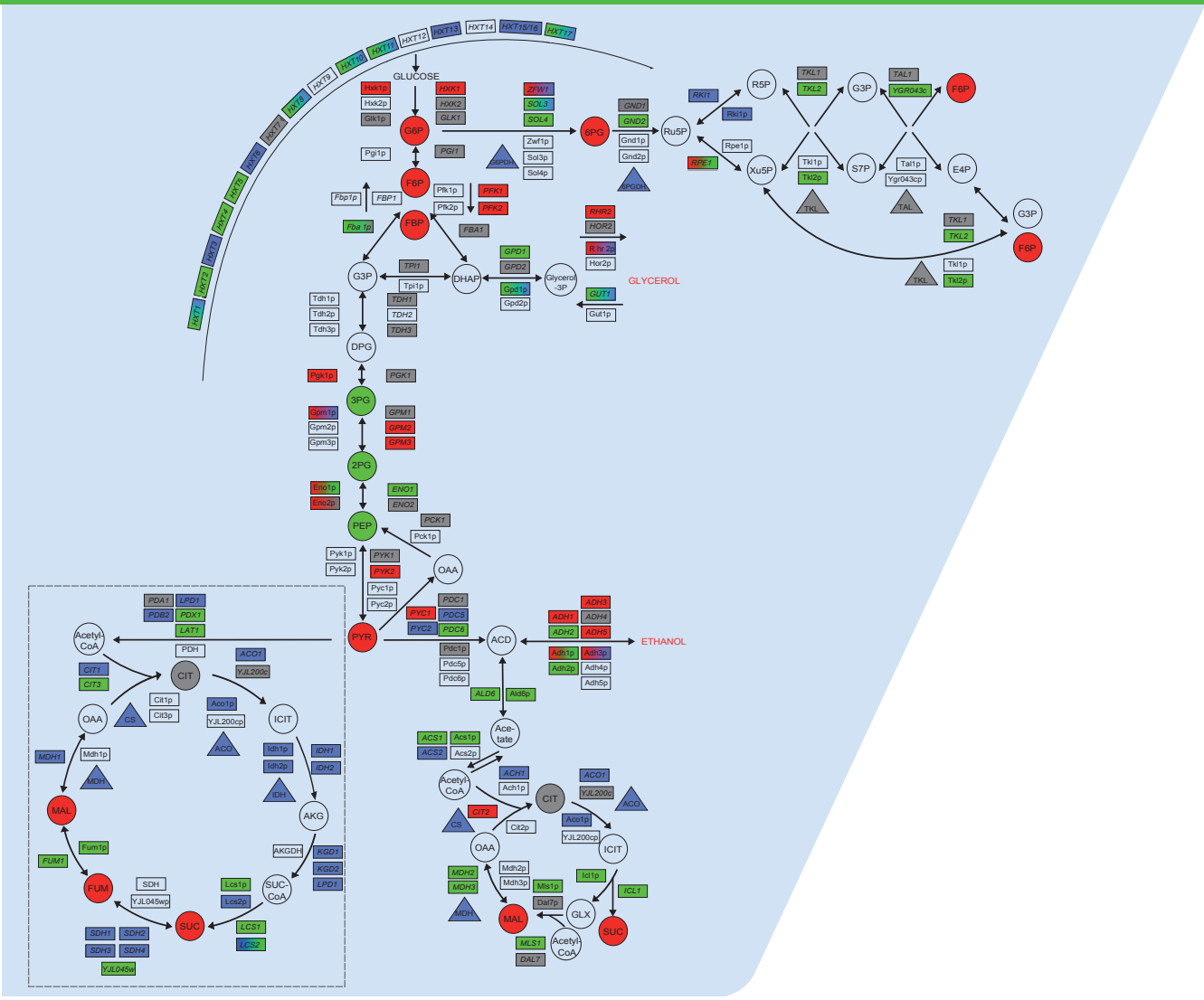


Eija Rintala

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

VTT PUBLICATIONS 747

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

Eija Rintala

VTT

Faculty of Biological and Environmental Sciences
Department of Biosciences
Division of General Microbiology
University of Helsinki, Finland

A dissertation for the degree of Doctor of Philosophy is to be presented, by permission of the Biological and Environmental Sciences, the University of Helsinki, for public examination and debate in Auditorium PIII in Porthania (Yliopistonkatu 3) Friday, November 26th 2010, at 12 noon.

ISBN 978-951-38-7413-1 (soft back ed.)
ISSN 1235-0621 (soft back ed.)

ISBN 978-951-38-7414-8 (URL: http://www.vtt.fi/publications/index.jsp)
ISSN 1455-0849 (URL: http://www.vtt.fi/publications/index.jsp)

JULKAISIJA – UTGIVARE – PUBLISHER

VTT, Vuorimiehentie 5, PL 1000, 02044 VTT
puh. vaihde 020 722 111, faksi 020 722 4374

VTT, Bergsmansvägen 5, PB 1000, 02044 VTT
tel. växel 020 722 111, fax 020 722 4374

VTT Technical Research Centre of Finland, Vuorimiehentie 5, P.O. Box 1000, FI-02044 VTT, Finland
phone internat. +358 20 722 111, fax + 358 20 722 4374

Technical editing Mirjami Pullinen

Edita Prima Oy, Helsinki 2010

Supervised by

Team Leader, PhD Laura Ruohonen
VTT Technical Research Centre of Finland
Espoo, Finland

Reviewed by

Professor Kalervo Hiltunen
Department of Biochemistry
Faculty of Science
University of Oulu, Finland

PhD Brian Gibson
VTT Technical Research Centre of Finland
Espoo, Finland

Opponent

Docent Christer Larsson
Department of Chemical and Biological Engineering
Chalmers University of Technology
Gothenburg, Sweden

Custos

Professor Dennis Bamford
Department of Biosciences
University of Helsinki, Finland

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 p. + app. 93 p.

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.

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

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.

Former Vice President R&D, Prof. Juha Ahvenainen, Vice President Anu Kaukovirta-Norja and Research Professor Hans Söderlund are thanked for the possibility to prepare this thesis and for creating excellent working facilities. Technology manager Tiina Nakari-Setälä is thanked for her supportive attitude towards this work.

I warmly thank my supervisor Team Leader Laura Ruohonen for guidance and encouragement over the years. Thank you Laura also for gently pushing me towards this thesis. To Research Professor Merja Penttilä I am especially greatful for the scientific guidance and interest towards this work.

I wish my deepest gratitude to my co-authors Merja Penttilä, Laura Ruohonen, Marilyn Wiebe, Mervi Toivari, Laura Salusjärvi, Juha-Pekka Pitkänen, Paula Jouhten, Hannu Maaheimo, Anu Tamminen, Anne Huuskonen, Helena Simolin, Juha Kokkonen Jari Kiuru and Raimo Ketola for their contributions to the research work and writing of the manuscripts. Without your valuable input this work would not have been possible.

Prof. Kalervo Hiltunen and Brian Gibson are thanked for the careful pre-examination of the thesis and their valuable comments to improve it. Michael Bailey is thanked for revising the English language.

I am greatful to everyone in the Metabolic Engineering team for the help I have received. I also thank everyone in the yeast and mold –lab for creating such a nice atmosphere to work in. In addition I thank all the people in various labs at Tietotie 2 in which I have shortly visited to use different equipment. My special thanks go to Pirjo Tähtinen, Seija Rissanen, Outi Könönen, Eila Leino and Tarja Laakso for the skillful technical assistance. Aili Grundström is thanked for all the help given especially during my first year at VTT.

I warmly thank my collegues Mervi, Laura, Mikko, Anne, Satu, Virve, Jari, Ritva, Outi and Mari for sharing the lunchbrakes and your lives with me. Mikko, Gopal and Brudy are thanked for the help on different bioinformatic matters.

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

Abstract 4

Tiivistelmä 6

Preface 8

List of publications 12

Abbreviations 13

1. Introduction 15
   - 1.1 Oxygen 15
   - 1.2 Fermentative and respiratory metabolism of *S. cerevisiae* 16
   - 1.3 Central carbon metabolism of *S. cerevisiae* 17
   - 1.4 Mitochondrial respiratory chain 20
   - 1.5 Growth under anaerobic conditions and oxygen-mediated transcriptional regulation 23
   - 1.6 Oxidative stress 26
   - 1.7 Genome-wide studies on responses to oxygen 27
   - 1.8 Glucose transport 28
   - 1.9 Aims of the study 31

2. Materials and methods 32
   - 2.1 Summary of methods 32
   - 2.2 Chemostat cultures 33

3. Results 34
   - 3.1 Cultivations and physiology (I) 34
   - 3.2 Intracellular metabolites (I) 36
   - 3.3 Transcriptional analyses (I, II, III, IV) 37
     - 3.3.1 Targeted analysis using TRAC (I,II) 37
       - 3.3.1.1 Analysis of central carbon metabolism (I) 37
       - 3.3.1.2 Analysis of hexose transporters (II) 38
     - 3.3.2 Global analysis using microarrays (III, IV) 39
       - 3.3.2.1 Different levels of oxygen provision (III) 39
       - 3.3.2.2 Change in oxygen provision (IV) 41
     - 3.3.3 Comparison of TRAC and microarray analyses (I, III, IV) 45
     - 3.3.4 Comparison of transcriptional, proteomics and enzyme activity analyses in different oxygen concentrations (III) 46

4. Discussion 48
   - 4.1 Physiological responses to oxygen 48
   - 4.2 Fermentative pathways, glucose transport and reserve carbohydrate metabolism 49
   - 4.3 The respiratory pathway and the pentose phosphate pathway 52
   - 4.4 Growth, protein degradation and stress 55
   - 4.5 Transport of sterols, phosphate and nitrogen-containing compounds 56
   - 4.6 TRAC vs. Affymetrix 57

5. Conclusions and future perspectives 58

References 60

Appendices

Publications I–IV

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].

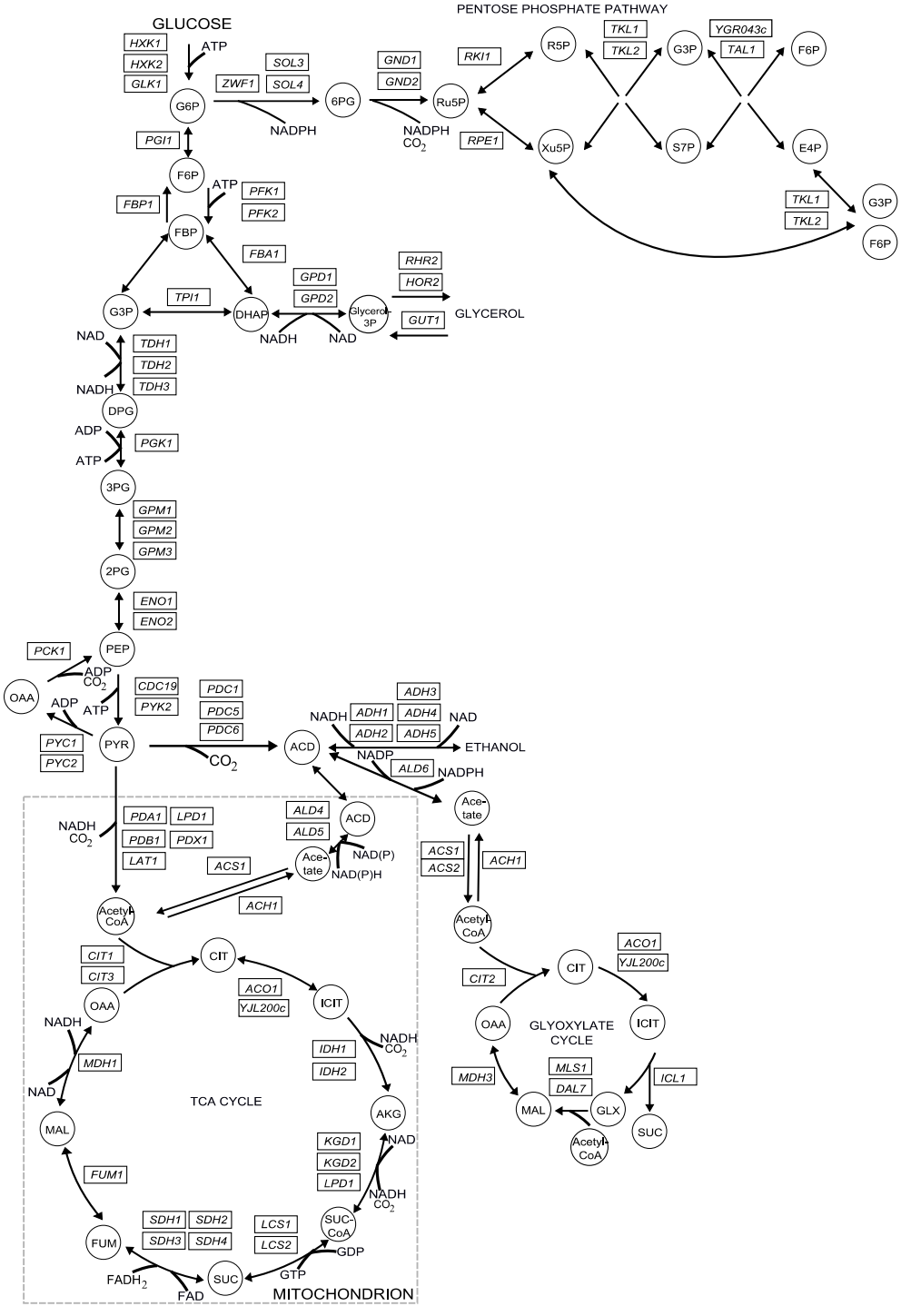


Figure 1. Genes encoding the enzymes of central carbon metabolism in *S. cerevisiae*.

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].

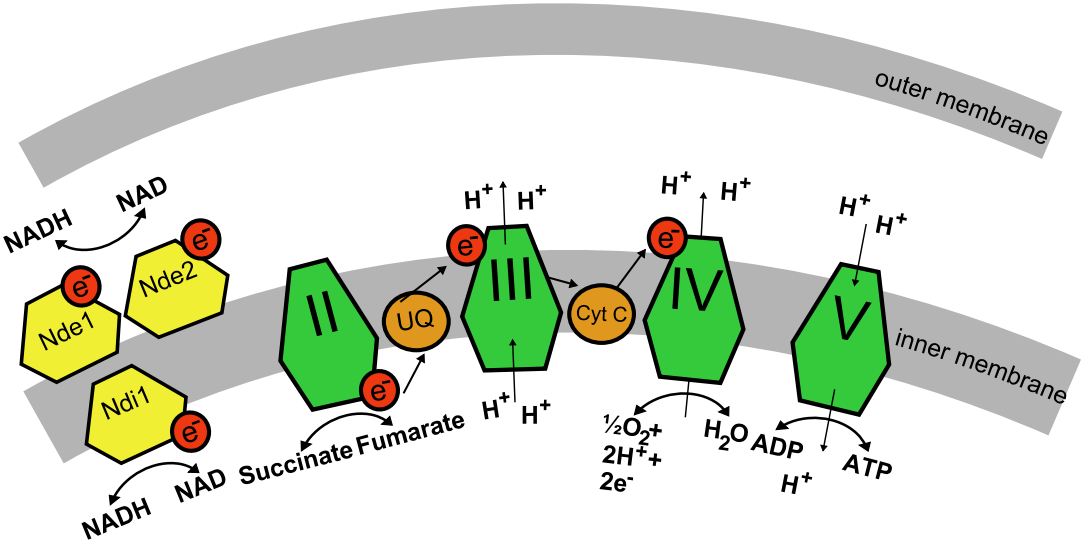


Figure 2. The mitochondrial respiratory chain of *S.cerevisiae*.

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].

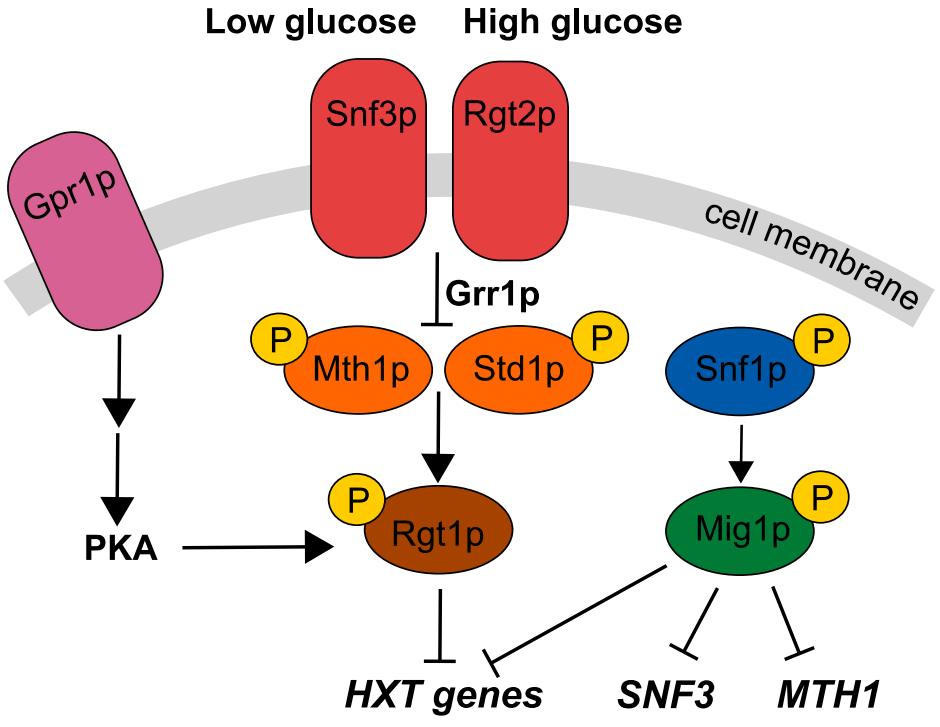


Figure 3. Regulation of genes encoding the major hexose transporters. Positive regulation is marked by a line with an arrowhead and negative regulation by a line with a bar at the end. The figure is adapted from Kim and Johnston 2006 [188].

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 *HXT*7. 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].

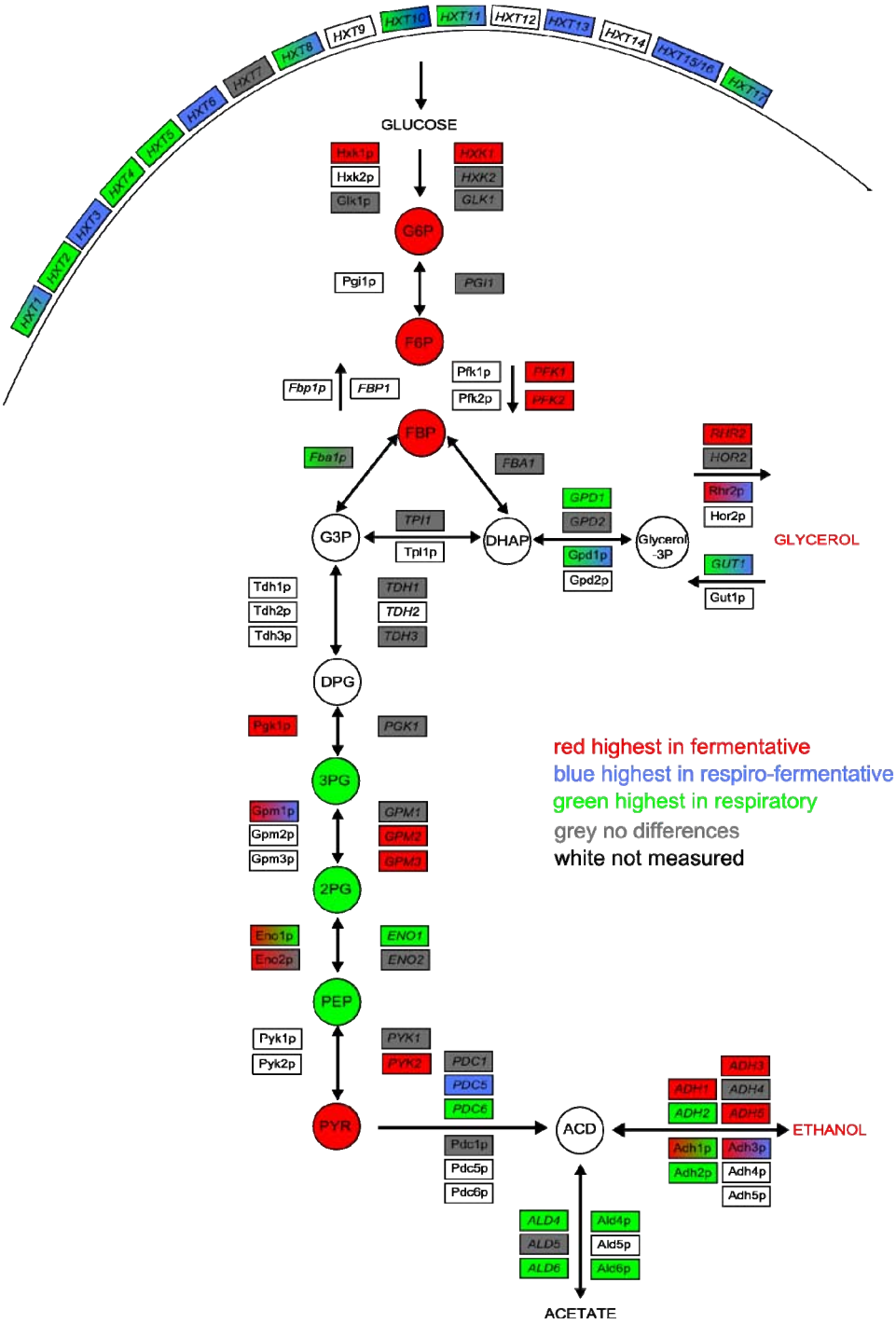


Figure 4. Relative levels of intra- and extracellular metabolites, gene expression and protein expression in the fermentative pathway. The gene expression data is derived from Affymetrix measurements except for *Hxt* genes, the data of which is derived from TRAC measurements.

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].

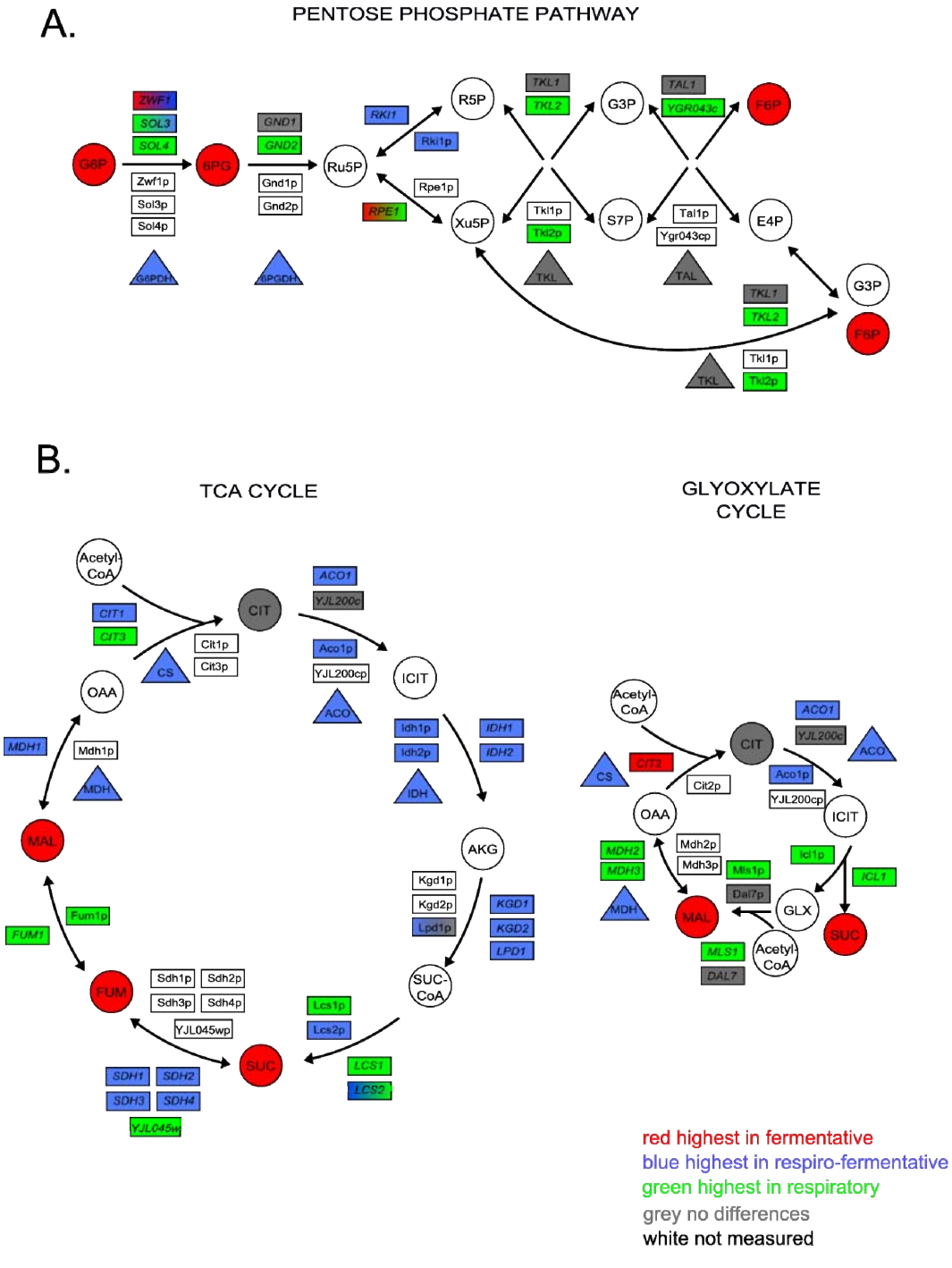


Figure 5. Relative levels of intracellular metabolites, gene expression, protein expression and enzyme activities in A. pentose phosphate pathway and B. TCA cycle and glyoxylate cycle. The gene expression data is derived from Affymetrix measurements.

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.

References

1. Canuto, V.M., Levine, J.,S., Augustsson, T.R., Imhoff, C.L. & Giampapa, M.S. The young Sun and the atmosphere and photochemistry of the early Earth. Nature 1983, Vol. 305, pp. 281–286.

2. Kasting, J.F. Earth's early atmosphere. Science (New York, N.Y.) 1993, Vol. 259, No. 5097, pp. 920–926.

3. Lyons, T.W. Palaeoclimate: oxygen's rise reduced. Nature 2007, Vol. 448, No. 7157, pp. 1005–1006.

4. Berner, R.A. Atmospheric oxygen over Phanerozoic time. Proceedings of the National Academy of Sciences of the United States of America 1999, Vol. 96, No. 20, pp. 10955–10957.

5. Falkowski, P.G., Katz, M.E., Milligan, A.J., Fennel, K., Cramer, B.S., Aubry, M.P., Berner, R.A., Novacek, M.J. & Zapol, W.M. The rise of oxygen over the past 205 million years and the evolution of large placental mammals. Science (New York, N.Y.) 2005, Vol. 309, No. 5744, pp. 2202–2204.

6. Han, T.M. & Runnegar, B. Megascopic eukaryotic algae from the 2.1-billion-year-old negaunee iron-formation, Michigan. Science (New York, N.Y.) 1992, Vol. 257, No. 5067, pp. 232–235.

7. Knoll, A.H. Proterozoic and early Cambrian protists: evidence for accelerating evolutionary tempo. Proceedings of the National Academy of Sciences of the United States of America 1994, Vol. 91, No. 15, pp. 6743–6750.

8. Brocks, J.J., Logan, G.A., Buick, R. & Summons, R.E. Archean molecular fossils and the early rise of eukaryotes. Science (New York, N.Y.) 1999, Vol. 285, No. 5430, pp. 1033–1036.

9. Acquisti, C., Kleffe, J. & Collins, S. Oxygen content of transmembrane proteins over macroevolutionary time scales. Nature 2007, Vol. 445, No. 7123, pp. 47–52.

10. Chen, L. L., Wang, G.Z. & Zhang, H.Y. Sterol biosynthesis and prokaryotes-to-eukaryotes evolution. Biochemical and biophysical research communications 2007, Vol. 363, No. 4, pp. 885–888.

11. Turrens, J.F. Mitochondrial formation of reactive oxygen species. The Journal of physiology 2003, Vol. 552, No. Pt 2, pp. 335–344.

12. Boveris, A., Oshino, N. & Chance, B. The cellular production of hydrogen peroxide. The Biochemical journal 1972, Vol. 128, No. 3, pp. 617–630.

13. Embley, T.M. Multiple secondary origins of the anaerobic lifestyle in eukaryotes. Philosophical transactions of the Royal Society of London.Series B, Biological sciences 2006, Vol. 361, No. 1470, pp. 1055–1067.

14. Andreasen, A.A. & Stier, T.J. Anaerobic nutrition of *Saccharomyces cerevisiae*. II. Unsaturated fatty acid requirement for growth in a defined medium. Journal of cellular physiology 1954, Vol. 43, No. 3, pp. 271–281.

15. Andreasen, A.A. & Stier, T.J. Anaerobic nutrition of *Saccharomyces cerevisiae*. I. Ergosterol requirement for growth in a defined medium. Journal of cellular physiology 1953, Vol. 41, No. 1, pp. 23–36.

16. Satyanarayana, T. & Kunze, G. . Yeast Biotechnology: Diversity and Applications. 2009.

17. Alfenore, S., Camelle yre, X., Benb adis, L., Bideau x, C., Uribelarre a, J.L., Goma, G., Molina-Jouve, C. & Guillouet, S.E. Aeration strategy : a need for very high ethanol performance in *Saccharomyces cerevisiae* fed-batch process. Applied microbiology and biotechnology 2004, Vol. 63, No. 5, pp. 537–542.

18. Blanco, C.A., Rayo, J. & Giralda, J.M. Improving industrial full-scale production of baker's yeast by optimizing aeration control. Journal of AOAC International 2008, Vol. 91, No. 3, pp. 607–613.

19. Snoek, I. & Steensma, H. Factors involved in anaerobic growth of *Saccharomyces cerevisiae*. Yeast 2007, Vol. 24, No. 1, pp. 1–10.

20. Piskur, J., Rozpedowska, E., Polakova, S., Merico, A. & Compag no, C. How did *Saccharomyces* evolve to become a good brewer? Trends in genetics 2006, Vol. 22, No. 4, pp. 183–186.

21. Thomson, J.M., Gaucher, E.A., Burgan, M.F., De Kee, D.W ., Li, T ., Aris, J.P. & Benner, S.A. Resurrecting ancestral alcohol dehydrogenases from yeast. Nature genetics 2005, Vol. 37, No. 6, pp. 630–635.

22. Kellis, M., Birren, B.W . & Lander, E.S. Proof and evolutionary analysis of ancient genome duplication in the yeast *Saccharomyces cerevisiae*. Nature 2004, Vol. 428, No. 6983, pp. 617–624.

23. Wolfe, K.H. & Shi elds, D.C. Molecular evidence for an ancient duplication of the entire yeast genome. Nature 1997, Vol. 387, No. 6634, pp. 708–713.

24. Gancedo, J.M. Yeast carbon catabolite repression. Microbiology and molecular biology reviews 1998, Vol. 62, No. 2, pp. 334–361.

25. van Dijken, J.P., Weusthuis, R.A. & Pronk, J.T. Kinetics of growth and sugar consumption in yeasts. Antonie van Leeuwenhoek 1993, Vol. 63, No. 3–4, pp. 343–352.

26. Raghevendran, V., Nielsen, J. & Olsson, L. Teaching microbial physiology using glucose repression phenomenon in baker's yeast as an example. Biochemistry and molecular biology education 2005, Vol. 33, No. 6, pp. 404–410.

27. Vemuri, G.N., Eiteman, M.A., McEwen, J.E., Olsson, L. & Nielsen, J. Increasing NADH oxidation reduces overflow metabolism in *Saccharomyces cerevisiae*. Proceedings of the National Academy of Sciences of the United States of America 2007, Vol. 104, No. 7, pp. 2402–2407.

28. Pronk, J.T., Yde Steensma, H. & Van Dijken, J.P. Pyruvate metabolism in *Saccharomyces cerevisiae*. Yeast (Chichester, England) 1996, Vol. 12, No. 16, pp. 1607–1633.

29. Nissen, T.L., Hamann, C.W., Kielland-Brandt, M.C., Nielsen, J. & Villadsen, J. Anaerobic and aerobic batch cultivations of *Saccharomyces cerevisiae* mutants impaired in glycerol synthesis. Yeast (Chichester, England) 2000, Vol. 16, No. 5, pp. 463–474.

30. Sierkstra, L.N., Verbakel, J.M. & Verrips, C.T. Analysis of transcription and translation of glycolytic enzymes in glucose-limited continuous cultures of *Saccharomyces cerevisiae*. Journal of general microbiology 1992, Vol. 138, No. 12, pp. 2559–2566.

31. Daran-Lapujade, P., Jansen, M.L., Daran, J.M., van Gulik, W., de Winde, J.H. & Pronk, J.T. Role of transcriptional regulation in controlling fluxes in central carbon metabolism of *Saccharomyces cerevisiae*. A chemostat culture study. The Journal of biological chemistry 2004, Vol. 279, No. 10, pp. 9125–9138.

32. van Hoek, P., van Dijken, J.P. & Pronk, J.T. Regulation of fermentative capacity and levels of glycolytic enzymes in chemostat cultures of *Saccharomyces cerevisiae*. Enzyme and microbial technology 2000, Vol. 26, No. 9–10, pp. 724–736.

33. Blazquez, M.A., Lagunas, R., Gancedo, C. & Gancedo, J.M. Trehalose-6-phosphate, a new regulator of yeast glycolysis that inhibits hexokinases. FEBS letters 1993, Vol. 329, No. 1–2, pp. 51–54.

34. Banuelos, M., Gancedo, C. & Gancedo, J.M. Activation by phosphate of yeast phosphofructokinase. The Journal of biological chemistry 1977, Vol. 252, No. 18, pp. 6394–6398.

35. Avigad, G. Stimulation of yeast phosphofructokinase activity by fructose 2,6-bisphosphate. Biochemical and biophysical research communications 1981, Vol. 102, No. 3, pp. 985–991.

36. Nissler, K., Otto, A., Schellenberger, W. & Hofmann, E. Similarity of activation of yeast phosphofructokinase by AMP and fructose-2,6-bisphosphate. Biochemical and biophysical research communications 1983, Vol. 111, No. 1, pp. 294–300.

37. Moore, P.A., Sagliocco, F.A., Wood, R.M. & Brown, A.J. Yeast glycolytic mRNAs are differentially regulated. Molecular and cellular biology 1991, Vol. 11, No. 10, pp. 5330–5337.

38. Hess, B. & Haeckel, R. Interaction between potassium-, ammonium- and fructose-1,6-diphosphate activation of yeast pyruvate kinase. Nature 1967, Vol. 214, No. 5090, pp. 848–849.

39. Diderich, J.A., Teusink, B., Valkier, J., Anjos, J., Spencer-Martins, I., van Dam, K. & Walsh, M.C. Strategies to determine the extent of control exerted by glucose transport on glycolytic flux in the yeast *Saccharomyces bayanus*. Microbiology (Reading, England) 1999, Vol. 145 (Pt 12), No. Pt 12, pp. 3447–3454.

40. Scott, E.W. & Baker, H.V. Concerted action of the transcriptional activators REB1, RAP1, and GCR1 in the high-level expression of the glycolytic gene *TPI*. Molecular and cellular biology 1993, Vol. 13, No. 1, pp. 543–550.

41. McAlister, L. & Holland, M.J. Differential expression of the three yeast glyceraldehyde-3-phosphate dehydrogenase genes. The Journal of biological chemistry 1985, Vol. 260, No. 28, pp. 15019–15027.

42. Cohen, R., Yokoi, T., Holland, J.P., Pepper, A.E. & Holland, M.J. Transcription of the constitutively expressed yeast enolase gene *ENO1* is mediated by positive and negative cis-acting regulatory sequences. Molecular and cellular biology 1987, Vol. 7, No. 8, pp. 2753–2761.

43. Daran-Lapujade, P., Rossell, S., van Gulik, W.M., Luttik, M.A., de Groot, M.J., Slijper, M., Heck, A.J., Daran, J.M., de Winde, J.H., Westerhoff, H.V., Pronk, J.T. & Bakker, B.M. The fluxes through glycolytic enzymes in *Saccharomyces cerevisiae* are predominantly regulated at posttranscriptional levels. Proceedings of the National Academy of Sciences of the United States of America 2007, Vol. 104, No. 40, pp. 15753–15758.

44. de Groot, M.J., Daran-Lapujade, P., van Breukelen, B., Knijnenburg, T.A., de Hulster, E.A., Reinders, M.J., Pronk, J.T., Heck, A.J. & Slijper, M. Quantitative proteomics and transcriptomics of anaerobic and aerobic yeast cultures reveals post-

transcriptional regulation of key cellular processes. Microbiology (Reading, England) 2007, Vol. 153, No. Pt 11, pp. 3864–3878.

45. Yin, Z., Wilson, S., Hauser, N.C., Tournu, H., Hoheisel, J.D. & Brown, A.J. Glucose triggers different global responses in yeast, depending on the strength of the signal, and transiently stabilizes ribosomal protein mRNAs. Molecular microbiology 2003, Vol. 48, No. 3, pp. 713–724.

46. DeRisi, J.L., Iyer, V.R. & Brown, P.O. Exploring the metabolic and genetic control of gene expression on a genomic scale. Science 1997, Vol. 278, No. 5338, pp. 680–686.

47. Blank, L.M. & Sauer, U. TCA cycle activity in *Saccharomyces cerevisiae* is a function of the environmentally determined specific growth and glucose uptake rates. Microbiology (Reading, England) 2004, Vol. 150, No. Pt 4, pp. 1085–1093.

48. Buschlen, S., Amillet, J.M., Guiard, B., Fournier, A., Marcireau, C. & Bolotin-Fukuhara, M. The *S. cerevisiae* HAP complex, a key regulator of mitochondrial function, coordinates nuclear and mitochondrial gene expression. Comparative and functional genomics 2003, Vol. 4, No. 1, pp. 37–46.

49. Liu, Z. & Butow, R.A. A transcriptional switch in the expression of yeast tricarboxylic acid cycle genes in response to a reduction or loss of respiratory function. Molecular and cellular biology 1999, Vol. 19, No. 10, pp. 6720–6728.

50. Gombert, A.K., Moreira dos Santos, M., Christensen, B. & Nielsen, J. Network identification and flux quantification in the central metabolism of *Saccharomyces cerevisiae* under different conditions of glucose repression. Journal of bacteriology 2001, Vol. 183, No. 4, pp. 1441–1451.

51. Brewster, N.K., Val, D.L., Walker, M.E. & Wallace, J.C. Regulation of pyruvate carboxylase isozyme (*PYC1, PYC2*) gene expression in *Saccharomyces cerevisiae* during fermentative and nonfermentative growth. Archives of biochemistry and biophysics 1994, Vol. 311, No. 1, pp. 62–71.

52. Yin, Z., Smith, R.J. & Brown, A.J. Multiple signalling pathways trigger the exquisite sensitivity of yeast gluconeogenic mRNAs to glucose. Molecular microbiology 1996, Vol. 20, No. 4, pp. 751–764.

53. Mercado, J.J., Smith, R., Sagliocco, F.A., Brown, A.J. & Gancedo, J.M. The levels of yeast gluconeogenic mRNAs respond to environmental factors. European journal of biochemistry / FEBS 1994, Vol. 224, No. 2, pp. 473–481.

54. Holzer, H. & Purwin, C. How does glucose initiate proteolysis of yeast fructose-1,6-bisphosphatase? Biomedica biochimica acta 1986, Vol. 45, No. 11–12, pp. 1657–1663.

55. Duntze, W., Neumann, D., Gancedo, J.M., Atzpodien, W. & Holzer, H. Studies on the regulation and localization of the glyoxylate cycle enzymes in *Saccharomyces cerevisiae*. European journal of biochemistry / FEBS 1969, Vol. 10, No. 1, pp. 83–89.

56. Hartig, A., Simon, M. M., Schuster, T., Daugherty, J.R., Yoo, H.S. & Cooper, T.G. Differentially regulated malate synthase genes participate in carbon and nitrogen metabolism of *S. cerevisiae*. Nucleic acids research 1992, Vol. 20, No. 21, pp. 5677–5686.

57. Foat, B.C., Houshmandi, S.S., Olivas, W.M. & Bussemaker, H.J. Profiling condition-specific, genome-wide regulation of mRNA stability in yeast. Proceedings of the National Academy of Sciences of the United States of America 2005, Vol. 102, No. 49, pp. 17675–17680.

58. Fernandez, E., Moreno, F. & Rodicio, R. The *ICL1* gene from *Saccharomyces cerevisiae*. European journal of biochemistry / FEBS 1992, Vol. 204, No. 3, pp. 983–990.

59. Stryer, L. Biochemistry. W.H.Freeman and Co., San Fransisco, CA., 1989.

60. Levy, H.R. Glucose-6-phosphate dehydrogenases. Advances in enzymology and related areas of molecular biology 1979, Vol. 48, pp. 97–192.

61. Vaseghi, S., Baumeister, A., Rizzi, M. & Reuss, M. In vivo dynamics of the pentose phosphate pathway in *Saccharomyces cerevisiae*. Metabolic engineering 1999, Vol. 1, No. 2, pp. 128–140.

62. Larochelle, M., Drouin, S., Robert, F. & Turcotte, B. Oxidative stress-activated zinc cluster protein Stb5 has dual activator/repressor functions required for pentose phosphate pathway regulation and NADPH production. Molecular and cellular biology 2006, Vol. 26, No. 17, pp. 6690–6701.

63. Lee, J., Godon, C., Lagniel, G., Spector, D., Garin, J., Labarre, J. & Toledano, M.B. Yap1 and Skn7 control two specialized oxidative stress response regulons in yeast. The Journal of biological chemistry 1999, Vol. 274, No. 23, pp. 16040–16046.

64. Gasch, A.P., Spellman, P.T., Kao, C.M., Carmel-Harel, O., Eisen, M.B., Storz, G., Botstein, D. & Brown, P.O. Genomic expression programs in the response of yeast cells to environmental changes. Molecular biology of the cell 2000, Vol. 11, No. 12, pp. 4241–4257.

65. Wyrick, J.J., Holstege, F.C., Jennings, E.G., Causton, H.C., Shore, D., Grunstein, M., Lander, E.S. & Young, R.A. Chromosomal landscape of nucleosome-dependent gene expression and silencing in yeast. Nature 1999, Vol. 402, No. 6760, pp. 418–421.

66. David, P.S. & Poyton, R.O. Effects of a transition from normoxia to anoxia on yeast cytochrome *c* oxidase and the mitochondrial respiratory chain: implications for hypoxic gene induction. Biochimica et biophysica acta 2005, Vol. 1709, No. 2, pp. 169–180.

67. Helbig, A.O., de Groot, M.J., van Gestel, R.A., Mohammed, S., de Hulster, E.A., Luttik, M.A., Daran-Lapujade, P., Pronk, J.T., Heck, A.J. & Slijper, M. A three-way proteomics strategy allows differential analysis of yeast mitochondrial membrane protein complexes under anaerobic and aerobic conditions. Proteomics 2009, Vol. 9, No. 20, pp. 4787–4798.

68. Rosenfeld, E., Schaeffer, J., Beauvoit, B. & Salmon, J.M. Isolation and properties of promitochondria from anaerobic stationary-phase yeast cells. Antonie van Leeuwenhoek 2004, Vol. 85, No. 1, pp. 9–21.

69. Joseph-Horne, T., Hollomon, D.W. & Wood, P.M. Fungal respiration: a fusion of standard and alternative components. Biochimica et biophysica acta 2001, Vol. 1504, No. 2–3, pp. 179–195.

70. Larsson, C., Pahlman, I.L., Ansell, R., Rigoulet, M., Adler, L. & Gustafsson, L. The importance of the glycerol 3-phosphate shuttle during aerobic growth of *Saccharomyces cerevisiae*. Yeast (Chichester, England) 1998, Vol. 14, No. 4, pp. 347–357.

71. Schagger, H. & Pfeiffer, K. Supercomplexes in the respiratory chains of yeast and mammalian mitochondria. The EMBO journal 2000, Vol. 19, No. 8, pp. 1777–1783.

72. Grandier-Vazeille, X., Bathany, K., Chaignepain, S., Camougrand, N., Manon, S. & Schmitter, J.M. Yeast mitochondrial dehydrogenases are associated in a supramolecular complex. Biochemistry 2001, Vol. 40, No. 33, pp. 9758–9769.

73. Oyedotun, K.S. & Lemire, B.D. The quaternary structure of the *Saccharomyces cerevisiae* succinate dehydrogenase. Homology modeling, cofactor docking, and molecular dynamics simulation studies. The Journal of biological chemistry 2004, Vol. 279, No. 10, pp. 9424–9431.

74. Brandt, U., Uribe, S., Schagger, H. & Trumpower, B.L. Isolation and characterization of *QCR10*, the nuclear gene encoding the 8.5-kDa subunit 10 of the *Sac-*

charomyces cerevisiae cytochrome bc1 complex. The Journal of biological chemistry 1994, Vol. 269, No. 17, pp. 12947–12953.

75. Burke, P.V., Raitt, D.C., Allen, L.A., Kellogg, E.A. & Poyton, R.O. Effects of oxygen concentration on the expression of cytochrome *c* and cytochrome *c* oxidase genes in yeast. The Journal of biological chemistry 1997, Vol. 272, No. 23, pp. 14705–14712.

76. Poyton, R.O., Goehring, B., Droste, M., Sevarino, K.A., Allen, L.A. & Zhao, X .J. Cytochrome-*c* oxidase from *Saccharomyces cerevisiae*. Methods in enzymology 1995, Vol. 260, pp. 97–116.

77. Waterland, R.A., Basu, A., Chance, B. & Poyton, R.O. The isoforms of yeast cytochrome *c* oxidase subunit V alter the in vivo kinetic properties of the holoenzyme. The Journal of biological chemistry 1991, Vol. 266, No. 7, pp. 4180–4186.

78. Hodge, M.R., Kim, G., Singh, K. & Cumsky, M.G. Inverse regulation of the yeast *COX5* genes by oxygen and heme. Molecular and cellular biology 1989, Vol. 9, No. 5, pp. 1958–1964.

79. Devenish, R.J., Prescott, M., Roucou, X. & Nagley, P. Insights into ATP synthase assembly and function through the molecular genetic manipulation of subunits of the yeast mitochondrial enzyme complex. Biochimica et biophysica acta 2000, Vol. 1458, No. 2–3, pp. 428–442.

80. Wright, R.M. & Poyton, R.O. Release of two *Saccharomyces cerevisiae* cytochrome genes, *COX6* and *CYC1*, from glucose repression requires the *SNF1* and *SSN6* gene products. Molecular and cellular biology 1990, Vol. 10, No. 3, pp. 1297–1300.

81. Lombardo, A., Ceregh ino, G.P. & Sc heffler, I.E. Control of mRNA turnover as a mechanism of glucose repression in *Saccharomyces cerevisiae*. Molecular and cellular biology 1992, Vol. 12, No. 7, pp. 2941–2948.

82. Forsburg, S.L. & Guarente, L. Identification and characterization of HAP4: a third component of the CCAAT-bound HAP2/HAP3 heteromer. Genes & development 1989, Vol. 3, No. 8, pp. 1166–1178.

83. Oura, E. Effect of aeration intensity on the biochemical composition of baker's yeast. II. Activities of the oxidative enzymes. Biotechnology and bioengineering 1974, Vol. 16, No. 9, pp. 1213–1225.

84. Hiltunen, J.K., Autio, K.J., Schonauer, M.S., Kursu, V.A., Dieckmann, C.L. & Kastaniotis, A.J. Mitochondrial fatty acid synthesis and respiration. Biochimica et biophysica acta 2010, , No. 6–7, pp. 1195–1202.

85. Lill, R. & Muhlenhoff, U. Iron-sulfur-protein biogenesis in eukaryotes. Trends in biochemical sciences 2005, Vol. 30, No. 3, pp. 133–141.

86. Zelenaya-Troitskaya, O., Perlman, P.S. & Butow, R.A. An enzyme in yeast mitochondria that catalyzes a step in branched-chain amino acid biosynthesis also functions in mitochondrial DNA stability. The EMBO journal 1995, Vol. 14, No. 13, pp. 3268–3276.

87. Glerum, D.M., Shtanko, A., Tzagoloff, A., Gorman, N. & Sinclair, P. R. Cloning and identification of *HEM14*, the yeast gene for mitochondrial protoporphyrinogen oxidase. Yeast (Chichester, England) 1996, Vol. 12, No. 14, pp. 1421–1425.

88. Newmeyer, D.D. & Ferguson-Miller, S. Mitochondria: releasing power for life and unleashing the machineries of death. Cell 2003, Vol. 112, No. 4, pp. 481–490.

89. Sickmann, A., Reinders, J., Wagner, Y., Joppich, C., Zahedi, R., Meyer, H.E., Schonfisch, B., Perschil, I., Chacinska, A., Guiard, B., Rehling, P., Pfanner, N. & Meisinger, C. The proteome of *Saccharomyces cerevisiae* mitochondria. Proceedings of the National Academy of Sciences of the United States of America 2003, Vol. 100, No. 23, pp. 13207–13212.

90. Reinders, J., Zahedi, R.P., Pfanner, N., Meisinger, C. & Sickmann, A. Toward the complete yeast mitochondrial proteome: multidimensional separation techniques for mitochondrial proteomics. Journal of proteome research 2006, Vol. 5, No. 7, pp. 1543–1554.

91. Ohlmeier, S., Kastaniotis, A.J., Hiltunen, J.K. & Bergmann, U. The yeast mitochondrial proteome, a study of fermentative and respiratory growth. The Journal of biological chemistry 2004, Vol. 279, No. 6, pp. 3956–3979.

92. Prokisch, H., Scharfe, C., Camp, D.G.,2nd, Xiao, W., David, L., Andreoli, C., Monroe, M.E., Moore, R.J., Gritsenko, M.A., Kozany, C., Hixson, K.K., Mottaz, H.M., Zischka, H., Ueffing, M., Herman, Z.S., Davis, R.W., Meitinger, T., Oefner, P.J., Smith, R.D. & Steinmetz, L.M. Integrative analysis of the mitochondrial proteome in yeast. PLoS biology 2004, Vol. 2, No. 6, pp. e160.

93. Ansell, R., Granath, K., Hohmann, S., Thevelein, J.M. & Adler, L. The two isoenzymes for yeast NAD+-dependent glycerol 3-phosphate dehydrogenase encoded by *GPD1* and *GPD2* have distinct roles in osmoadaptation and redox regulation. Embo journal 1997, Vol. 16, No. 9, pp. 2179–2187.

94. Camarasa, C., Faucet, V. & Dequin, S. Role in anaerobiosis of the isoenzymes for *Saccharomyces cerevisiae* fumarate reductase encoded by *OSM1* and *FRDS1*. Yeast (Chichester, England) 2007, Vol. 24, No. 5, pp. 391–401.

95. Tzagoloff, A., Jang, J., Glerum, D.M. & Wu, M. FLX 1 codes for a carrier pr otein involved in maintaining a pr oper balance of flavin n ucleotides in yeast mitochondria. The Journal of biological chemistry 1996, Vol. 271, No. 13, pp. 7392–7397.

96. Bafunno, V., Giancaspero, T.A., Brizio, C., Bufano, D., Passarella, S., Boles, E. & Barile, M. Rib oflavin uptake and FAD s ynthesis in *Saccharomyces cerevisiae* mitochondria: involvement of the Flx1p carrier in FAD export. The Journal of biological chemistry 2004, Vol. 279, No. 1, pp. 95–102.

97. Ma chado, A., Nun ez de Ca stro, I. & May or, F. Iso citrate dehydrogenases and oxoglutarate dehydrogenase activities of baker's yeast grown in a variety of hypoxic conditions. Molecular and cellular biochemistry 1975, Vol. 6, No. 2, pp. 93–100.

98. Camarasa, C., Grivet, J.P. & Dequin, S. Investigation by 13C-NMR and tricar boxylic acid (T CA) deleti on mut ant ana lysis of path ways for succin ate formation i n *Saccharomyces cerevisiae* during anaerobic fermentation. Microbiology 2003, Vol. 149, No. Pt 9, pp. 2669–2678.

99. Nagy, M., Lacroute, F. & Thomas, D. Divergent evolution of pyrimidine biosynthesis between anaerobic and aerobic yeasts. Proceedings of t he National Academy of Sciences of the United States of America 1992, Vol. 89, No. 19, pp. 8966–8970.

100. Kolberg, M., Strand, K.R., Graff, P. & Andersson, K.K. Stru cture, function, an d mechanism of ribonucleotide reductases. Biochimica et biophysica acta 2004, Vol. 1699, No. 1–2, pp. 1–34.

101. Krawiec, Z., Swiecilo, A. & Bilinski, T. Heme s ynthesis in yeast d oes n ot requ ire oxygen as an obligatory e lectron accept or. Acta Biochim ica Polo nica 2000, Vol. 47, No. 4, pp. 1027–1035.

102. Nurminen, T., Konttinen, K. & Suomalainen, H. Ne utral li pids in t he cells and cell envelope fractions of aerobic baker's yeast and anaerobic brewer's yeast. Chemistry and physics of lipids 1975, Vol. 14, No. 1, pp. 15–32.

103. Gojkovic, Z., Knecht, W., Zameitat, E., Warneboldt, J., Coutelis, J.B., Pynyaha, Y., Neuveglise, C., Moller, K., Loffler, M. & Piskur, J. Horizontal ge ne tran sfer promoted ev olution of the a bility to propa gate under an aerobic cond itions in yeasts. Molecular genetics and genomics 2004, Vol. 271, No. 4, pp. 387–393.

104. Elledge, S.J. & Davis, R.W. Two genes differentially regulated in the cell cycle and by DNA-damaging agents encode alternative regulatory subunits of ribonucleotide reductase. Genes & development 1990, Vol. 4, No. 5, pp. 740–751.

105. Nguyen, H.H., Ge, J., Perlstein, D.L. & Stubb e, J. Purificati on of ribo nucleotide reductase su bunits Y1, Y 2, Y3, and Y4 fro m yeast: Y4 pl ays a key r ole in d iiron

cluster assembly. Proceedings of the National Academy of Sciences of the United States of America 1999, Vol. 96, No. 22, pp. 12339–12344.

106. Stukey, J.E., McDonough, V.M. & Martin, C.E. The *OLE1* gene of *Saccharomyces cerevisiae* encodes the delta 9 fatty acid desaturase and can be functionally replaced by the rat stearoyl-CoA desaturase gene. The Journal of biological chemistry 1990, Vol. 265, No. 33, pp. 20144–20149.

107. Rosenfeld, E. & Beauvoit, B. Role of the non-respiratory pathways in the utilization of molecular oxygen by *Saccharomyces cerevisiae*. Yeast (Chichester, England) 2003, Vol. 20, No. 13, pp. 1115–1144.

108. Kwast, K.E., Lai, L.C., Menda, N., James, D.T.,3rd, Aref, S. & Burke, P.V. Genomic analyses of anaerobically induced genes in *Saccharomyces cerevisiae*: functional roles of Rox1 and other factors in mediating the anoxic response. Journal of bacteriology 2002, Vol. 184, No. 1, pp. 250–265.

109. Abramova, N.E., Cohen, B.D., Sertil, O., Kapoor, R., Davies, K.J. & Lowry, C.V. Regulatory mechanisms controlling expression of the DAN/TIR mannoprotein genes during anaerobic remodeling of the cell wall in *Saccharomyces cerevisiae*. Genetics 2001, Vol. 157, No. 3, pp. 1169–1177.

110. Klis, F.M., Mol, P., Hellingwerf, K. & Brul, S. Dynamics of cell wall structure in *Saccharomyces cerevisiae*. FEMS microbiology reviews 2002, Vol. 26, No. 3, pp. 239–256.

111. Alimardani, P., Regnacq, M., Moreau-Vauzelle, C., Ferreira, T., Rossignol, T., Blondin, B. & Berges, T. *SUT1*-promoted sterol uptake involves the ABC transporter Aus1 and the mannoprotein Dan1 whose synergistic action is sufficient for this process. The Biochemical journal 2004, Vol. 381, No. Pt 1, pp. 195–202.

112. Vik, A. & Rine, J. Upc2p and Ecm22p, dual regulators of sterol biosynthesis in *Saccharomyces cerevisiae*. Molecular and cellular biology 2001, Vol. 21, No. 19, pp. 6395–6405.

113. Davies, B.S. & Rine, J. A role for sterol levels in oxygen sensing in *Saccharomyces cerevisiae*. Genetics 2006, Vol. 174, No. 1, pp. 191–201.

114. Bourot, S. & Karst, F. Isolation and characterization of the *Saccharomyces cerevisiae SUT1* gene involved in sterol uptake. Gene 1995, Vol. 165, No. 1, pp. 97–102.

115. Ness, F., Bourot, S., Regnacq, M., Spagnoli, R., Berges, T. & Karst, F. *SUT1* is a putative Zn[II]2Cys6-transcription factor whose upregulation enhances both sterol uptake and synthesis in aerobically growing *Saccharomyces cerevisiae* cells. European journal of biochemistry / FEBS 2001, Vol. 268, No. 6, pp. 1585–1595.

116. Hon, T., Dodd, A., Dirmeier, R., Gorman, N., Sinclair, P.R., Z hang, L. & Poy ton, R.O. A mechanism of oxy gen sensing in yeast. Multiple oxygen-responsive steps in the heme bi osynthetic pat hway affect Hap1 activity. T he Jo urnal of biological chemistry 2003, Vol. 278, No. 50, pp. 50771–50780.

117. Lowry, C.V. & Zitomer, R.S. Oxygen regul ation of ana erobic an d aerob ic ge nes mediated by a common factor in yeast. Pro ceedings of th e Nati onal Ac ademy of Sciences of the United States of America 1984, Vol. 81, No. 19, pp. 6129–6133.

118. Becerra, M., Lombar dia-Ferreira, L. J., Hauser, N.C., Hoheis el, J .D., Tizon, B. & Cerdan, M.E. The yeast transcriptome in aerobic and hypoxic conditions: effects of hap1, rox1, rox3 and srb10 deletions. Molecular microbiology 2002, Vol. 43, No. 3, pp. 545–555.

119. Zhang, L. & Guarente, L. *HAP1* is nu clear but is bo und to a ce llular factor in the absence of heme. The Journal of biological chemistry 1994, Vol. 269, No. 20, p p. 14643–14647.

120. Ter Linde, J.J. & Stee nsma, H.Y. A microarray-assisted screen for potential Hap1 and Rox1 target genes in *Saccharomyces cerevisiae*. Yeast (Chichester, England) 2002, Vol. 19, No. 10, pp. 825–840.

121. Hickman, M.J. & Winston, F. Heme lev els s witch the functi on of Ha p1 of *Saccharomyces c erevisiae* b etween transcri ptional activ ator and transcri ptional re p-ressor. Molecular and cellular biology 2007, Vol. 27, No. 21, pp. 7414–7424.

122. Kwast, K.E., Burke, P.V. & Poyton, R.O. Oxy gen s ensing and the transcriptional regulation of oxyg en-responsive gen es in yeast. The Journal of experim ental biol-ogy 1998, Vol. 201, No. Pt 8, pp. 1177–1195.

123. Raghevendran, V., Patil, K.R., Olsson, L. & Ni elsen, J. Hap4 is n ot essentia l for activation of re spiration at lo w specific gro wth rates in *Saccharomyces cerevisiae*. The Journal of biological chemistry 2006, Vol. 281, No. 18, pp. 12308–12314.

124. Olesen, J.T. & Guarente, L. T he H AP2 sub unit o f yeast CCA AT transcription al activator contains adjacent domains for subunit ass ociation and DNA r ecognition: model for the HAP2/3/4 complex. Genes & development 1990, Vol. 4, No . 10, pp. 1714–1729.

125. Poyton, R.O. Models for oxy gen sensing in yeast: implications for oxy gen-regulated gene expression in higher eucaryotes. Respiration physiology 1999, Vol. 115, No. 2, pp. 119–133.

126. Castro-Prego, R., L amas-Maceiras, M., Soeng as, P., F ernandez-Leiro, R., Carneiro, I., Becerra, M., Gonzalez-Sis o, M.I. & Cerdan, M.E. Ixr1p regulates oxy-

gen-dependent *HEM13* transcription. FEMS yeast research 2010, Vol. 10, No. 3, pp. 309–321.

127. Lambert, J.R., Bilanchone, V.W. & Cumsky, M.G. The *ORD1* gene encodes a transcription factor involved in oxygen regulation and is identical to *IXR1*, a gene that confers cisplatin sensitivity to *Saccharomyces cerevisiae*. Proceedings of the National Academy of Sciences of the United States of America 1994, Vol. 91, No. 15, pp. 7345–7349.

128. Castro-Prego, R., Lamas-Maceiras, M., Soengas, P., Carneiro, I., Gonzalez-Siso, I. & Cerdan, M.E. Regulatory factors controlling transcription of *Saccharomyces cerevisiae IXR1* by oxygen levels: a model of transcriptional adaptation from aerobiosis to hypoxia implicating *ROX1* and *IXR1* cross-regulation. The Biochemical journal 2009, Vol. 425, No. 1, pp. 235–243.

129. Vasconcelles, M.J., Jiang, Y., McDaid, K., Gilooly, L., Wretzel, S., Porter, D.L., Martin, C.E. & Goldberg, M.A. Identification and characterization of a low oxygen response element involved in the hypoxic induction of a family of *Saccharomyces cerevisiae* genes. Implications for the conservation of oxygen sensing in eukaryotes. The Journal of biological chemistry 2001, Vol. 276, No. 17, pp. 14374–14384.

130. Kwast, K.E., Burke, P.V., Staahl, B.T. & Poyton, R.O. Oxygen sensing in yeast: evidence for the involvement of the respiratory chain in regulating the transcription of a subset of hypoxic genes. Proceedings of the National Academy of Sciences of the United States of America 1999, Vol. 96, No. 10, pp. 5446–5451.

131. Ferreira, T.C., Hertzberg, L., Gassmann, M. & Campos, E.G. The yeast genome may harbor hypoxia response elements (HRE). Comparative Biochemistry and Physiology – Part C: Toxicology & Pharmacology 2007, Vol. 146, No. 1–2, pp. 255–263.

132. Wang, G.L. & Semenza, G.L. Desferrioxamine induces erythropoietin gene expression and hypoxia-inducible factor 1 DNA-binding activity: implications for models of hypoxia signal transduction. Blood 1993, Vol. 82, No. 12, pp. 3610–3615.

133. SCHATZ, G. Subcellular particles carrying mitochondrial enzymes in anaerobically-grown cells of *Saccharomyces cerevisiae*. Biochimica et biophysica acta 1965, Vol. 96, pp. 342–345.

134. Criddle, R.S. & Schatz, G. Promitochondria of anaerobically grown yeast. I. Isolation and biochemical properties. Biochemistry 1969, Vol. 8, No. 1, pp. 322–334.

135. Egner, A., Jakobs, S. & Hell, S.W. Fast 100-nm resolution three-dimensional microscope reveals structural plasticity of mitochondria in live yeast. Proceedings

of the National Academy of Sciences of the United States of America 2002, Vol. 99, No. 6, pp. 3370–3375.

136. Plattner, H. & Schatz, G. Promitochondria of anaerobically grown yeast. 3. Morphology. Biochemistry 1969, Vol. 8, No. 1, pp. 339–343.

137. Visser, W., van Spronsen, E.A., Nanninga, N., Pronk, J.T., Gijs Kuenen, J. & van Dijken, J.P. Effects of growth conditions on mitochondrial morphology in *Saccharomyces cerevisiae*. Antonie van Leeuwenhoek 1995, Vol. 67, No. 3, pp. 243–253.

138. Nunnari, J., Marshall, W.F., Straight, A., Murray, A., Sedat, J.W. & Walter, P. Mitochondrial transmission during mating in *Saccharomyces cerevisiae* is determined by mitochondrial fusion and fission and the intramitochondrial segregation of mitochondrial DNA. Molecular biology of the cell 1997, Vol. 8, No. 7, pp. 1233–1242.

139. Paumard, P., Vaillier, J., Coulary, B., Schaeffer, J., Soubannier, V., Mueller, D.M., Brethes, D., di Rago, J.P. & Velours, J. The ATP synthase is involved in generating mitochondrial cristae morphology. The EMBO journal 2002, Vol. 21, No. 3, pp. 221–230.

140. Church, C. & Poyton, R.O. Neither respiration nor cytochrome c oxidase affects mitochondrial morphology in *Saccharomyces cerevisiae*. The Journal of experimental biology 1998, Vol. 201, No. Pt 11, pp. 1729–1737.

141. Reiner, S., Micolod, D., Zellnig, G. & Schneiter, R. A genomewide screen reveals a role of mitochondria in anaerobic uptake of sterols in yeast. Molecular biology of the cell 2006, Vol. 17, No. 1, pp. 90–103.

142. Nohl, H., Kozlov, A.V., Gille, L. & Staniek, K. Cell respiration and formation of reactive oxygen species: facts and artefacts. Biochemical Society transactions 2003, Vol. 31, No. Pt 6, pp. 1308–1311.

143. Jamieson, D.J. Oxidative stress responses of the yeast *Saccharomyces cerevisiae*. Yeast (Chichester, England) 1998, Vol. 14, No. 16, pp. 1511–1527.

144. Bunn, H.F., Gu, J., Huang, L.E., Park, J.W. & Zhu, H. Erythropoietin: a model system for studying oxygen-dependent gene regulation. The Journal of experimental biology 1998, Vol. 201, No. Pt 8, pp. 1197–1201.

145. Bunn, H.F. & Poyton, R.O. Oxygen sensing and molecular adaptation to hypoxia. Physiological reviews 1996, Vol. 76, No. 3, pp. 839–885.

146. Guzy, R.D., Mack, M.M. & Schumacker, P.T. Mitochondrial complex III is required for hypoxia-induced ROS production and gene transcription in yeast. Antioxidants & redox signaling 2007, Vol. 9, No. 9, pp. 1317–1328.

147. Dirmeier, R., O'Brien, K.M., Engle, M., Dodd, A., Spears, E. & Poyton, R.O. Exposure of yeast cells to anoxia induces transient oxidative stress. Implications for the induction of hypoxic genes. The Journal of biological chemistry 2002, Vol. 277, No. 38, pp. 34773–34784.

148. Jamieson, D.J. *Saccharomyces cerevisiae* has distinct adaptive responses to both hydrogen peroxide and menadione. Journal of bacteriology 1992, Vol. 174, No. 20, pp. 6678–6681.

149. Oura, E. Effect of aeration intensity on the biochemical composition of baker's yeast. I. Factors affecting the type of metabolism. Biotechnology and bioengineering 1974, Vol. 16, No. 9, pp. 1197–1212.

150. Collinson, L.P. & Dawes, I.W. Inducibility of the response of yeast cells to peroxide stress. Journal of general microbiology 1992, Vol. 138, No. 2, pp. 329–335.

151. Davies, J.M., Lowry, C.V. & Davies, K.J. Transient adaptation to oxidative stress in yeast. Archives of biochemistry and biophysics 1995, Vol. 317, No. 1, pp. 1–6.

152. Jamieson, D.J., Rivers, S.L. & Stephen, D.W. Analysis of *Saccharomyces cerevisiae* proteins induced by peroxide and superoxide stress. Microbiology (Reading, England) 1994, Vol. 140, No. Pt 12, pp. 3277–3283.

153. Flattery-O'Brien, J., Collinson, L.P. & Dawes, I.W. *Saccharomyces cerevisiae* has an inducible response to menadione which differs from that to hydrogen peroxide. Journal of general microbiology 1993, Vol. 139, No. 3, pp. 501–507.

154. Juhnke, H., Krems, B., Kotter, P. & Entian, K.D. Mutants that show increased sensitivity to hydrogen peroxide reveal an important role for the pentose phosphate pathway in protection of yeast against oxidative stress. Molecular & general genetics 1996, Vol. 252, No. 4, pp. 456–464.

155. Causton, H.C., Ren, B., Koh, S.S., Harbison, C.T., Kanin, E., Jennings, E.G., Lee, T.I., True, H.L., Lander, E.S. & Young, R.A. Remodeling of yeast genome expression in response to environmental changes. Molecular biology of the cell 2001, Vol. 12, No. 2, pp. 323–337.

156. Morgan, B.A., Banks, G.R., Toone, W.M., Raitt, D., Kuge, S. & Johnston, L.H. The Skn7 response regulator controls gene expression in the oxidative stress response of the budding yeast *Saccharomyces cerevisiae*. The EMBO journal 1997, Vol. 16, No. 5, pp. 1035–1044.

157. Kuge, S., Jones, N. & Nomoto, A. Regulation of yAP-1 nuclear localization in response to oxidative stress. The EMBO journal 1997, Vol. 16, No. 7, pp. 1710–1720.

158. Snoek, I.S. & Steens ma, H.Y. W hy do es *Kluyver omyces lactis* not grow un der anaerobic con ditions? Com parison of esse ntial a naerobic genes of *Saccharomyces cerevisiae* with the *Kluyveromyces lactis* genome. FEMS yeast research 2006, Vol. 6, No. 3, pp. 393–403.

159. Salusjärvi, L., Poutanen, M., Pitkänen, J.P., Koivistoinen, H., Aristid ou, A., Kalkkinen, N., Ruo honen, L. & Penttilä, M. Pr oteome an alysis of recombi nant xylos e-fermenting *Saccharomyces cerevisiae*. Yeast (Chichester, England) 2003, Vol. 20, No. 4, pp. 295–314.

160. Tai, S.L., Boer, V.M., Daran-Lapujade, P., W alsh, M.C., de W inde, J.H., Daran, J.M. & Pronk, J.T. Two-dimensional transcriptome an alysis in chemostat cultures. Combinatorial effects of oxygen availability and macr onutrient lim itation in *Saccharomyces cerevisiae*. The Journal of biological chemistry 2005, Vol. 280, No. 1, pp. 437–447.

161. ter Linde, J.J., Liang, H., Davis, R.W., Steensma, H.Y., van Dijken , J.P. & Pronk, J.T. Genome- wide transcr iptional an alysis of aerob ic and anaer obic ch emostat cultures of *Saccharomyces cerevisiae*. Journal of bacteriology 1999, Vol. 181, No. 24, pp. 7409–7413.

162. Piper, M.D., Daran-Lapujade, P., Bro, C., Regenberg, B., Knudsen, S., Nielsen, J. & Pronk, J.T. Reproducibility of oligonucleotide microarray transcriptome analyses. An interl aboratory comparison usin g chem ostat cultures of *Saccharomyces cerevisiae*. T he Jo urnal of bio logical ch emistry 2002, Vol. 27 7, No. 40, pp. 3700 1–37008.

163. Lai, L.C., Kosorukoff, A.L., Burke, P.V. & Kwast, K.E. Dynamical remodeling of the transcriptome during short-term anaerobiosis in *Saccharomyces cerevisiae*: differential respo nse and rol e of Msn2 an d/or Msn4 and other factors in gal actose and glucose media. Mol Cell Biol 2005, Vol. 25, No. 10, pp. 4075–4091.

164. Lai, L.C., Kosorukoff, A.L., Burke, P .V. & K wast, K.E. Metabolic-s tate-dependent remodeling of the transcriptome in response to anoxia and subsequent reoxygenation in *Saccharomyces cerev isiae*. Euk aryotic cell 2 006, Vol. 5, No. 9, p p. 146 8–1489.

165. Lai, L.C., Kissinger, M.T., Burke, P.V. & K wast, K.E. Compar ison of the transcr iptomic ' stress respo nse' evok ed b y antimycin A an d o xygen d eprivation in *Saccharomyces cerevisiae*. BMC genomics 2008, Vol. 9, No. 1, pp. 627.

166. Kundaje, A., Xin, X., Lan, C., Lia noglou, S., Z hou, M., Zhang, L. & Leslie, C. A predictive model of the oxygen and heme regulatory network in yeast. PLoS computational biology 2008, Vol. 4, No. 11, pp. e1000224.

167. Salusjärvi, L., Kankainen, M., Soliymani, R., Pitkänen, J.P., Penttilä, M. & Ruohonen, L. Regulation of xylose metabolism in recombinant *Saccharomyces cerevisiae*. Microbial cell factories 2008, Vol. 7, pp. 18.

168. Bruckmann, A., Hensbergen, P.J., Balog, C.I., Deelder, A.M., Brandt, R., Snoek, I.S., Steensma, H.Y. & van Heusden, G.P. Proteome analysis of aerobically and anaerobically grown *Saccharomyces cerevisiae* cells. Journal of proteomics 2009, Vol. 71, No. 6, pp. 662–669.

169. Schuller, H.J. Transcriptional control of nonfermentative metabolism in the yeast *Saccharomyces cerevisiae*. Current genetics 2003, Vol. 43, No. 3, pp. 139–160.

170. van Urk, H., Postma, E., Scheffers, W.A. & van Dijken, J.P. Glucose transport in crabtree-positive and crabtree-negative yeasts. Journal of general microbiology 1989, Vol. 135, No. 9, pp. 2399–2406.

171. Weusthuis, R.A., Pronk, J.T., van den Broek, P.J. & van Dijken, J.P. Chemostat cultivation as a tool for studies on sugar transport in yeasts. Microbiological reviews 1994, Vol. 58, No. 4, pp. 616–630.

172. Gasnier, B. Characterization of low- and high-affinity glucose transports in the yeast *Kluyveromyces marxianus*. Biochimica et biophysica acta 1987, Vol. 903, No. 3, pp. 425–433.

173. Kruckeberg, A.L. The hexose transporter family of *Saccharomyces cerevisiae*. Archives of microbiology 1996, Vol. 166, No. 5, pp. 283–292.

174. Özcan, S., Dover, J., Rosenwald, A.G., Wolfl, S. & Johnston, M. Two glucose transporters in *Saccharomyces cerevisiae* are glucose sensors that generate a signal for induction of gene expression. Proceedings of the National Academy of Sciences of the United States of America 1996, Vol. 93, No. 22, pp. 12428–12432.

175. Reifenberger, E., Boles, E. & Ciriacy, M. Kinetic characterization of individual hexose transporters of *Saccharomyces cerevisiae* and their relation to the triggering mechanisms of glucose repression. European journal of biochemistry 1997, Vol. 245, No. 2, pp. 324–333.

176. Verwaal, R., Arako, M., Kapur, R., Verkleij, A.J., Verrips, C.T. & Boonstra, J. *HXT5* expression is under control of STRE and HAP elements in the *HXT5* promoter. Yeast 2004, Vol. 21, No. 9, pp. 747–757.

177. Diderich, J.A., Schuurmans, J.M., Van Gaalen, M.C., Kruckeberg, A.L. & Van Dam, K. Functional analysis of the hexose transporter homologue *HXT5* in *Saccharomyces cerevisiae*. Yeast 2001, Vol. 18, No. 16, pp. 1515–1524.

178. Wieczorke, R., Krampe, S., Weierstall, T., Freidel, K., Hollenberg, C.P. & Boles, E. Concurrent knock-out of at least 20 transporter genes is required to block uptake of hexoses in *Saccharomyces cerevisiae*. FEBS letters 1999, Vol. 464, No. 3, pp. 123–128.

179. Reifenberger, E., Freidel, K. & Ciriacy, M. Identification of novel *HXT* genes in *Saccharomyces cerevisiae* reveals the impact of individual hexose transporters on glycolytic flux. Molecular microbiology 1995, Vol. 16, No. 1, pp. 157–167.

180. Liang, H. & Gaber, R.F. A novel signal transduction pathway in *Saccharomyces cerevisiae* defined by Snf3-regulated expression of *HXT6*. Molecular biology of the cell 1996, Vol. 7, No. 12, pp. 1953–1966.

181. Özcan, S. Two different signals regulate repression and induction of gene expression by glucose. The Journal of biological chemistry 2002, Vol. 277, No. 49, pp. 46993–46997.

182. Özcan, S., Dover, J., Rosenwald, A.G., Wolfl, S. & Johnston, M. Two glucose transporters in *Saccharomyces cerevisiae* are glucose sensors that generate a signal for induction of gene expression. Proceedings of the National Academy of Sciences of the United States of America 1996, Vol. 93, No. 22, pp. 12428–12432.

183. Özcan, S. & Johnston, M. Function and regulation of yeast hexose transporters. Microbiology and molecular biology reviews 1999, Vol. 63, No. 3, pp. 554–569.

184. Özcan, S., Dover, J. & Johnston, M. Glucose sensing and signaling by two glucose receptors in the yeast *Saccharomyces cerevisiae*. Embo journal 1998, Vol. 17, No. 9, pp. 2566–2573.

185. Schmidt, M.C., McCartney, R.R., Zhang, X., Tillman, T.S., Solimeo, H., Wolfl, S., Almonte, C. & Watkins, S.C. Std1 and Mth1 proteins interact with the glucose sensors to control glucose-regulated gene expression in *Saccharomyces cerevisiae*. Molecular and cellular biology 1999, Vol. 19, No. 7, pp. 4561–4571.

186. Kim, J.H., Brachet, V., Moriya, H. & Johnston, M. Integration of transcriptional and posttranslational regulation in a glucose signal transduction pathway in *Saccharomyces cerevisiae*. Eukaryot cell 2006, Vol. 5, No. 1, pp. 167–173.

187. Lakshmanan, J., Mosley, A.L. & Ozcan, S. Repression of transcription by Rgt1 in the absence of glucose requires Std1 and Mth1. Current genetics 2003, Vol. 44, No. 1, pp. 19–25.

188. Kim, J.H. & Johnston, M. Two glucose-sensing pathways converge on Rgt1 to regulate expression of glucose transporter genes in *Saccharomyces cerevisiae*. Journal of biological chemistry 2006, Vol. 281, No. 36, pp. 26144–26149.

189. Özcan, S., Leong, T. & Johnston, M. Rgt1p of *Saccharomyces cerevisiae*, a key regulator of glucose-induced genes, is both an activator and a repressor of transcription. Molecular and cellular biology 1996, Vol. 16, No. 11, pp. 6419–6426.

190. Kaniak, A., Xue, Z., Macool, D., Kim, J.H. & Johnston, M. Regulatory network connecting two glucose signal transduction pathways in *Saccharomyces cerevisiae*. Eukaryot cell 2004, Vol. 3, No. 1, pp. 221–231.

191. Diderich, J.A., Schepper, M., van Hoek, P., Luttik, M.A., van Dijken, J.P., Pronk, J.T., Klaassen, P., Boelens, H.F., de Mattos, M.J., van Dam, K. & Kruckeberg, A.L. Glucose uptake kinetics and transcription of *HXT* genes in chemostat cultures of *Saccharomyces cerevisiae*. J Biol Chem 1999, Vol. 274, No. 22, pp. 15350–15359.

192. Nourani, A., Wesolowski-Louvel, M., Delaveau, T., Jacq, C. & Delahodde, A. Multiple-drug-resistance phenomenon in the yeast *Saccharomyces cerevisiae*: involvement of two hexose transporters. Molecular and cellular biology 1997, Vol. 17, No. 9, pp. 5453–5460.

193. Liu, Z., Boles, E. & Rosen, B.P. Arsenic trioxide uptake by hexose permeases in *Saccharomyces cerevisiae*. The Journal of biological chemistry 2004, Vol. 279, No. 17, pp. 17312–17318.

194. Gross, C., Kelleher, M., Iyer, V.R., Brown, P.O. & Winge, D.R. Identification of the copper regulon in *Saccharomyces cerevisiae* by DNA microarrays. The Journal of biological chemistry 2000, Vol. 275, No. 41, pp. 32310–32316.

195. Jungmann, J., Reins, H.A., Lee, J., Romeo, A., Hassett, R., Kosman, D. & Jentsch, S. MAC1, a nuclear regulatory protein related to Cu-dependent transcription factors is involved in Cu/Fe utilization and stress resistance in yeast. The EMBO journal 1993, Vol. 12, No. 13, pp. 5051–5056.

196. Greatrix, B.W. & van Vuuren, H.J. Expression of the *HXT13, HXT15* and *HXT17* genes in *Saccharomyces cerevisiae* and stabilization of the *HXT1* gene transcript by sugar-induced osmotic stress. Current genetics 2006, Vol. 49, No. 4, pp. 205–217.

197. Wiebe, M., Rintala, Eija, Tamminen, Anu, Simolin, H., Salusjärvi, L., Toivari, M., Jouhten, P., Huuskonen, A., Maaheimo, H., Ruohonen, L. & Penttilä, M. The response of anaerobically grown *Saccharomyces cerevisiae* to low and high oxygen in glucose–limited chemostat cultures. Helsinki, Finland: 2007, June 13– 16, p. 116.

198. Köhler, T., Wesche, S., Taheri, N., Braus, G.H. & Mosch, H.U. Dual role of the *Saccharomyces cerevisiae* TEA/ATTS family transcription factor Tec1p in regula-

tion of gene expression and cellular development. Eukaryotic cell 2002, Vol. 1, No. 5, pp. 673–686.

199. Dolan, J.W., Kirkman, C. & Fields, S. The yeast STE12 protein binds to the DNA sequence mediating pheromone induction. Proceedings of the National Academy of Sciences of the United States of America 1989, Vol. 86, No. 15, pp. 5703–5707.

200. Karpichev, I.V. & Small, G.M. Global regulatory functions of Oaf1p and Pip2p (Oaf2p), transcription factors that regulate genes encoding peroxisomal proteins in *Saccharomyces cerevisiae*. Molecular and cellular biology 1998, Vol. 18, No. 11, pp. 6560–6570.

201. Cohen, B.D., Sertil, O., Abramova, N.E., Davies, K.J. & Lowry, C.V. Induction and repression of *DAN1* and the family of anaerobic mannoprotein genes in *Saccharomyces cerevisiae* occurs through a complex array of regulatory sites. Nucleic acids research 2001, Vol. 29, No. 3, pp. 799–808.

202. Mai, B. & Breeden, L. Xbp1, a stress-induced transcriptional repressor of the *Saccharomyces cerevisiae* Swi4/Mbp1 family. Molecular and cellular biology 1997, Vol. 17, No. 11, pp. 6491–6501.

203. Martinez-Pastor, M.T., Marchler, G., Schuller, C., Marchler-Bauer, A., Ruis, H. & Estruch, F. The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). The EMBO journal 1996, Vol. 15, No. 9, pp. 2227–2235.

204. Zhang, N., Wu, J. & Oliver, S.G. Gis1 is required for transcriptional reprogramming of carbon metabolism and the stress response during transition into stationary phase in yeast. Microbiology (Reading, England) 2009, Vol. 155, No. Pt 5, pp. 1690–1698.

205. Oliveira, A.P., Patil, K.R. & Nielsen, J. Architecture of transcriptional regulatory circuits is knitted over the topology of bio-molecular interaction networks. BMC systems biology 2008, Vol. 2, pp. 17.

206. Patil, K.R. & Nielsen, J. Uncovering transcriptional regulation of metabolism by using metabolic network topology. Proceedings of the National Academy of Sciences of the United States of America 2005, Vol. 102, No. 8, pp. 2685–2689.

207. Jouhten, P., Rintala, E., Huuskonen, A., Tamminen, A., Toivari, M., Wiebe, M., Ruohonen, L., Penttila, M. & Maaheimo, H. Oxygen dependence of metabolic fluxes and energy generation of *Saccharomyces cerevisiae* CEN.PK113-1A. BMC systems biology 2008, Vol. 2, p. 60.

208. Braun, E. & Brenner, N. Transient responses and adaptation to steady state in a eukaryotic gene regulation system. Physical biology 2004, Vol. 1, No. 1–2, pp. 67–76.

209. Rautio, J.J., Smit, B.A., Wiebe, M., Penttila, M. & Saloheimo, M. Transcriptional monitoring of steady state and effects of anaerobic phases in chemostat cultures of the filamentous fungus Trichoderma reesei. BMC genomics 2006, Vol. 7, p. 247.

210. Davies, S.E. & Brindle, K.M. Effects of overexpression of phosphofructokinase on glycolysis in the yeast *Saccharomyces cerevisiae*. Biochemistry 1992, Vol. 31, No. 19, pp. 4729–4735.

211. Ernandes, J.R., De Meirsman, C., Rolland, F., Winderickx, J., de Winde, J., Brandao, R.L. & Thevelein, J.M. During the initiation of fermentation overexpression of hexokinase PII in yeast transiently causes a similar deregulation of glycolysis as deletion of Tps1. Yeast (Chichester, England) 1998, Vol. 14, No. 3, pp. 255–269.

212. Fell, D.A. & Thomas, S. Physiological control of metabolic flux: the requirement for multisite modulation. The Biochemical journal 1995, Vol. 311 (Pt 1), No. Pt 1, pp. 35–39.

213. Reijenga, K.A., Snoep, J.L., Diderich, J.A., van Verseveld, H.W., Westerhoff, H.V. & Teusink, B. Control of glycolytic dynamics by hexose transport in *Saccharomyces cerevisiae*. Biophysical journal 2001, Vol. 80, No. 2, pp. 626–634.

214. Fiaux, J., Cakar, Z.P., Sonderegger, M., Wuthrich, K., Szyperski, T. & Sauer, U. Metabolic-flux profiling of the yeasts *Saccharomyces cerevisiae* and *Pichia stipitis*. Eukaryotic cell 2003, Vol. 2, No. 1, pp. 170–180.

215. Franzen, C.J. Metabolic flux analysis of RQ-controlled microaerobic ethanol production by *Saccharomyces cerevisiae*. Yeast (Chichester, England) 2003, Vol. 20, No. 2, pp. 117–132.

216. van Winden, W.A., van Dam, J.C., Ras, C., Kleijn, R.J., Vinke, J.L., van Gulik, W.M. & Heijnen, J.J. Metabolic–flux analysis of *Saccharomyces cerevisiae* CEN.PK113–7D based on mass isotopomer measurements of (13)C–labeled primary metabolites. FEMS yeast research 2005, Vol. 5, No. 6–7, pp. 559–568.

217. Hohmann, S., Bell, W., Neves, M.J., Valckx, D. & Thevelein, J.M. Evidence for trehalose-6-phosphate-dependent and -independent mechanisms in the control of sugar influx into yeast glycolysis. Molecular microbiology 1996, Vol. 20, No. 5, pp. 981–991.

218. Parrou, J.L., Teste, M.A. & Francois, J. Effects of various types of stress on the metabolism of reserve carbohydrates in *Saccharomyces cerevisiae*: genetic evidence for a stress-induced recycling of glycogen and trehalose. Microbiology (Reading, England) 1997, Vol. 143 (Pt 6), No. Pt 6, pp. 1891–1900.

219. Hottiger, T., Schmutz, P. & Wiemken, A. Heat-induced accumulation and futile cycling of trehalose in *Saccharomyces cerevisiae*. Journal of bacteriology 1987, Vol. 169, No. 12, pp. 5518–5522.

220. Villas-Boas, S.G., Moxley, J.F., Akesson, M., Stephanopoulos, G. & Nielsen, J. High-throughput metabolic state analysis: the missing link in integrated functional genomics of yeasts. Biochemical journal 2005, Vol. 388, No. Pt 2, pp. 669–677.

221. Epstein, C.B., Waddle, J.A., Hale, W.,4th, Dave, V., Thornton, J., Macatee, T.L., Garner, H.R. & Butow, R.A. Genome-wide responses to mitochondrial dysfunction. Molecular biology of the cell 2001, Vol. 12, No. 2, pp. 297–308.

222. Krems, B., Charizanis, C. & Entian, K. D. Mutants of *Saccharomyces cerevisiae* sensitive to oxidative and osmotic stress. Current genetics 1995, Vol. 27, No. 5, pp. 427–434.

223. Nogae, I. & Johnston, M. Isolation and characterization of the *ZWF1* gene of *Saccharomyces cerevisiae* , encoding glucose-6-phosphate dehydrogenase. Gene 1990, Vol. 96, No. 2, pp. 161–169.

224. Brauer, M.J., Huttenhower, C., Airoldi, E.M., Rosenstein, R., Matese, J.C., Gresham, D., Boer, V.M., Troyanskaya, O.G. & Botstein, D. Coordination of growth rate, cell cycle, stress response, and metabolic activity in yeast. Molecular biology of the cell 2008, Vol. 19, No. 1, pp. 352–367.

225. Regenberg, B., Grotkjaer, T., Winther, O., Fausboll, A., Åkesson, M., Bro, C., Hansen, L.K., Brunak, S. & Nielsen, J. Growth-rate regulated genes have profound impact on interpretation of transcriptome profiling in *Saccharomyces cerevisiae*. Genome biology 2006, Vol. 7, No. 11, p. R107.

226. Fazio, A., Jewett, M.C., Daran-Lapujade, P., Mustacchi, R., Usaite, R., Pronk, J.T., Workman, C.T. & Nielsen, J. Transcription factor control of growth rate dependent genes in *Saccharomyces cerevisiae*: a three factor design. BMC genomics 2008, Vol. 9, p. 341.

227. Levy, S., Ihmels, J., Carmi, M., Weinberger, A., Friedlander, G. & Barkai, N. Strategy of transcription regulation in the budding yeast. PLoS ONE 2007, Vol. 2, No. 2, p. e250.

228. Grigull, J., Mnaimneh, S., Pootoolal, J., Robinson, M.D. & Hughes, T.R. Genome-wide analysis of mRNA stability using transcription inhibitors and microarrays reveals posttranscriptional control of ribosome biogenesis factors. Molecular and cellular biology 2004, Vol. 24, No. 12, pp. 5534–5547.

229. Lindquist, S. Regulation of protein synthesis during heat shock. Nature 1981, Vol. 293, No. 5830, pp. 311–314.

230. Romero-Santacreu, L., Moreno, J., Perez-Ortin, J.E. & Alepuz, P. Specific and global regulation of mRNA stability during osmotic stress in *Saccharomyces cerevisiae*. RNA (New York, N.Y.) 2009, Vol. 15, No. 6, pp. 1110–1120.

231. Gerber, A.P., Herschlag, D. & Brown, P.O. Extensive association of functionally and cytotopically related mRNAs with Puf family RNA-binding proteins in yeast. PLoS biology 2004, Vol. 2, No. 3, p. E79.

232. Shianna, K.V., Dotson, W.D., Tove, S. & Parks, L.W. Identification of a UPC2 homolog in *Saccharomyces cerevisiae* and its involvement in aerobic sterol uptake. Journal of bacteriology 2001, Vol. 183, No. 3, pp. 830–834.

233. Rosenfeld, E., Beauvoit, B., Blondin, B. & Salmon, J.M. Oxygen consumption by anaerobic *Saccharomyces cerevisiae* under enological conditions: effect on fermentation kinetics. Applied and environmental microbiology 2003, Vol. 69, No. 1, pp. 113–121.

234. Gonzalez, B., de Graaf, A., Renaud, M. & Sahm, H. Dynamic in vivo (31)P nuclear magnetic resonance study of *Saccharomyces cerevisiae* in glucose-limited chemostat culture during the aerobic-anaerobic shift. Yeast (Chichester, England) 2000, Vol. 16, No. 6, pp. 483–497.

235. Wang, Y., Barbacioru, C., Hyland, F., Xiao, W., Hunkapiller, K.L., Blake, J., Chan, F., Gonzalez, C., Zhang, L. & Samaha, R.R. Large scale real-time PCR validation on gene expression measurements from two commercial long-oligonucleotide microarrays. BMC genomics 2006, Vol. 7, pp. 59.

236. Etienne, W., Meyer, M.H., Peppers, J. & Meyer, R.A.,Jr Comparison of mRNA gene expression by RT-PCR and DNA microarray. BioTechniques 2004, Vol. 36, No. 4, pp. 618–620, 622, 624–626.

237. Rajeevan, M.S., Vernon, S.D., Taysavang, N. & Unger, E.R. Validation of array-based gene expression profiles by real-time (kinetic) RT-PCR. The Journal of molecular diagnostics 2001, Vol. 3, No. 1, pp. 26–31.

Publication I

Central carbon metabolism of *Saccharomyces cerevisiae* in anaerobic, oxygen-limited and fully aerobic steady-state conditions and following a shift to anaerobic conditions

In: FEMS Yest Research 2007: 8(1).
Pp. 140–157.

Central carbon metabolism of *Saccharomyces cerevisiae* in anaerobic, oxygen-limited and fully aerobic steady-state conditions and following a shift to anaerobic conditions

Marilyn G. Wiebe[1], Eija Rintala[1], Anu Tamminen[1], Helena Simolin[1], Laura Salusjärvi[1], Mervi Toivari[1], Juha T. Kokkonen[1], Jari Kiuru[1], Raimo A. Ketola[2], Paula Jouhten[1], Anne Huuskonen[1], Hannu Maaheimo[1], Laura Ruohonen[1] & Merja Penttilä[1]

[1]VTT Technical Research Centre of Finland, Finland; and [2]Drug Discovery and Development Technology Center, Division of Pharmaceutical Chemistry, University of Helsinki, Helsinki, Finland

Correspondence: Marilyn G. Wiebe, VTT Technical Research Centre of Finland, PO Box 1000, FI-02044 VTT, Finland. Tel.: + 358 20 722 5139; fax: + 358 20 722 2103; e-mail: marilyn.wiebe@vtt.fi

Received 30 November 2006; revised 8 February 2007; accepted 13 February 2007.

DOI:10.1111/j.1567-1364.2007.00234.x

Editor: Teun Boekhout

Keywords
Saccharomyces cerevisiae; oxygen; hypoxic transient; gene transcription; metabolites; systems biology.

Abstract

Saccharomyces cerevisiae CEN.PK113-1A was grown in glucose-limited chemostat culture with 0%, 0.5%, 1.0%, 2.8% or 20.9% O_2 in the inlet gas ($D = 0.10\ h^{-1}$, pH 5, 30 °C) to determine the effects of oxygen on 17 metabolites and 69 genes related to central carbon metabolism. The concentrations of tricarboxylic acid cycle (TCA) metabolites and all glycolytic metabolites except 2-phosphoglycerate + 3-phosphoglycerate and phosphoenolpyruvate were higher in anaerobic than in fully aerobic conditions. Provision of only 0.5–1% O_2 reduced the concentrations of most metabolites, as compared with anaerobic conditions. Transcription of most genes analyzed was reduced in 0%, 0.5% or 1.0% O_2 relative to cells grown in 2.8% or 20.9% O_2. Ethanol production was observed with 2.8% or less O_2. After steady-state analysis in defined oxygen concentrations, the conditions were switched from aerobic to anaerobic. Metabolite and transcript levels were monitored for up to 96 h after the transition, and this showed that more than 30 h was required for the cells to fully adapt to anaerobiosis. Levels of metabolites of upper glycolysis and the TCA cycle increased following the transition to anaerobic conditions, whereas those of metabolites of lower glycolysis generally decreased. Gene regulation was more complex, with some genes showing transient upregulation or downregulation during the adaptation to anaerobic conditions.

Introduction

The physiology of *Saccharomyces cerevisiae* under fermentative, respiratory and respirofermentative conditions has always attracted considerable attention, both because it is one of the few eukaryotic organisms that can grow in truly anaerobic conditions, and because of the industrial importance of growth in anaerobic or aerobic conditions for the production of ethanol, proteins, cell biomass, and other products. The ability of *S. cerevisiae* to ferment glucose to ethanol even in aerobic conditions (the Crabtree effect) has made the understanding of respirofermentative growth important for optimizing aerobic processes in which ethanol is an undesirable byproduct. Furthermore, the development of *S. cerevisiae* as a host organism for the production of novel products such as lactic acid or 3-hydroxypropanoic acid may lead to an increase in the application of well-controlled, oxygen-restricted industrial processes, because the energy balance is probably negative under completely anaerobic conditions, whereas high oxygen concentrations lead to a loss in product yield (van Maris *et al.*, 2004). Inadequate mixing in high-cell-density and very high-cell-density cultures may result in regions of very low oxygen provision, to which processes operating under oxygen-restricted conditions will be particularly vulnerable, and it is important to understand how cells respond to small changes in oxygen concentration and variable oxygen provision.

Although respirofermentative physiology and the transition between respirofermentative and purely fermentative growth can be studied in batch cultures (e.g. Burke *et al.*, 1997; Lai *et al.*, 2006), it is difficult to separate the effects of changing nutrient concentrations and specific growth rate from the effects of oxygen provision. Furthermore, purely

respiratory growth in batch cultures is obtained by growing cells on nonfermentable or poorly fermentable carbon sources, adding carbon source to the variables. Respirofermentative physiology has also been obtained by growing cells in glucose-limited chemostat cultures, in which respirofermentative growth occurs at high specific growth rates, and respiratory growth at low specific growth rates (e.g. Frick & Wittmann, 2005). However, in this case, respirofermentative physiology is not distinguished from growth rate-related physiology. Franzén (2003) determined metabolic fluxes in *S. cerevisiae* in anaerobic and very low oxygen [0.1–2 mmol O_2 (g biomass)$^{-1}$ h^{-1}] continuous flow cultures at various dilution rates, but there is a general lack of information on the effects of low oxygen concentration on yeast cell physiology in constant conditions.

In order to understand the response of *S. cerevisiae* to oxygen, we assessed the physiology of CEN.PK113-1A cells in terms of 17 metabolites and 69 genes related to central carbon metabolism during glucose-limited steady states with various concentrations of provided oxygen. After assessment of the steady states, anaerobic conditions were imposed in the same cultures to determine how the cells achieved their new, anaerobic steady state. By maintaining the cells under otherwise constant conditions, the effects of glucose repression and changes in specific growth rate that occur in batch cultures were minimized.

This work also highlights some of the problems and possibilities of identifying biomarkers for aerobic/anaerobic physiology. The increasing demand for information on microbial physiology that can be obtained directly from a production system, and then used to optimize the actual production process, requires the identification of one or a few marker genes or metabolites that would be indicative of the critical physiology for the specific process. Data obtained from both steady-state and transient conditions will be useful for their identification.

Materials and methods

Strain and medium

Saccharomyces cerevisiae CEN.PK113-1A (*MATα*, *URA3*, *HIS3*, *LEU2*, *TRP1*, *MAL2-8c*, *SUC2*) was kindly provided by Dr P. Kötter (Institut für Mikrobiologie, J.W. Goethe Universität Frankfurt, Germany; de Jong-Gubbels *et al.*, 1998) and stored in glycerol (30% v/v) at $-80\,^{\circ}$C.

Yeasts were grown in the defined minimal medium described by Verduyn *et al.* (1992), with 10 g glucose L^{-1} as carbon source, and supplemented with 10 mg ergosterol L^{-1} and 420 mg Tween-80 L^{-1}. BDH silicone antifoam (0.5 mL L^{-1}) was used to prevent foam production in the cultures.

Culture conditions

Cells were grown in 0.8–1 L of medium in B. Braun Biotech International (Sartorius AG, Germany) Biostat CT (2.5 L working volume) bioreactors. Cultures were inoculated to an initial $OD_{600\,nm}$ of *c.* 0.5, and maintained as batch cultures for 6–9 h, after which continuous medium feed was started while the cells were still growing exponentially. Chemostat cultures were maintained at $D = 0.10 \pm 0.02$ h^{-1}, pH 5.0, and 30 °C, with 1.5 volume gas [volume culture]$^{-1}$ min^{-1} (vvm). For cultures that received $< 20.9\%$ O_2 in the gas stream, O_2 was replaced with the equivalent volume of N_2, so that total gas flow was kept constant for all experiments. The k_La (overall oxygen transfer coefficient) for the bioreactor under these conditions was 0.035–0.039 s^{-1} (in pure water), and the solubility of oxygen under these conditions is given in Table 1, to facilitate comparison with other published data. The dissolved oxygen tension in cultures receiving 20.9% O_2 was 83%, but it was 0% in cultures receiving 2.8% O_2 or less. Steady-state samples were taken after the cultures had been in constant conditions for a minimum of four residence times (six generations). Steady states were assessed over four to nine residence times (six to 13 generations) for constant biomass production, carbon dioxide evolution and oxygen uptake rates (CER and OUR), alkali utilization, and extracellular metabolites, as well as constant intracellular metabolites and gene transcription.

Cultures that were fed 2.8% or 20.9% O_2 were subject to oscillations. To prevent these, *c.* 5% of the total cell concentration in the bioreactor was added to the culture as cells in mid-exponential to late exponential phase at the time when continuous medium feed was started (Zamamiri *et al.*, 2001).

The gas concentration (CO_2, $^{13}CO_2$, O_2, N_2, and Ar) was analyzed continuously in an Omnistar quadrupole mass spectrometer (Balzers AG, Liechtenstein), calibrated with 3% CO_2 in Ar. Washout kinetics were determined as described by Esener *et al.* (1981).

Biomass determination

Biomass was measured as $OD_{600\,nm}$ and as cell dry weight (DW). For DW determination, cells were collected by centrifugation and washed with one to two sample volumes of distilled water. Duplicate (5 mL) or triplicate (2 mL) samples were taken for all DW measurements. Cells were dried to a constant weight at 100 °C.

Metabolite and chemical analyses

Extracellular metabolites (ethanol, glycerol, pyruvate, and acetate) and glucose were analyzed by HPLC on an Aminex HPX-87 H column (BioRad Laboratories, Hercules, CA) with 2.5 mM H_2SO_4 as eluant and a flow rate of

Table 1. Biomass concentration, yield on glucose (moles of carbon, Cmol, in biomass per mole of carbon in substrate) and specific rates for oxygen uptake (OUR), glucose consumption, carbon dioxide evolution (CER), and ethanol and glycerol production in steady-state glucose-limited chemostat cultures ($D = 0.10\,h^{-1}$, pH 5.0, 30 °C, 1.5 vvm gas flow) of *Saccharomyces cerevisiae* CEN.PK113-1A

O_2 provided (%)	O_2 solubility (μM)*	Biomass ($g\,L^{-1}$)	Yield ($_{X/C}$) ($Cmol\,Cmol^{-1}$)	Specific OUR [$mmol\,(g\,DW)^{-1}\,h^{-1}$]	Specific CER [$mmol\,(g\,DW)^{-1}\,h^{-1}$]	Specific glucose consumption rate [$Cmmol\,(g\,DW)^{-1}\,h^{-1}$]	Specific ethanol production rate [$Cmmol\,(g\,DW)^{-1}\,h^{-1}$]	Specific glycerol production rate [$Cmmol\,(g\,DW)^{-1}\,h^{-1}$]†
20.9	250	5.0 ± 0.05	0.60 ± 0.01	2.7 ± 0.04	2.6 ± 0.03	6.6 ± 0.5	0	ND
2.8	34	4.8 ± 0.05	0.56 ± 0.01	2.5 ± 0.04	3.0 ± 0.03	8.0 ± 0.3	0.2 ± 0.01	ND
1.0	12	3.0 ± 0.03	0.36 ± 0.01	1.7 ± 0.02	3.7 ± 0.04	11.4 ± 0.5	3.2 ± 0.2	ND
0.5	6	2.1 ± 0.02	0.27 ± 0.01	1.2 ± 0.02	4.6 ± 0.06	14.3 ± 1.1	5.5 ± 0.5	ND
0	0	1.0 ± 0.02	0.12 ± 0.03	0	11.3 ± 0.30	37.1 ± 3.0	16.7 ± 1.6	3.0 ± 0.3

Values are mean ± SEM ($n = 33$–88 for biomass and yield, 658–4199 for OUR and CER, 11–23 for ethanol and glycerol, and 13–24 for glucose, from steady states during two or four cultivations, using compound SEM for specific rates). Cmmol, mmole carbon.
*Solubility of O_2 in pure water, provided for comparison with, for example, Lai *et al.* (2005).
†ND indicates not detectable, i.e. < 0.04 g glycerol L^{-1} and < 0.03–0.06 Cmmol $(g\,DW)^{-1}\,h^{-1}$, depending on biomass concentration.

0.5 mL min^{-1}. The column was maintained at 55 °C. Peaks were detected using a Waters 410 differential refractometer and a Waters 2487 dual-wavelength UV (210 nm) detector. Glucose concentrations were also sometimes determined enzymatically using the Roche (Germany) Glucose GOD-PAP measurement kit (Cat. no. 1448676 216).

Intracellular metabolites were extracted from cells that had been transferred to 60% v/v methanol at −40 °C immediately after their removal from the bioreactor, collected by centrifugation at 2000 ***g*** at −19 °C for 5 min, washed once with 60% v/v cold (−40 °C) methanol at −19 °C (de Koning & van Dam, 1992), frozen in liquid N_2, and stored at −80 °C. Metabolites were subsequently extracted in boiling ethanol (Gonzalez *et al.*, 1997), and analyzed by liquid chromatography (LC)-MS/MS (van Dam *et al.*, 2002) using a Waters HT-Alliance HPLC coupled with a Micromass Quattro Micro triple quadrupole mass spectrometer. Internal standards were derived from a [^{13}C]glucose fed-batch culture, as described by Wu *et al.* (2005).

Adenosine nucleotides (ATP, ADP, and AMP) were separated and quantified using ion-pairing LC–electrospray ionization (ESI)–MS with diisopropyl amine (DIPA) as the ion-pairing reagent. HPLC was carried out in an Agilent 1100 (Santa Clara, CA) with an Xterra MS C_{18} (1 × 150 mm) column (Waters, Milford MA). Mobile phases were: (1) 60 mM DIPA (pH 7) (solution A), and (2) methanol/DIPA (6 mM) 80 : 20 (pH 7) (solution B), the pH being adjusted with formic acid. Elution was carried out with 5% (v/v) solution B at 80 μL min^{-1}, for a 5-μL injection volume. Isopropanol (40 μL min^{-1}) was then added to enhance the ionization efficiency in the mass spectrometer. The nucleotides were detected by ESI-MS (positive ionization mode) with a Micromass Quattro II triple quadrupole mass spectrometer (UK). The adduct ions formed by DIPA with nucleotides in ESI-MS were used for quantification: single ion monitoring quantification ions were 551 *m/z* for AMP (AMP+2 DIPA+H^+), 732 *m/z* for ADP (ADP+2 DIPA+H^+), and 812 *m/z* for ATP (ATP+2 DIPA+H^+).

Transcript analysis

Transcriptional analysis was performed with the TRanscript analysis with aid of Affinity Capture (TRAC) assay described by Rautio *et al.* (2006a) for 69 genes involved in central carbon and related metabolism. mRNA was extracted from 10-mL samples (10–50 mg DW) that had been rapidly frozen in liquid N_2 and stored at −80 °C. GeneScan-120LIZ size standard (Applied Biosystems, Foster City, CA) was added to each sample to calibrate the separation of the detection probes by size. In addition, *in vitro* synthesized mRNA (MEGAscript transcription kit;

Ambion, Austin, TX) of the *Escherichia coli traT* gene was also added to each sample [1.5 fmol $(100\,\mu L)^{-1}$] so that the results for each probe in any analysis could be correlated with this internal standard, eliminating experimental variation in different hybridizations and samples. Probes were divided into seven probe pools with eight to 11 probes per pool. The identity of the probes was determined by the migration behavior, and the quantity by the peak area.

Total polyA RNA was quantified from the cell lysate after elution of polyA RNA in dimethyl pyrocarbonate-treated H_2O, using the RiboGreen RNA quantification kit (Molecular Probes, Leiden, the Netherlands). mRNA expression levels are given as the standardized (using *traT* internal standard) amount per total polyA RNA.

Statistical analyses

Data are given as mean ± SEM. Where appropriate, values were compared by ANOVA, and significant differences were determined using Fisher's multiple range test.

Results and discussion

Steady-state metabolite and transcript levels in aerobic (20.9% O_2) and anaerobic cultures

The physiology of *S. cerevisiae* CEN.PK113-1A was strongly affected by changes in oxygen concentration, as expected. The metabolite concentrations observed during aerobic steady states (Fig. 1) were similar to those reported by Mashego *et al.* (2005), Wu *et al.* (2005) and Visser *et al.* (2004) for *S. cerevisiae* CEN.PK113-7D (the *MAT*a strain equivalent to CEN.PK113-1A) in glucose-limited chemostats at $D = 0.05\,h^{-1}$ in the presence of 31 mM ethanol. Metabolites of upper glycolysis [glucose 6-phosphate (G6P), fructose 6-phosphate (F6P), and fructose 1,6-bisphosphate (F1,6BP)] were maintained at significantly higher concentrations in anaerobic than in aerobic conditions, as was pyruvate (Fig. 1). 2-Phosphoglycerate (2PG)+3-phosphoglycerate (3PG) and phosphoenolpyruvate (PEP) concentrations were significantly lower in anaerobic than in aerobic conditions (Fig. 1). A similar increase in the concentrations of hexose phosphates and reduction in the

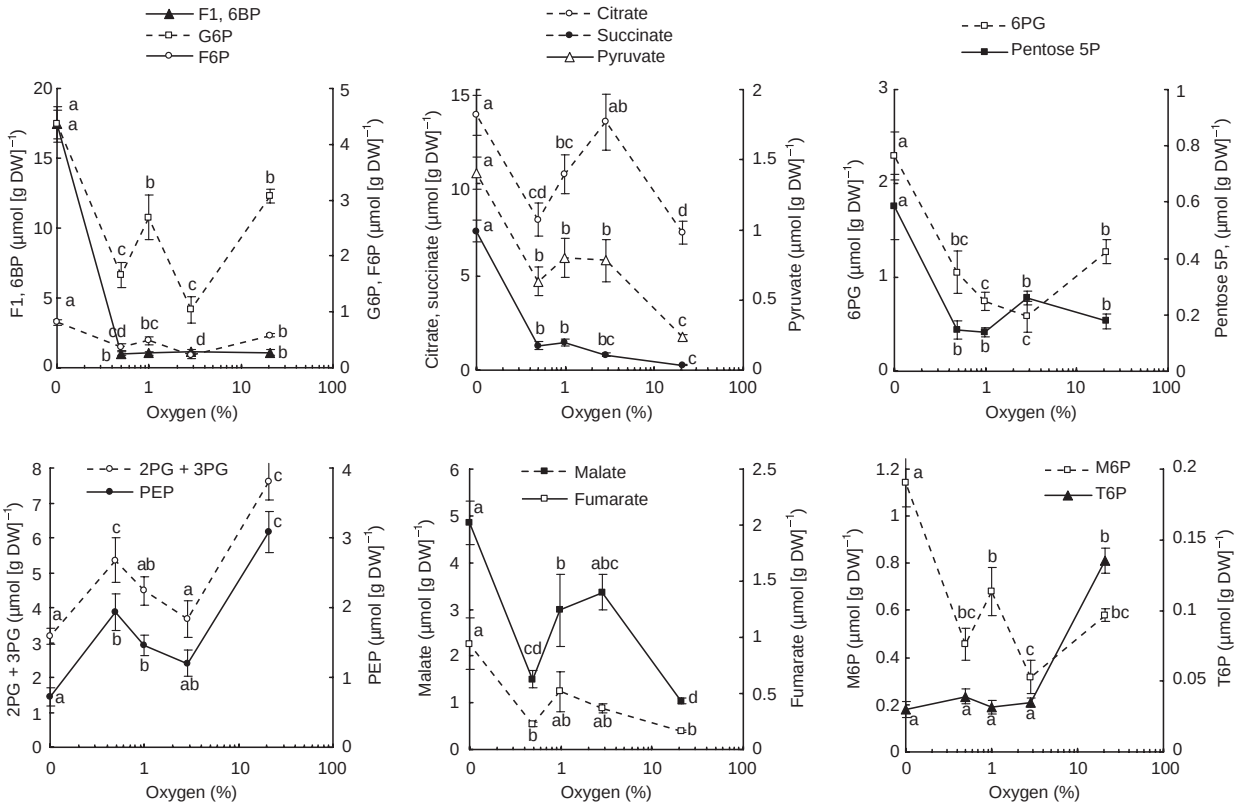


Fig. 1. Metabolite concentrations in steady-state glucose-limited chemostat cultures ($D = 0.10\,h^{-1}$, pH 5.0, 30 °C, 1.5 vvm gas flow) of *Saccharomyces cerevisiae* CEN.PK113-1A. Error bars indicate ± SEM for seven to 24 samples taken during steady states in two (0.5% and 2.8% O_2) or four (0%, 1.0% and 20.9% O_2) cultivations. Data points with the same letter (a–e) did not differ significantly ($P > 0.05$, Fisher's multiple range test) from data points for the same metabolite showing the same letter. M6P, maltose 6-phosphate; T6P, trehalose 6-phosphate.

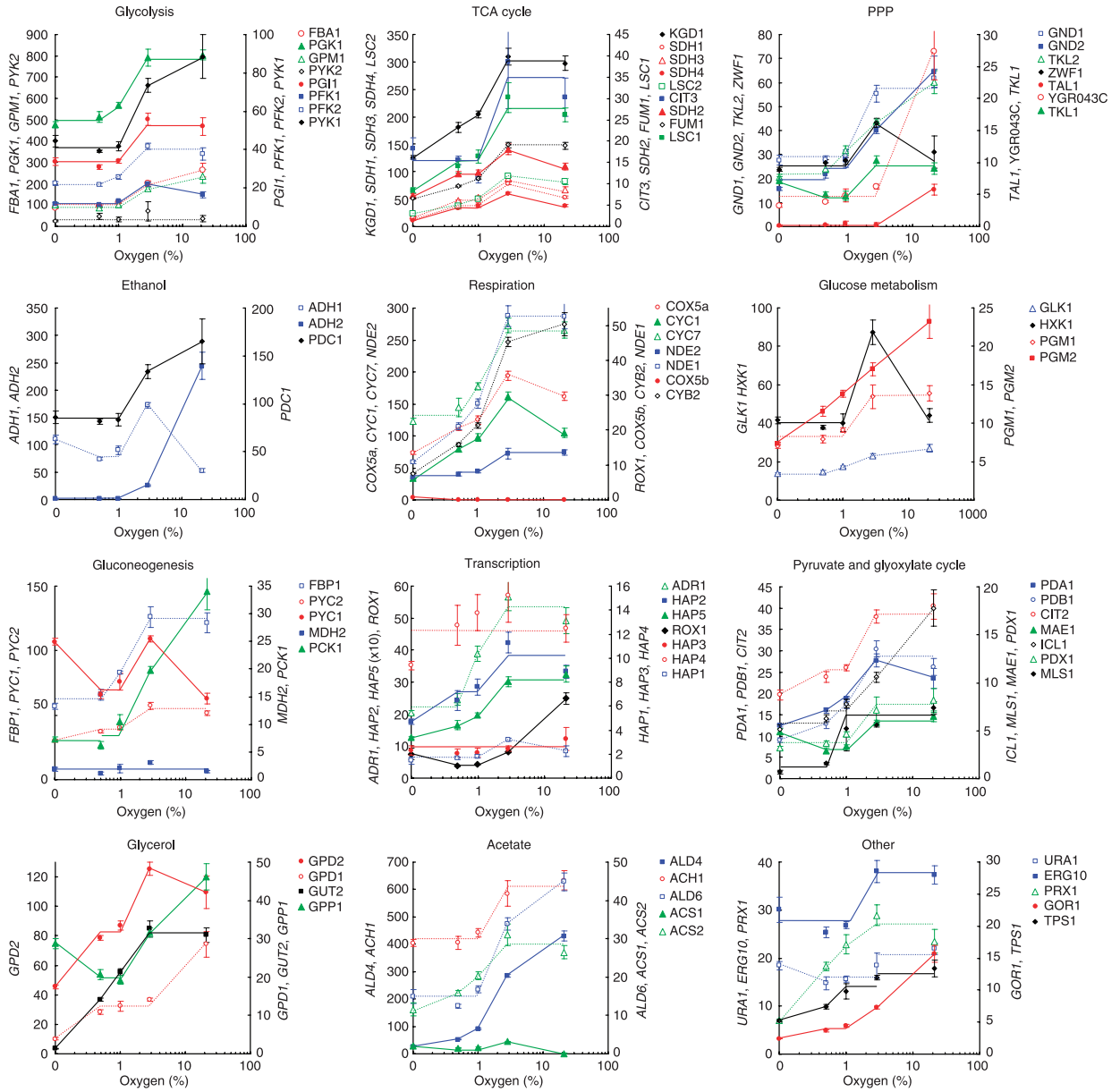


Fig. 2. Relative mRNA levels for genes involved in central carbon metabolism, and some genes involved in transcription, redox regulation and stress responses in *Saccharomyces cerevisiae* CEN.PK113-1A in steady-state, glucose-limited chemostat cultures ($D = 0.10\,h^{-1}$, pH 5.0, 30 °C, 1.5 vvm gas flow) with various concentrations of oxygen provision. Error bars indicate ± SEM for four to eight samples taken during steady states in duplicate cultivations. Lines indicate (adjacent) data points that did (lines with positive or negative slope; $P < 0.05$, Fisher's multiple range test) or did not (lines with slope = 0; $P > 0.05$) differ significantly from each other.

concentrations of 2PG+3PG and PEP is observed during respirofermentative growth following addition of a pulse of glucose (Visser *et al.*, 2004; Wu *et al.*, 2005), when the low concentrations of 2PG+3PG and PEP are thought to reflect a dynamic response to F1,6BP regulation of pyruvate kinase.

Tricaboxylic acid cycle (TCA) metabolites (citrate, succinate, fumarate, and malate) concentrations were also significantly higher in anaerobic than in aerobic conditions (Fig. 1), as observed by Villas-Bôas *et al.* (2005a) in anaerobic, as compared to aerobic, batch cultures of CEN.PK113-7D. The high concentrations of TCA cycle metabolites in anaerobic conditions are maintained while there is low flux through the pathway (Nissen *et al.*, 1997; Franzén, 2003) and low or zero oxoglutarate dehydrogenase, isocitrate dehydrogenase (Machado *et al.*, 1975) and succinate dehydrogenase (Camarasa *et al.*, 2003) activities. TCA

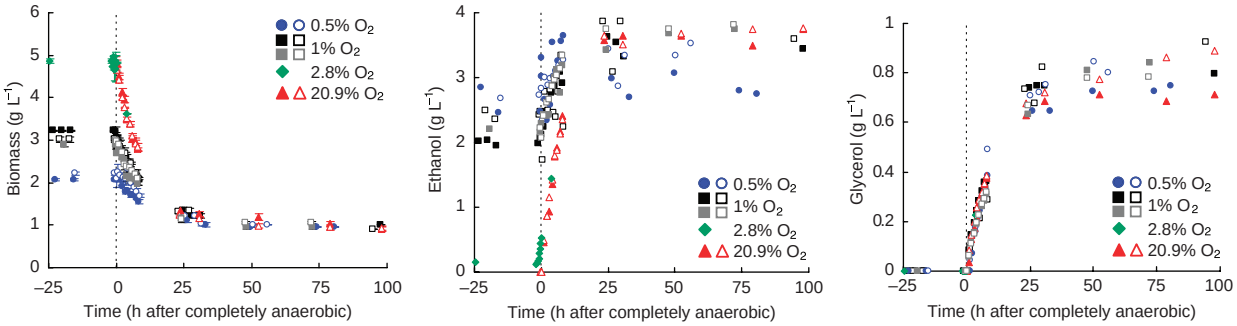


Fig. 3. Biomass, ethanol and glycerol concentrations during glucose-limited chemostat cultures of *Saccharomyces cerevisiae* CEN.PK113-1A following a shift from aerobic (0.5%, 1.0%, 2.8% or 20.9% O_2) to anaerobic conditions. Cultures were maintained at $D = 0.10\ h^{-1}$, pH 5.0, 30 °C, and 1.5 vvm gas flow throughout the culture, with N_2 replacing air in the gas to maintain a constant gas flow. Error bars for biomass indicate ± SEM ($n = 3$–5).

cycle metabolites provide precursors for amino acid biosynthesis during anaerobic growth, but it is not clear why relatively high concentrations of these metabolites are maintained during anaerobic growth.

Individual pentose phosphates were not separated by the LC-MS/MS method used here, but together showed higher concentrations under anaerobic than in any aerobic condition (Fig. 1).

The adenylate energy charge [AEC, calculated as ([ATP]+1/2[ADP])/([ATP]+[ADP]+[AMP])] was the same (0.83 ± 0.01; $P > 0.05$) under anaerobic and aerobic conditions, and comparable to values observed in both anaerobic and aerobic batch (0.8–0.9; Ball & Atkinson, 1975) and aerobic chemostat (0.8–0.9; $D = 0.05\ h^{-1}$; Mashego *et al.*, 2005) cultures of *S. cerevisiae*.

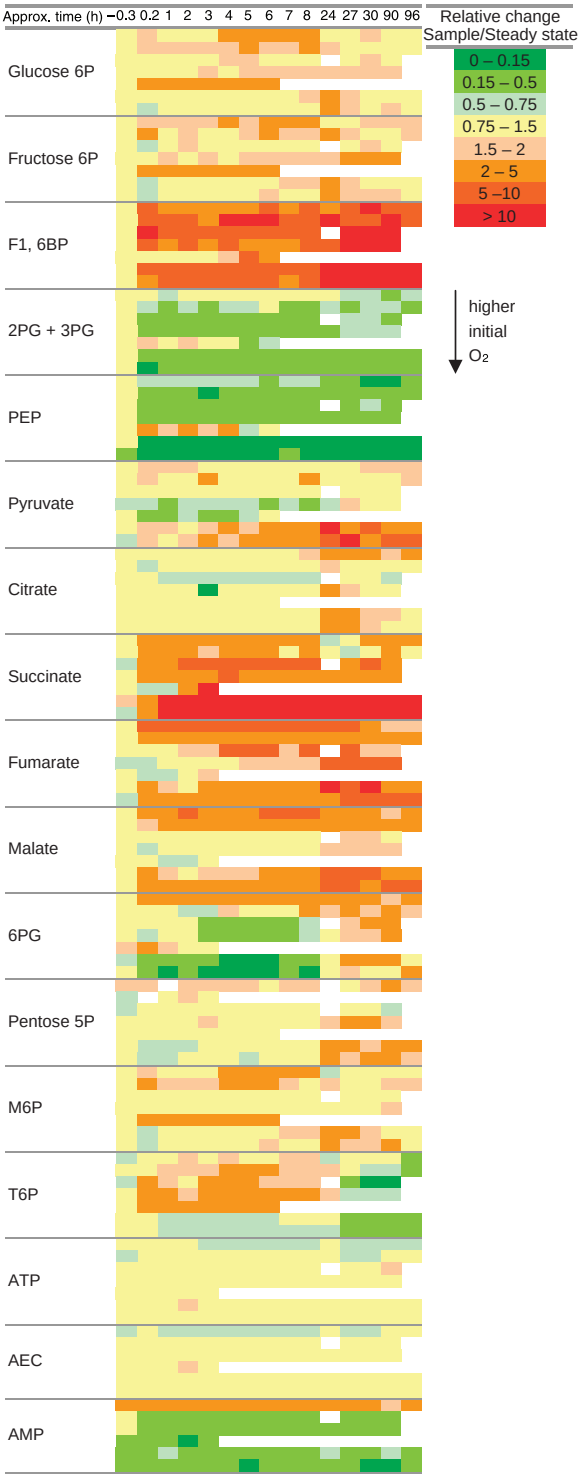
Using TRAC analysis, we found that all except six (*HAP3*, *HAP4*, *HXK1*, *MDH2*, *PYK2*, and *URA1*) of the 69 genes related to central carbon metabolism that were considered here showed significant differences ($P < 0.05$) in expression in aerobic and anaerobic cultures (Fig. 2). Only *ADH1*, *COX5b*, *ACS1* and *PYC1* were more highly expressed in anaerobic than in aerobic (20.9% O_2) steady states (Fig. 2), whereas the other 59 genes showed lower expression in the anaerobic conditions. ter Linde *et al.* (1999) identified 219 genes that showed higher transcription in aerobic glucose-limited chemostat cultures of *S. cerevisiae* CEN.PK113-7D than in anaerobic cultures, and 140 genes that had lower expression in the aerobic glucose-limited chemostat cultures. However, of the 69 genes considered here, ter Linde *et al.* (1999) observed significant differences in expression between aerobic and anaerobic glucose-limited chemostat cultures for only 26, of which five (*ADH1*, *CIT2*, *COX5b*, *MAE1*, and *GPP1*) showed higher expression under anaerobic than under aerobic conditions. Thus, both studies identified *ADH1* and *COX5b* as genes more strongly expressed in anaerobic than aerobic conditions. We did find that both *GPP1* and *MAE1* were more highly expressed in anaerobic conditions than with 0.5% or 1.0% O_2, although they had higher expression in fully aerobic conditions. The relatively high expression of *PYC1*, which was not identified by ter Linde *et al.* (1999) as an anaerobically upregulated gene (less than twofold induction), that we observed in anaerobic conditions presumably reflects its role in providing C4 intermediates for growth (Brewster *et al.*, 1994). None of these anaerobically upregulated genes was mentioned in the study published by Piper *et al.* (2002), which did not list anaerobic genes with less than an eightfold increase in expression, and would thus not include this subset. Low expression of *ADH2*, *CIT3* and *LSC1* in anaerobic, as compared to aerobic, conditions was observed by both Piper *et al.* (2002) and ter Linde *et al.* (1999), as well as in this study (Fig. 2), and the low expression of most genes involved in the TCA cycle in anaerobic conditions was also observed by Piper *et al.* (2002).

Biomass concentration and specific consumption or production of glucose, oxygen, ethanol and glycerol during steady-state growth of *S. cerevisiae* CEN.PK113-1A in glucose-limited culture are given in Table 1.

Respirofermentative growth in low oxygen

Provision of only 0.5% O_2 resulted in significantly lower concentrations of the metabolites of upper glycolysis, such as G6P, F6P, and F1,6BP, in comparison with anaerobic cultures, whereas the concentrations of the lower glycolysis metabolites 2PG+3PG and PEP were increased (Fig. 1). The concentrations of TCA cycle metabolites such as citrate, succinate, malate and fumarate decreased as compared to anaerobic cultures (Fig. 1). Glycolytic gene transcription, in contrast to metabolite concentration, was largely unaffected (relative to anaerobic cultures) by provision of up to 1% O_2, whereas most of the TCA cycle genes were upregulated with provision of only 0.5% O_2 (Fig. 2).

Reduction of the oxygen input from 20.9% to 2.8% was sufficient to allow net ethanol production, even though there was sufficient oxygen to maintain a high yield of



biomass on glucose and a high OUR (Table 1). The high level of expression of *ADH1* did not result in substantial ethanol production, but *ADH2* was strongly repressed, so it seemed unlikely that substantial simultaneous consumption was occurring (Fig. 2). Approximately 50% of the 69 genes considered here showed either significantly higher (23%) or lower (31%) expression with 2.8% O_2, as compared to 20.9% O_2 (Fig. 2). All glycolytic and TCA cycle genes analyzed and most genes of the pentose phosphate pathway (PPP) showed higher expression levels with 2.8% O_2, as compared to more anaerobic conditions. However, the metabolite pools of the cells grown in 2.8% O_2 were more similar to those of cells grown in 0.5% or 1.0% O_2 than that of cells grown in 20.9% O_2 (Fig. 1). Cells growing in sufficient but low oxygen concentration were clearly very different from cells growing in abundant oxygen.

The difference in the metabolic condition of cells growing with 2.8% O_2 as compared to those grown with 20.9% O_2 was also apparent in the low AEC, which was only 0.54 ± 0.03 when 2.8% O_2 was supplied ($D = 0.10\,h^{-1}$, pH 5.0). Unlike *E. coli*, which requires an AEC of at least 0.8 for growth (Chapman *et al.*, 1971), *S. cerevisiae* remains able to grow in conditions supporting an AEC as low as 0.4 (Polakis & Bartley, 1966).

Shifting from aerobic conditions to anaerobic conditions

When conditions were switched from aerobic (0.5–20.8% O_2) to anaerobic (0% O_2), ethanol and glycerol concentrations in the culture started to increase within an hour of the shift, requiring *c.* 36 h to reach the steady-state values observed in anaerobic cultures (Fig. 3). Biomass concentrations also began to decrease almost immediately (Fig. 3). Although biomass concentration decreased after the switch to anaerobic conditions, cells continued to grow at *c.* 0.06 h^{-1} during the washout.

On the basis of the steady-state concentrations, most glycolytic and TCA cycle metabolites were expected to increase to reach steady-state anaerobic concentrations, following a switch to anaerobic conditions, as was observed (Figs 4 and 5). An initial increase in metabolite concentration occurred within < 10 min, except for citrate, but new

Fig. 4. Relative changes in the concentration of intracellular metabolites during glucose-limited chemostat cultures of *Saccharomyces cerevisiae* CEN.PK113-1A, following a shift from aerobic (0.5%, 1.0%, 2.8% or 20.9% O_2) to anaerobic conditions, with time increasing from left to right. For each metabolite, the bars indicate relative concentrations in cultures that initially received oxygen as follows: top two bars 0.5%, the next two bars 1.0%, the next bar 2.8%, and the bottom two bars 20.9%. Yellow indicates no change in concentration. Red indicates increasing concentrations of the metabolite (from light red indicating a 1.5–2-fold increase, to bright red indicating a $>$ 10-fold increase), and green indicates reduced concentrations (from light green indicating 1.4–2-fold lower concentration, to bright green indicating $>$ sevenfold lower). Cultures were maintained at $D = 0.10\,h^{-1}$, pH 5.0, 30 °C, and 1.5 vvm gas flow throughout the culture, with N_2 replacing air in the gas to maintain a constant gas flow.

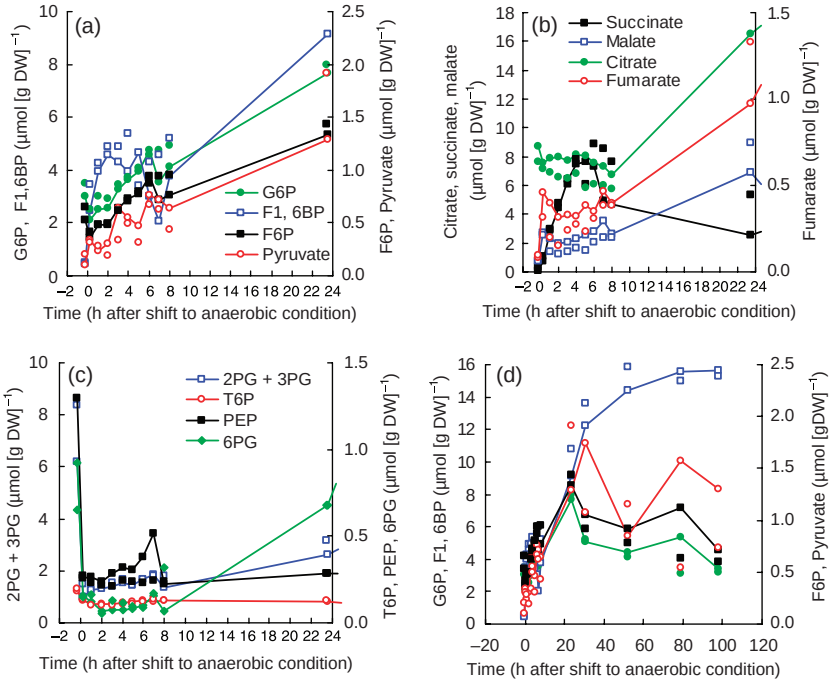


Fig. 5. Metabolite concentrations in *Saccharomyces cerevisiae* CEN.PK113-1A after a shift from growth in 20.9% O_2 to 0% O_2 in glucose-limited chemostat cultures ($D = 0.10\,h^{-1}$, pH 5.0, 30 °C, 1.5 vvm gas flow). (a) Glycolytic metabolites that increase. (b) TCA cycle metabolites that increase. (c) Metabolites that decrease. (d) Glycolytic metabolites, G6P green, F1,6BP blue, F6P black, pyruvate red. Data from two cultures are shown (connecting lines indicate data from one culture, and separate data points data from the other culture).

steady-state concentrations generally required at least 30 h (i.e. three retention times or four to five generations) to become established (Fig. 5). 2PG+3PG and PEP of lower glycolysis both decreased in concentration within 10 min of the switch from aerobic to anaerobic conditions (Fig. 5). The gradual adjustment of most metabolites to the anaerobic steady-state concentration was generally independent of the initial oxygen concentration provided, reflecting the fact that metabolite concentrations generally differed significantly between anaerobic conditions and any level of oxygen provision (Fig. 1).

For several of the genes considered here, the shift to anaerobic conditions had no effect on transcription during the first hour (e.g. *SDH1*, *ADR1*, *PDA1*, and *PDB1*), and in some cases even for 8 h (e.g. *HAP1* and *KGD1*), after the change (Fig. 6), being comparable to the 'chronic' response to anaerobiosis described by Lai *et al.* (2005) for batch cultures of *S. cerevisiae* JM43. We also observed that several genes involved in carbohydrate utilization and reserve energy metabolism (e.g. *GLK1*, *HXK1*, *PGI1*, and *TPS1*) showed weak transient responses to the anaerobic shift in the glucose-limited chemostats. These genes showed transient upregulation in response to anaerobiosis in batch cultures with galactose (but not glucose) as the carbon source (Lai *et al.*, 2005), leading to the suggestion that these genes could be regulated by oxygen provision when glucose was not repressing this. Our data support this suggestion while confirming the importance of balancing the energy supply during the transition to fermentative growth.

The three genes that showed higher expression in anaerobic than aerobic chemostat cultures (*COX5b*, *ADH1*, and *PYC1*; Fig. 2) also showed stable upregulation following a transition from 20.9% O_2 to anaerobic conditions (Figs 6 and 7). However, many other genes showed transient upregulation (Figs 6 and 7), typically during the first 8 h (approximately one generation) after the change. Genes with higher expression in 2.8% than in 20.9% O_2 (e.g. *GPD2*, *SDH2*, *CYC1*, *PDA1*, and *HAP1*) did not exhibit transient upregulation following the shift to anaerobic conditions, indicating that cells did not pass through a physiological condition corresponding to that observed in the steady state with 2.8% O_2 when adjusting to anaerobiosis. Transient downregulation was also observed (Figs 6 and 7), with rapid downregulation, but more gradual subsequent recovery. The duration of transient responses was affected by the initial oxygen concentration before the change (Fig. 6).

The effect of oxygen provision on glycolysis and the PPP

Although glycolytic genes were generally more highly expressed with 2.8% or 20.9% O_2 than with less or no O_2

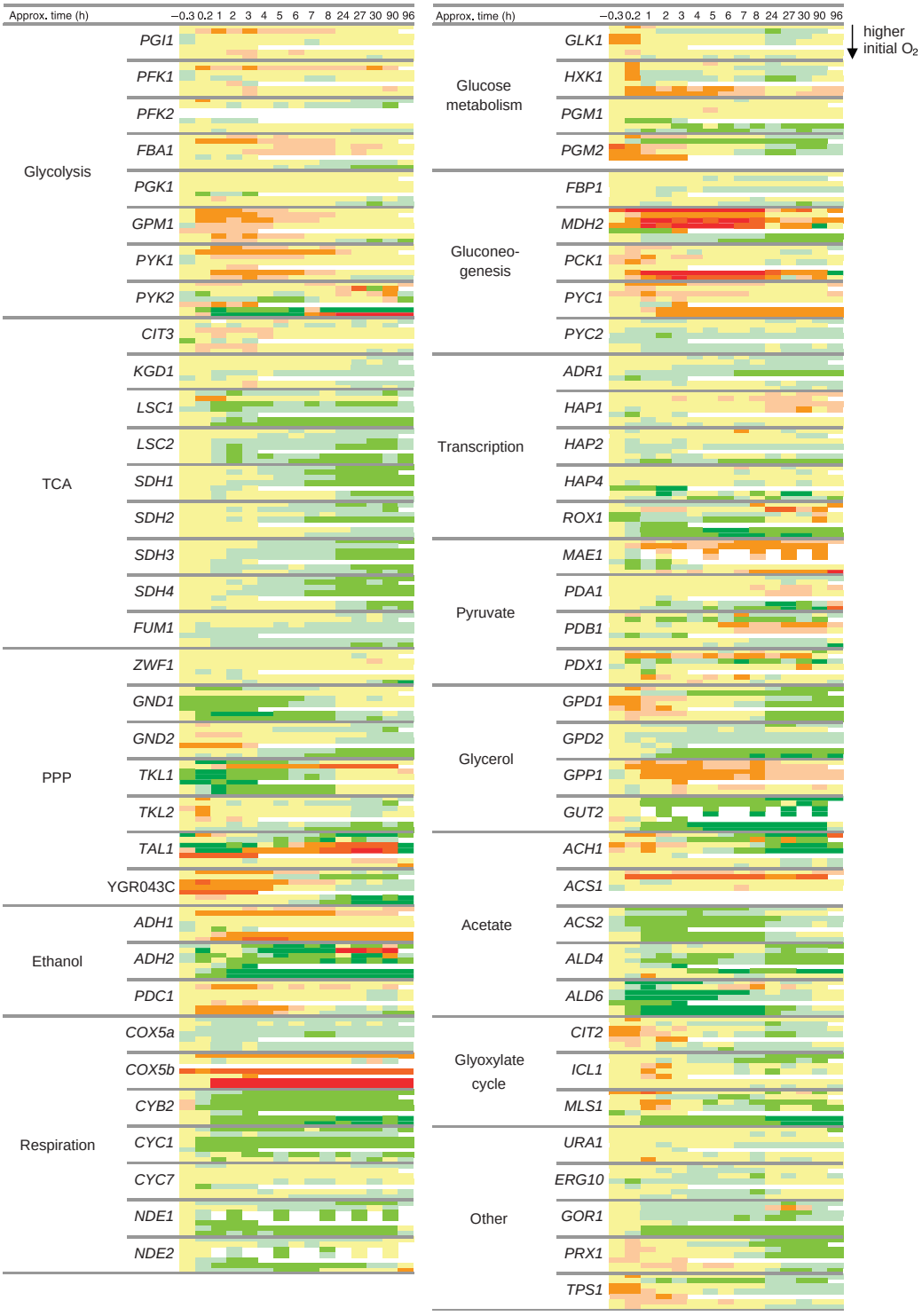


Fig. 6. Relative changes in the level of mRNA expression for genes involved in central carbon metabolism, transcription or respiration in glucose-limited chemostat cultures of *Saccharomyces cerevisiae* CEN.PK113-1A following a shift from aerobic (0.5%, 1.0%, 2.8% or 20.9% O_2) to anaerobic conditions, with time increasing from left to right. For each gene, the top two bars indicate relative transcription in cultures that initially received 0.5% O_2, the next two bars cultures that initially received 1.0% O_2, the next bar a culture that initially received 2.8% O_2, and the bottom two bars cultures that initially received 20.9% O_2. Yellow indicates no change in expression. Red indicates increasing levels of expression (from light red indicating a 1.5–2-fold increase, to bright red indicating a $>$ 10-fold increase), and green indicates decreased expression (from light green indicating 1.4–2-fold lower expression, to bright green indicating $>$ sevenfold lower expression), as illustrated in Fig. 4. Cultures were maintained at $D = 0.10\,h^{-1}$, pH 5.0, 30 °C, and 1.5 vvm gas flow throughout the culture, with N_2 replacing air in the gas to maintain a constant gas flow.

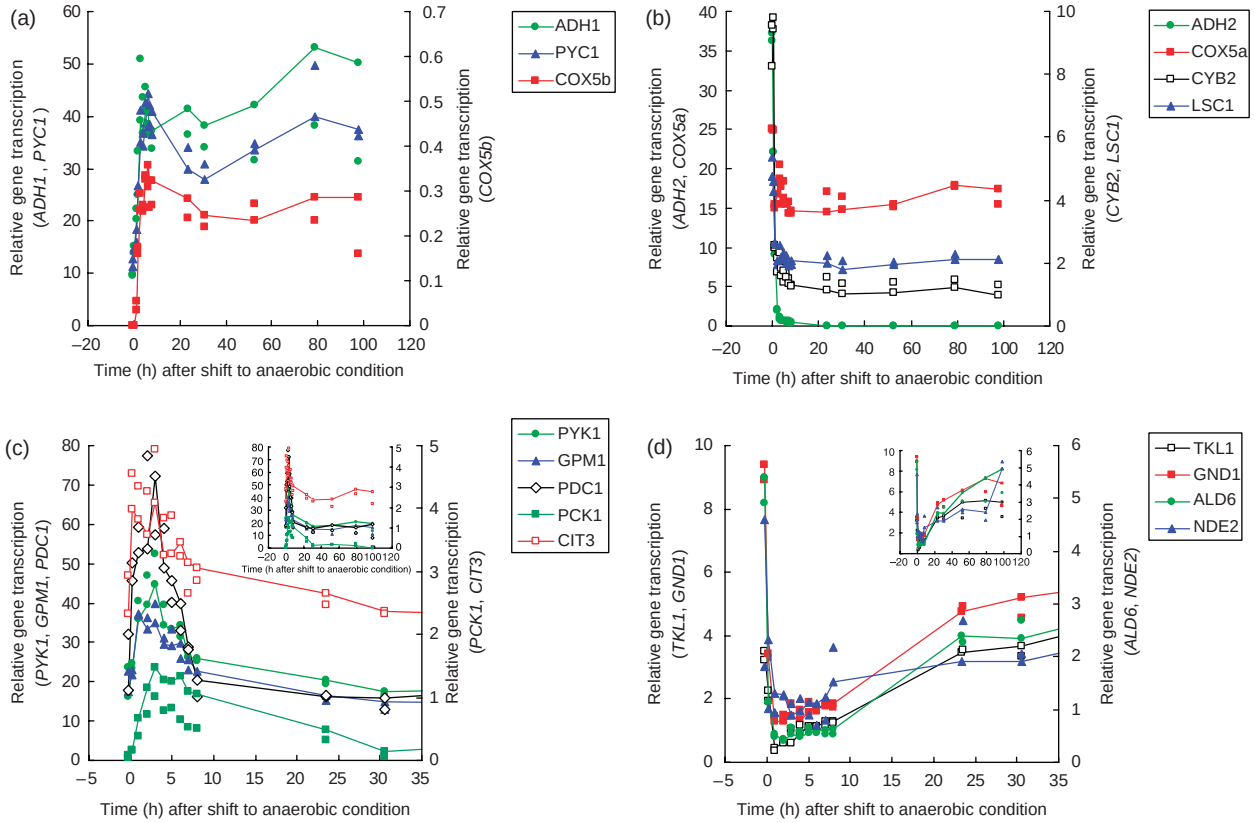


Fig. 7. Changes in relative gene expression in *Saccharomyces cerevisiae* CEN.PK113-1A after a shift from growth in 20.9% O_2 to growth in 0% O_2 during glucose-limited chemostat cultures ($D = 0.10\ h^{-1}$, pH 5.0, 30 °C, 1.5 vvm gas flow), showing: (a) upregulation, (b) downregulation, (c) transient upregulation, and (d) transient downregulation. Insets for (c) and (d) show extended (120 h) time frames. Data from two cultures are shown (connecting lines indicate data from one culture, and separate data points data from the other culture).

(Fig. 2), these genes generally showed no downregulation following a shift from aerobic to anaerobic conditions until at least 24 h after the shift. *GPM1* and *PYK1*, in fact, were initially upregulated following the shift (Fig. 6). In contrast, most genes involved in the TCA cycle were downregulated, as expected, but generally only after 2 or 3 h in anaerobic conditions. Downregulation of PPP genes also occurred (Fig. 6).

As the amount of oxygen available for cellular metabolism is reduced, the relative flux through the PPP diminishes (cf. Gombert *et al.*, 2001; Fiaux *et al.*, 2003; Franzén, 2003; van Winden *et al.*, 2005), and the flux through glycolysis increases. However, mRNA transcripts of the genes involved in glycolysis and those involved in the PPP are maintained at higher levels in conditions in which oxygen is readily available than in conditions with little or no oxygen (Fig. 2). Thus, although the slight increase in transcription of glycolytic genes following a shift from aerobic to anaerobic conditions may reflect the cells' initial means of increasing the flux through the pathway and increasing energy provision through ethanol production, it can only be transitory, as transcription levels need to be reduced to achieve the anaerobic steady state. The downregulation of PPP genes presumably contributes to the shift in flux towards glycolysis, but much of the control for the glycolytic flux must occur posttranscriptionally, as is also implied by the sharp changes in glycolytic metabolite concentrations, particularly those of lower glycolysis. Daran-Lapujade *et al.* (2004) also concluded that regulation of glycolytic genes for CEN.PK113-7D grown aerobically on different carbon sources was primarily posttranscriptional, whereas there was evidence that both transcriptional and posttranscriptional regulation occurred for genes of the PPP. This conclusion is further supported by the observation that glycolytic enzyme activity is higher under anaerobic conditions than under aerobic conditions in glucose-limited chemostat cultures of *S. cerevisiae* CEN.PK113-7D (van Hoek *et al.*, 2000).

It is also interesting to note that the expression of *GND1*, *TKL1*, and *ALD6*, which are all subject to Stb5p induction (Larochelle *et al.*, 2006), showed similar responses (transient downregulation) to a change from aerobic (20.9%, 1.0% or

0.5% O_2) to anaerobic conditions, whereas other genes under Stb5p regulation (*GND2*, *TAL1*, *ALD4*, *GOR1*, and *PGI1*) were either unaffected by the change (*PGI1*, *ZWF1* and *TAL1* in 20.9% O_2) or downregulated (*ALD4*, *GOR1* and *TAL1* in 0.5% and 1.0% O_2).

Induction and repression of oxygen-dependent genes

Several genes (e.g. *COX5a*, *COX5b*, *CYC1*, *CYC7*, and *ROX1*) were included in this study because their expression was known or expected to be strongly influenced by extracellular oxygen. However, expression of these genes did not necessarily respond to oxygen concentration as predicted on the basis of previous reports.

COX5b and *CYC7* have been highlighted as anaerobic genes that are only expressed when oxygen solubility is below 0.5 μM (Burke *et al.*, 1997). *COX5b* was indeed only detected in anaerobic cultures (0.5% O_2, resulting in < 6 μM solubility, should be sufficient to repress expression); however, expression levels were very low, and reduced expression would not have been detectable even if some expression still occurred. Others in our laboratory have observed higher expression levels for *COX5b* in an industrial brewer's yeast, even when oxygen was available (J.J. Rautio, pers. commun.), which suggests that expression of this gene is subject to strain variation, with CEN.PK113-1A not being the optimal strain in which to study its response to oxygen, because of its low anaerobic expression. Furthermore, *CYC7* was more highly expressed in aerobic than in anaerobic conditions (Fig. 2), rather than being repressed by the presence of oxygen, as in batch cultures of *S. cerevisiae* JM43 (Burke *et al.*, 1997). Similarly, ter Linde *et al.* (1999) did not observe induction of *CYC7* expression in CEN.PK113-7D in anaerobic conditions, and nor was *CYC7* subject to transient induction following the shift from aerobic to anaerobic conditions (Fig. 6). This difference in *CYC7* expression may reflect differences in gene regulation between batch (Burke *et al.*, 1997) and chemostat cultures, but may also again indicate strain differences. Such variation, however, has implications for the selection of biomarkers for assessment of industrial processes.

CYC1 and *COX5a* were more highly expressed in aerobic (2.8% and 20.9% O_2) than in low (0.5% or 1.0%) or no O_2, as expected (Burke *et al.*, 1997; ter Linde *et al.*, 1999), and downregulation was observed following a shift from aerobic to anaerobic conditions. Expression was reduced in 1.0% (*COX5a*) or 0.5% (*CYC1*) O_2, providing soluble oxygen at a concentration (6–12 μM) comparable to that in which reduced expression of these genes was observed in batch cultures (Burke *et al.*, 1997). The regulatory gene *ROX1* only had high expression in 20.9% O_2. However, although genes such as *CYC7* and *COX5b* are under *ROX1* regulation, a high level of expression of *CYC7* was observed in 20.9% O_2 when *ROX1* expression was also high, and *COX5b* remained undetectable in 0.5–2.8% O_2, even though *ROX1* expression was low in these conditions.

Another pair of genes that have been described as aerobic or nonaerobic are the acetyl coenzyme A synthetase genes *ACS1* and *ACS2* (van den Berg *et al.*, 1996). In this case, the function of the enzymes encoded by the genes is linked to anaerobic conditions, rather than the expression, with *ACS2*, the 'nonaerobic' form, being expressed both aerobically and anaerobically, with higher anaerobic expression than *ACS1* (van den Berg *et al.*, 1996). We found that *ACS1* was not highly expressed in CEN.PK113-1A, and *ACS2* expression was always higher than that of *ACS1* (Fig. 2). *ACS2* expression was lower in cultures receiving 0%, 0.5% or 1.0% O_2 than in cultures receiving 2.8% or 20.9% O_2 (Fig. 2), and was downregulated when conditions were changed from aerobic to anaerobic (Fig. 6). Thus, if ACS activity was reduced in anaerobic as compared to aerobic glucose-limited chemostats in CDN.PK113-1A, as has been observed in strain T2-3D (van den Berg *et al.*, 1996), this would be explained by the reduction in *ACS2* expression, rather than a reduction in *ACS1* expression.

All four of the dehydrogenases located in the mitochondrial intermembrane space (*NDE1*, *NDE2*, *GUT2*, and *CYB2*) that are considered here had low expression under anaerobic conditions and were downregulated following a shift from aerobic to anaerobic conditions.

Transcriptional regulation of ethanol and byproduct formation

Ethanol production occurred in conditions of low (0.5–2.8%) and no O_2, as expected (Fig. 3; Table 1). However, *ADH1* expression did not simply increase with decreasing oxygen provision, as noted above, and upregulation of *ADH1* following a switch to anaerobic conditions was only seen when the initial oxygen concentration was high (20.9%) or very low (0.5%).

Genes involved in ethanol consumption were consistently downregulated, as were genes involved in respiration (*ROX1*, *COX5a*, *CYB2*, and *CYC1*) and some genes involved in acetate metabolism (*ALD4*, *ALD6*, *ACS2*, and *ACH1*). Low expression of genes involved in ethanol consumption (*ADH2* and *ALD4*) has also been observed during respiro-fermentative growth at high specific growth rates in comparison with respiratory growth at lower specific growth rates (Regenberg *et al.*, 2006). Regulation of genes involved in ethanol consumption (and respiration) following a shift to anaerobic conditions followed the pattern suggested for *HAP4*-regulated genes (Raghevendran *et al.*, 2006), even though *HAP4* itself was not transcriptionally regulated with change in oxygen provision (Figs 2 and 6).

No extracellular acetate was observed under any condition. Glycerol was produced only in completely anaerobic conditions (Table 1; Fig. 3), and was not produced in proportion with ethanol production, indicating that 0.5% O_2 still provided sufficient oxygen to avoid cytoplasmic NADH accumulation. *GPD1* and *GPP1* showed transient upregulation following the shift to anaerobic conditions, but expression of *GPD2* was reduced (Fig. 6), even though its expression increases following a shift to anaerobic conditions in batch cultures and it is the primary glycerol-3-phosphate dehydrogenase involved in redox balancing in respiratory-deficient mutants (Ansell *et al.*, 1997; Valadi *et al.*, 2004). Both *GPD1* and *GPD2* had lower expression in anaerobic than in aerobic conditions (Figs 2 and 6).

Biomarkers

With the development of cost-efficient, sensitive methods for analysis of transcripts and metabolites, bioprocess monitoring can be extended to include monitoring of process-specific biomarkers from these groups of compounds. Optimally, such biomarkers would be used to identify when a culture was shifting towards an unproductive physiology, in order to facilitate intervention to correct the problem. Identification of suitable biomarkers is thus important for the full exploitation of physiologic data.

From the steady-state data, it is easy to identify gene transcripts whose expression level is unique to specific environmental conditions, with the clearest marker genes indicating only the presence of too much (for anaerobic or low-air cultures) or too little oxygen (for high-air or low-air cultures). In particular, genes such as *COX5b*, *TAL1*, YGR043C, *ACH1*, *CIT3* and *ADH2* would appear to be suitable for distinguishing cultures experiencing higher or lower oxygen supply than expected. This may be most relevant in cultures receiving limiting concentrations of oxygen, such that fluctuations would not be seen in the dissolved oxygen tension, which is always zero, or when assessing the extent to which oxygen-poor regions may exist in a large bioreactor. However, in either situation, the conditions of low oxygen are likely to be experienced only periodically (over intervals of a few minutes in poorly mixed bioreactors, to several hours when oxygen provision is variable), and it is necessary to consider how these genes respond to changing conditions. Thus, *TAL1* and YGR043C, whose expression showed transient upregulation and downregulation following a shift to anaerobic conditions, would not be suitable, as a high expression level could be indicative either of a high oxygen supply, or of a low oxygen supply or poor oxygen mixing. *ADH2* and *COX5b*, on the other hand, showed consistent downregulation and upregulation, respectively, in response to the switch to anaerobiosis, and would give more reliable information. In addition, the fact that many genes show transient responses to change means that it would be difficult to identify genes that would serve as markers for a range of conditions (the actual concentration of available oxygen) rather than just the contrast between two (too much or too little). *GUT2* and *GOR1*, with decreasing expression with decreasing oxygen concentration below 2.8% O_2 and consistent downregulation following a shift to anaerobic conditions, might be suitable biomarkers. Genes that did show transient upregulation or downregulation (Fig. 7) in response to the shift to anaerobic conditions, but did not show large expression differences in steady states, may be suitable candidates as markers for poor mixing and localized variation in conditions within the bioreactor (e.g. *GPM1* and *NDE2*). High (or low) expression levels would indicate that cells were experiencing periodic transient conditions. As the recovery is somewhat quicker (Fig. 7), upregulated genes (e.g. *PYK1*) would appear to be better biomarkers for heterogeneous conditions than downregulated genes, but it should be noted that none of the genes considered here would be expected to serve as an effective biomarker for transients of duration < 10 min.

The same considerations apply to the choice of a metabolite as a biomarker, but the choice should be wider, as transient changes were less frequently observed (Fig. 4). F1,6BP and succinate appear to be the best biomarkers for distinguishing between cells experiencing low and no air. Pyruvate may be a marker for intermediate conditions, although concentration changes following the shift to anaerobic conditions were dependent on the initial oxygen concentration. From this work, it is not clear how metabolite concentrations are affected in cells experiencing periodic exposure to varying oxygen concentrations. It is also apparent from this work that the current methodology for transcript analysis (TRAC as shown here, but also reverse transcriptase-PCR) is more robust and more readily available than metabolite analyses, making transcripts more likely to be successful biomarkers than metabolites, despite the problems inherent in transient regulation. Metabolite analyses still suffer from questions related to quenching, leaking of cell contents during quenching, extraction method, and separation methods (Villas-Bôas *et al.*, 2005b). Metabolic fingerprinting, either by MS or by nuclear magnetic resonance, may offer better prospects for bioprocess monitoring than quantitative metabolite measurements.

Conclusions

The results presented here show that, although *S. cerevisiae* is able to respond rapidly to changes in oxygen provision, considerable time (approximately four to five generations, i.e. 28–35 h) is required for cellular metabolism to fully adapt to anaerobic conditions, even when cells have been growing in the presence of only low amounts of air. Thus,

when interpreting experiments based on short-term analysis of the transcriptome (or metabolome) following a shift in external conditions, such as a transition from aerobic to anaerobic conditions in batch culture (Lai *et al.*, 2005), it is worth noting that physiological adaptation is likely to occur throughout the duration of the experiment. When fewer than four or five generations are monitored, the data cannot provide a direct comparison of the transcriptome in the conditions before and after the shift (e.g. in aerobic and anaerobic conditions), but rather provides an indication of the ongoing cellular response to the shift, relative to the stable condition prior to it. It is also worth noting that *S. cerevisiae* appears to require more time to adjust to a change in conditions than does the filamentous fungus *Trichoderma reesei*, which is able to attain transcriptional steady state within less than one generation (Rautio *et al.*, 2006b). The adaptation to anaerobic conditions (four to five generations) was, however, more rapid than the time needed for *S. cerevisiae* to achieve transcriptional steady state following a shift from growth on galactose to growth on glucose ($>$ 10 generations; $>$ 43 h at $D = 0.16\,h^{-1}$; Braun & Brenner, 2004). On the other hand, as the response to new conditions requires several generations, *S. cerevisiae* is likely to be very robust in conditions of inadequate mixing. Indeed Ronen & Botstein (2006) observed that steady-state glucose-limited cells exposed to pulses of excess glucose required only one generation (*c.* 3.5 h at $D = 0.2\,h^{-1}$) or less to regain transcriptional steady state. Further work should consider the effects of periodic exposure to altered oxygen provision, to determine how quickly cells recover from short-term oxygen shortage. Whereas metabolite concentrations responded more rapidly to removal of oxygen from the culture, metabolite concentrations also continued to change for up to five generations (35 h) following the change (Fig. 5d).

This work demonstrates how provision of limiting concentrations of oxygen to *S. cerevisiae* CEN.PK113-1A resulted in distinctly different cellular physiology, measured in terms of metabolite pools and gene transcription. In particular, 2.8% O_2 provided only limited oxygen (dissolved oxygen tension = 0%), but was sufficient for high biomass production. However, the levels of gene transcription in the presence of 2.8% O_2 were often higher than would have been predicted on the basis of data from aerobic and anaerobic cultures, whereas gene transcription in cultures receiving less O_2 (0.5% or 1.0%) was more predictable. This is also reflected in the fact that, for many genes involved in central carbon metabolism, up to 1.0% O_2 could be supplied to the culture with no effect as compared to transcription levels in anaerobic conditions. This information will be useful in the design of cultures requiring low oxygen input.

The role of oxygen in regulating cellular physiology is complex, as is also indicated by the extensive research on the topic (cf. Lascaris *et al.*, 2004; Tai *et al.*, 2005; Lai *et al.*, 2006) and the diversity of approaches used. It will be important to further identify those responses that are truly oxygen dependent and those that are strain, medium or culture system specific.

Acknowledgements

We thank Outi Könönen, Pirjo Tähtinen, Eila Leino and Tarja Hakkarainen for technical assistance. We thank Tekes, the Finnish Funding Agency for Technology and Innovation (Project numbers 40135/04 and 40537/05) and the Academy of Finland (Project number 202409, and SYSBIO program, project number 207435) for financial support.

References

Ansell R, Granath K, Hohmann S, Thevelein JM & Adler L (1997) The two isoenzymes for yeast NAD^+-dependent glycerol 3-phosphate dehydrogenase encoded by *GPD1* and *GPD2* have distinct roles in osmoadaptation and redox regulation. *EMBO J* **16**: 2179–2187.

Ball WJ & Atkinson DE (1975) Adenylate energy charge in *Saccharomyces cerevisiae* during starvation. *J Bacteriol* **121**: 975–982.

Braun E & Brenner N (2004) Transient responses and adaptation to steady state in a eukaryotic gene regulation system. *Physical Biol* **1**: 67–76.

Brewster NK, Val DL, Walker ME & Wallace JC (1994) Regulation of pyruvate carboxylase isozyme (PYC1, PYC2) gene expression in *Saccharomyces cerevisiae* during fermentative and nonfermentative growth. *Arch Biochem Biophys* **311**: 62–71.

Burke PV, Raitt DC, Allen LA, Kellogg EA & Poyton RO (1997) Effects of oxygen concentration on the expression of cytochrome *c* and cytochrome *c* oxidase genes in yeast. *J Biol Chem* **272**: 14705–14712.

Camarasa C, Grivet JP & Dequin S (2003) Investigation by ^{13}C-NMR and tricarboxylic acid (TCA) deletion mutant analysis of pathways for succinate formation in *Saccharomyces cerevisiae* during anaerobic fermentation. *Microbiology* **149**: 2669–2678.

Chapman AG, Fall L & Atkinson DE (1971) Adenylate energy charge in *Escherichia coli* during growth and starvation. *J Bacteriol* **108**: 1072–1086.

Daran-Lapujade P, Jansen ML, Daran JM, van Gulik W, de Winde JH & Pronk JT (2004) Role of transcriptional regulation in controlling fluxes in central carbon metabolism of *Saccharomyces cerevisiae*. A chemostat culture study. *J Biol Chem* **279**: 9125–9138.

de Jong-Gubbels P, Bauer J, Niederberger P, Stückrath I, Kötter P, van Dijken JP & Pronk JT (1998) Physiological characterisation of a pyruvate-carboxylase-negative *Saccharomyces cerevisiae* mutant in batch and chemostat cultures. *Antonie van Leeuwenhoek* **74**: 253–263.

de Koning W & van Dam K (1992) A method for the determination of changes of glycolytic metabolites in yeast on

a subsecond time scale using extraction at neutral pH. *Anal Biochem* **204**: 118–123.

Esener AA, Roels JA, Kossen NWF & Roozenburg JWH (1981) Description of microbial growth behaviour during the wash-out phase; determination of the maximum specific growth rate. *Eur J Appl Microbiol Biotechnol* **13**: 141–144.

Fiaux J, Çakar ZP, Sonderegger M, Wüthrich K, Szyperski T & Sauer U (2003) Metabolic flux profiling of the yeasts *Saccharomyces cerevisiae* and *Pichia stipitis*. *Eukaryot Cell* **2**: 170–180.

Franzén CJ (2003) Metabolic flux analysis of RQ-controlled microaerobic ethanol production by *Saccharomyces cerevisiae*. *Yeast* **20**: 117–132.

Frick O & Wittmann C (2005) Characterization of the metabolic shift between oxidative and fermentative growth in *Saccharomyces cerevisiae* by comparative ^{13}C flux analysis. *Microb Cell Fact* **4**: 30.

Gombert AK, dos Santos MM, Christensen B & Nielsen J (2001) Network identification and flux quantification in the central metabolism of *Saccharomyces cerevisiae* under different conditions of glucose repression. *J Bacteriol* **183**: 1441–1451.

Gonzalez B, Francois J & Renaud M (1997) A rapid and reliable method for metabolite extraction in yeast using boiling buffered ethanol. *Yeast* **13**: 1347–1355.

Lai L-C, Kosorukoff AL, Burke PV & Kwast KE (2005) Dynamical remodeling of the transcriptome during short-term anaerobiosis in *Saccharomyces cerevisiae*: differential response and role of Msn2 and/or Msn4 and other factors in galactose and glucose media. *Mol Cell Biol* **25**: 4075–4091.

Lai L-C, Kosorukoff AL, Burke PV & Kwast KE (2006) Metabolic-state-dependent remodeling of the transcriptome in response to anoxia and subsequent reoxygenation in *Saccharomyces cerevisiae*. *Eukaryot Cell* **5**: 1468–1489.

Larochelle M, Drouin S, Robert F & Turcotte B (2006) Oxidative stress-activated zinc cluster protein Stb5 has dual activator/repressor functions required for pentose phosphate pathway regulation and NADPH production. *Mol Cell Biol* **26**: 6690–6701.

Lascaris R, Piwowarski J, van der Spek H, Teixeira de Mattos J, Grivell L & Blom J (2004) Overexpression of *HAP4* in glucose-derepressed yeast cells reveals respiratory control of glucose-regulated genes. *Microbiology* **150**: 929–934.

Machado A, Nuñez de Castro I & Mayor F (1975) Isocitrate dehydrogenases and oxoglutarate dehydrogenase activities of baker's yeast grown in a variety of hypoxic conditions. *Mol Cell Biochem* **6**: 93–100.

Mashego MR, Jansen MLA, Vinke JL, van Gulik WM & Heijnen JJ (2005) Changes in the metabolome of *Saccharomyces cerevisiae* associated with evolution in aerobic glucose-limited chemostats. *FEMS Yeast Res* **5**: 419–430.

Nissen TL, Schulze U, Nielsen J & Villadsen J (1997) Flux distributions in anaerobic, glucose-limited continuous cultures of *Saccharomyces cerevisiae*. *Microbiology* **143**: 203–218.

Piper MDW, Daran-Lapujade P, Bro C, Regenberg B, Knudsen S, Nielsen J & Pronk JT (2002) Reproducibility of oligonucleotide microarray transcriptome analyses. *J Biol Chem* **277**: 37001–37008.

Polakis ES & Bartley W (1966) Changes in the intracellular concentrations of adenosine phosphates and nicotinamide nucleotides during the aerobic growth cycle of yeast on different carbon sources. *Biochem J* **99**: 521–533.

Rautio JJ, Kataja K, Satokari R, Penttilä M, Soderlund H & Saloheimo M (2006a) Rapid and multiplexed transcript analysis of microbial cultures using capillary electrophoresis-detectable oligonucleotide probe pools. *J Microbiol Methods* **65**: 404–416.

Rautio JJ, Smit BA, Wiebe M, Penttilä M & Saloheimo M (2006b) Transcriptional monitoring of steady state and effects of anaerobic phases in chemostat cultures of the filamentous fungus *Trichoderma reesei*. *BMC Genomics* **7**: 247.

Regenberg B, Grotkjær T, Winther O, Fausbøl A, Åkesson M, Bro C, Hansen LK, Brunak S & Nielsen J (2006) Growth-rate regulated genes have profound impact on interpretation of transcriptome profiling in *Saccharomyces cerevisiae*. *Genome Biol* **7**: R107.1–R107.13.

Raghevendran V, Patil KR, Olsson L & Nielsen J (2006) *Hap4* is not essential for activation of respiration at low specific growth rates in *Saccharomyces cerevisiae*. *J Biol Chem* **281**: 12308–12314.

Ronen M & Botstein D (2006) Transcriptional response of steady-state yeast cultures to transient perturbations in carbon source. *Proc Natl Acad Sci USA* **103**: 389–394.

Tai SL, Boer VM, Daran-Lapujade P, Walsh MC, de Winde JH, Daran J-M & Pronk JT (2005) Two-dimensional transcriptome analysis in chemostat cultures. *J Biol Chem* **280**: 437–447.

ter Linde JJM, Liang H, Davis RW, Steensma HY, van Dijken JP & Pronk JT (1999) Genome-wide transcriptional analysis of aerobic and anaerobic chemostat cultures of *Saccharomyces cerevisiae*. *J Bacteriol* **181**: 7409–7413.

Valadi A, Granath K, Gustafsson L & Adler L (2004) Distinct intracellular localization of Gpd1p and Gpd2p, the two yeast isoforms of NAD+-dependent glycerol-3-phosphate dehydrogenase, explains their different contributions to redox-driven glycerol production. *J Biol Chem* **279**: 39677–39685.

van Dam JC, Eman MR, Frank J, Lange HC, van Dedem GWK & Heijnen SJ (2002) Analysis of glycolytic intermediates in *Saccharomyces cerevisiae* using anion exchange chromatography and electrospray ionization with tandem mass spectrometric detection. *Anal Chim Acta* **460**: 209–218.

van den Berg MA, de Jong-Gubbels P, Kortland CJ, van Dijken JP, Pronk JT & Steensma HY (1996) The two acetyl-coenzyme A synthetases of *Saccharomyces cerevisiae* differ with respect to kinetic properties and transcriptional regulation. *J Biol Chem* **271**: 28953–28959.

van Hoek P, van Dijken JP & Pronk JT (2000) Regulation of fermentative capacity and levels of glycolytic enzymes in

chemostat cultures of *Saccharomyces cerevisiae*. *Enzyme Microb Technol* **26**: 724–736.

van Maris AJA, Konings WN, van Dijken JP & Pronk JT (2004) Microbial export of lactic and 3-hydroxypropanoic acid: implications for industrial fermentation processes. *Metab Eng* **6**: 245–255.

van Winden WA, van Dam JC, Ras C, Kleijn RJ, Vinke JL, van Gulik WM & Heijnen JJ (2005) Metabolic-flux analysis of *Saccharomyces cerevisiae* CEN. PK113-7D based on mass isotopomer measurements of ^{13}C-labeled primary metabolites. *FEMS Yeast Res* **5**: 559–568.

Verduyn C, Postma E, Scheffers WA & van Dijken JP (1992) Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation. *Yeast* **8**: 501–517.

Villas-Bôas SG, Moxley JF, Åkesson M, Stephanopoulos G & Nielsen J (2005a) High-throughput metabolic state analysis: the missing link in integrated functional genomics of yeasts. *Biochem J* **388**: 669–677.

Villas-Bôas SG, Højer-Pedersen J, Åkesson M, Smedsgaard J & Nielsen J (2005b) Global metabolite analysis of yeast: evaluation of sample preparation methods. *Yeast* **22**: 1155–1169.

Visser D, van Zuylen GA, van Dam JC, Eman MR, Pröll A, Wu L, van Gulik WM & Heijnen JJ (2004) Analysis of in vivo kinetics of glycolysis in aerobic *Saccharomyces cerevisiae* by application of glucose and ethanol pulses. *Biotechnol Bioeng* **88**: 157–167.

Wu L, Mashego MR, van Dam JC, Proell AM, Vinke JL, Ras C, van Winden WA, van Gulik WM & Heijnen JJ (2005) Quantitative analysis of the microbial metabolome by isotope dilution mass spectrometry using uniformly ^{13}C-labeled cell extracts as internal standards. *Anal Biochem* **336**: 164–171.

Zamamiri A-QM, Birol G & Hjortsø MA (2001) Multiple stable states and hysteresis in continuous, oscillating cultures of budding yeast. *Biotechnol Bioeng* **75**: 305–312.

Publication II

Transcription of hexose transporters of *Saccharomyces cerevisiae* is affected by change in oxygen provision

In: BMC Microbiology 2008, 8:53.

BMC Microbiology

BioMed Central

Research article

Transcription of hexose transporters of *Saccharomyces cerevisiae* is affected by change in oxygen provision

Eija Rintala*, Marilyn G Wiebe, Anu Tamminen, Laura Ruohonen and Merja Penttilä

Address: VTT, Technical Research Centre of Finland, P.O. Box 1000, FI-02044 VTT, Finland

Email: Eija Rintala* - eija.rintala@vtt.fi; Marilyn G Wiebe - marilyn.wiebe@vtt.fi; Anu Tamminen - anu.tamminen@vtt.fi; Laura Ruohonen - laura.ruohonen@vtt.fi; Merja Penttilä - merja.penttila@vtt.fi

* Corresponding author

Published: 28 March 2008

BMC Microbiology 2008, **8**:53 doi:10.1186/1471-2180-8-53

Received: 16 October 2007
Accepted: 28 March 2008

This article is available from: http://www.biomedcentral.com/1471-2180/8/53

Abstract

Background: The gene family of hexose transporters in *Saccharomyces cerevisiae* consists of 20 members; 18 genes encoding transporters (*HXT1-HXT17*, *GAL2*) and two genes encoding sensors (*SNF3*, *RGT2*). The effect of oxygen provision on the expression of these genes was studied in glucose-limited chemostat cultivations (D = 0.10 h^{-1}, pH 5, 30°C). Transcript levels were measured from cells grown in five steady state oxygen levels (0, 0.5, 1, 2.8 and 20.9% O_2), and from cells under conditions in which oxygen was introduced to anaerobic cultures or removed from cultures receiving oxygen.

Results: The expression pattern of the *HXT* gene family was distinct in cells grown under aerobic, hypoxic and anaerobic conditions. The transcription of *HXT2*, *HXT4* and *HXT5* was low when the oxygen concentration in the cultures was low, both under steady state and non-steady state conditions, whereas the expression of *HXT6*, *HXT13* and *HXT15/16* was higher in hypoxic than in fully aerobic or anaerobic conditions. None of the *HXT* genes showed higher transcript levels in strictly anaerobic conditions. Expression of *HXT9*, *HXT14* and *GAL2* was not detected under the culture conditions studied.

Conclusion: When oxygen becomes limiting in a glucose-limited chemostat cultivation, the glucose uptake rate per cell increases. However, the expression of none of the hexose transporter encoding genes was increased in anaerobic conditions. It thus seems that the decrease in the moderately low affinity uptake and consequently the relative increase of high affinity uptake may itself allow the higher specific glucose consumption rate to occur in anaerobic compared to aerobic conditions.

Background

The hexose transporter gene family in *Saccharomyces cerevisiae* contains the sugar transporter genes *HXT1* to *HXT17, GAL2* and the glucose sensor genes *SNF3* and *RGT2* [1,2]. The proteins encoded by *HXT1* to *HXT4* and *HXT6* to *HXT7* are considered to be the major hexose transporters in *S. cerevisiae*. The MC996A yeast strain lacking these six transporters and the protein encoded by *HXT5* (referred to as a *hxt* null mutant) is unable to grow on glucose [3]. Based on their K_m values and their ability

to restore growth on glucose when the respective genes are expressed individually in the *hxt* null mutant strain, these transporters have been classified as high (Hxt6p, Hxt7p), moderately low (Hxt2p, Hxt4p) and low (Hxt1p, Hxt3p) affinity transporters. However, Hxt2p in cells grown on low glucose concentrations exhibits both high and low affinity transport kinetics [4]. Gal2p is able to transport glucose with high affinity but the gene encoding it is expressed only when galactose is present [4,5]. In the CEN.PK2-1C strain, deletion of only *HXT1* to *HXT7* is not enough to completely abolish growth on glucose, fructose or mannose [6]. Only deletion of all seventeen of the *HXT* genes and *GAL2* produces a strain unable to grow on these sugars, and overexpression of any individual gene encoding a hexose transporter, except *HXT12* in this multiple deletant restores growth on at least one of the hexoses: glucose, fructose, mannose or galactose [6].

The major glucose transporters are regulated at transcriptional level by the extracellular glucose concentration [7]. The transcription factor Rgt1p represses the genes encoding these transporters in the absence of glucose, but it is also required for full induction of *HXT1* on high levels of glucose [8]. Paralogous proteins Mth1p and Std1p are necessary for Rgt1p to act as a repressor. Release of the repression requires removal of Mth1p and Std1p as well as phosphorylation of Rgt1. The glucose signal mediated by the Snf3p and Rgt2p sensors stimulates the degradation of Mth1p and Std1p, while the glucose signal mediated by the G-protein-coupled receptor Gpr1p leads to activation of protein kinase A, which phosphorylates Rgt1 and releases the repression of *HXT* genes [9-15]. In addition, high affinity transporters are repressed in high levels of glucose via the Snf1p-Mig1p glucose repression pathway. Mig1p binds directly to the promoters of *HXT2* and *HXT4* and also represses the expression of *MTH1* and *SNF3* [15,16].

Expression of *HXT5* is regulated by growth rate rather than the external glucose concentration [17,18]. It is expressed upon decrease in the growth rate of cells in glucose batch cultivations, at low dilution rates in glucose-limited chemostat cultivations, on non-fermentable carbon sources and during sporulation [17,19]. The regulation is mediated by STRE and HAP elements in the promoter region of *HXT5*. The STRE elements are needed for induction at low growth rates and during growth on ethanol, and the HAP elements for growth on ethanol or glycerol [17]. The Hxt5p transporter shows moderately low affinity for glucose [19].

The hexose transporters encoded by *HXT8* to *HXT17* have not been studied to the same extent as the major *HXT* genes. Many of the functions assigned to these transporters do not directly relate to sugar utilisation. Diderich and co-workers [20] were not able to detect the expression of these genes when probing total RNA from glucose-limited chemostat cultivations. It is known, however, that the regulation of all but *HXT11* and *HXT12* is controlled by glucose [7]. The transporters encoded by *HXT9* and *HXT11* are also shown to be involved in pleiotropic drug resistance [21], and the promoter of *HXT17* is a target for Mac1p transcription factor, which regulates high-affinity copper uptake genes under copper-deficient conditions [22,23]. Greatrix and co-workers [24] demonstrated that *HXT5*, *HXT13* and *HXT15* are induced in the presence of non-fermentable carbon sources, and that *HXT17* is upregulated when yeast cells are grown on medium containing raffinose and galactose at pH 7.7 but not at pH 4.7. In addition, Hxt9p and Hxt10p are able to transport arsenic trioxide into the cell, as do the major hexose transporters [25].

In addition to regulation at transcriptional level, inactivation of Hxt proteins takes place under certain conditions such as starvation or in the presence of high concentrations of glucose [7]. Degradation via endocytosis, autophaghy, and transport to the vacuole has been shown for Hxt2p, Hxt5p, Hxt6p and Hxt7p [26-30].

In glucose-limited chemostats, the specific glucose consumption rate is inversely related to the oxygen provided to the system [31,32]. Under oxygen-limited or anaerobic conditions, energy is provided by respiro-fermentative and fermentative metabolism, respectively. The biomass yield on glucose is lower and the cells must transport more glucose per unit time to be able to grow at the same rate in anaerobic or oxygen-limited conditions as when the metabolism is fully respirative. It is not clear what modifications in the cell are responsible for the higher glucose uptake. In addition, in spite of numerous studies, the mechanism(s) controlling glycolysis in yeast is still unknown. Control of individual steps or enzymes of this pathway, such as the phosphorylation of glucose and the control of phosphofructokinase have been extensively studied [33-35]. However, it is likely that the control is distributed over a number of steps, since the overexpression of individual glycolytic enzymes, or of all, did not enhance glycolytic flux [36-38]. Glucose transport has been suggested to play a major role in the control of glycolysis [39], and shown to be highly important for the dynamics of glycolysis with substantial control over the frequency of glycolytic oscillations [38]. Additionally, glucose transport may control the flux of glycolysis, even at high external glucose concentrations if transport capacity is reduced, as is the case in a strain that expresses a chimera between Hxt1p and Hxt7p and no other hexose transporter [40,41].

Previously, we have assessed the role of oxygen in the physiology of *S. cerevisiae* by studying the metabolite and transcript levels related to central carbon metabolism [32]. In the present study we extend the analysis to glucose transport to determine if and how hexose transporters are affected when oxygen becomes limiting for growth. We probed cells from glucose-limited chemostat cultivations of CEN.PK113-1A at five different oxygen levels for the expression of *HXT1* to *HXT11*, *HXT13* to *HXT17*, *GAL2*, *SNF3* and *RGT2*. In addition, we studied the expression of these genes under conditions in which oxygen was removed from cultivations receiving it or introduced to anaerobic cultivations.

Results

Transcription of HXT *genes in cells at steady state exposed to different oxygen levels*

Five different inflow gas oxygen levels (20.9, 2.8, 1.0, 0.5 and 0%) were used to study the response in expression of genes encoding hexose transporters and glucose sensors to external oxygen in glucose-limited chemostat cultures of CEN.PK113-1A. The specific glucose consumption rates were 6.6, 8.0, 11.4, 14.3 and 37.1 Cmmol g biomass^{-1} h^{-1} for chemostat cultures receiving 0.5, 1.0, 2.8 and 20.9% oxygen, respectively [32]. Extracellular glucose was not detected. The strongest expression signals were obtained from *HXT6*, *HXT7* and *HXT10*. The signals of *HXT9*, *HXT14* and *GAL2* transcripts were below the detection limit in these conditions.

Statistical differences ($p < 0.05$) in the expression levels of all the hexose transporter encoding genes were detected between at least some of the oxygen concentrations studied (Figure 1). *HXT2*, *HXT4* and *HXT5* were significantly more highly expressed in aerobic conditions compared to hypoxic (2.8, 1.0 and 0.5% O_2) or anaerobic conditions. Each of these genes showed some increase in expression already at 2.8% O_2. *HXT2* also had higher transcript levels in anaerobic conditions compared to the hypoxic conditions of 1.0 and 0.5% O_2. *HXT1*, *HXT8*, *HXT11* and *HXT17* had higher expression in 2.8% O_2 and fully aerobic conditions compared to lower oxygen levels, whereas *HXT10* had higher expression at 1% or more O_2. The signals from *HXT6*, *HXT13* and combined *HXT15* and *HXT16* (*HXT15/16*) were higher in at least two out of three hypoxic conditions than in either aerobic or anaerobic conditions. The highest transcript levels of *HXT6* and *HXT3* were seen in 2.8% O_2, whereas the expression of *HXT15/16* was reduced at this level of oxygen provision. *HXT13* had similar high expression in 1 and 2.8% O_2. None of the hexose transporter genes were upregulated in anaerobic compared to aerobic conditions. The signal for the gene encoding the Snf3p sensor was higher in aerobic compared to anaerobic conditions, and highest in 2.8% O_2, whereas the signal of the *RGT2* transcripts was lowest under the hypoxic conditions of 0.5 and 1% O_2.

Expression of transporter encoding genes after a change between aerobic and anaerobic conditions

The expression of hexose transporter and glucose sensor genes following a change in oxygen provision was studied by switching oxygen provision off or on, in aerobic and anaerobic cultivations, respectively. Following the change from aerobic to anaerobic conditions, samples were taken until a new steady state was achieved (Figure 2A). After the switch from anaerobic to aerobic conditions, the cultivations started to oscillate after 25 hours and the steady state could not be reached. The results are thus reported only for the first 25 hours (Figure 2B).

Clear changes as a response to the removal of oxygen from respiratory cells were seen in the transcript levels of *HXT2* to *HXT7*, although the time scales of the responses varied (Figures 2A and 3). Two of these genes (*HXT3* and *HXT6)* were upregulated and four (*HXT2*, *HXT4*, *HXT5* and *HXT7)* downregulated either transiently or permanently as a response to lack of oxygen. The transcript levels of *HXT3*, *HXT4*, *HXT6* and *HXT7* had changed already during the first 10 minutes, while a response in *HXT2* expression was seen only after 10 min but within one hour. Changes observed in *HXT5* expression were the slowest to occur; a significant response was seen after 6 hours.

When oxygen was introduced to the anaerobic cultivations (Figures 2B and 3), two of the genes encoding major hexose transporters (*HXT3* and *HXT6)* were downregulated and two (*HXT2* and *HXT4)* were upregulated. *HXT5* was upregulated only after a transient downregulation during the first 4 hours after oxygen was provided. *HXT7* was slightly upregulated after initial (10 min after oxygen was provided) downregulation. Expression of *HXT3*, *HXT4*, *HXT6* and *HXT7* exhibited rapid responses to a change in environmental oxygen, i.e within 10 min, the earliest sample taken after the change.

The responses of the transporter genes *HXT8* to *HXT17* were weaker than those of the major hexose transporters (Figures 2A, 2B and 3). In general, these genes were upregulated when oxygen was introduced to anaerobic cultivations, and either downregulated or did not respond when oxygen was removed from aerobic cultures. The expression of *HXT15/16*, increased only transiently when the culture was adapting to aerobic conditions and permanently following the shift to anaerobic conditions. Of the sensor genes, expression of *SNF3* was not affected by change in the oxygen concentration, but *RGT2* was downregulated when the cultures experienced lack of oxygen.

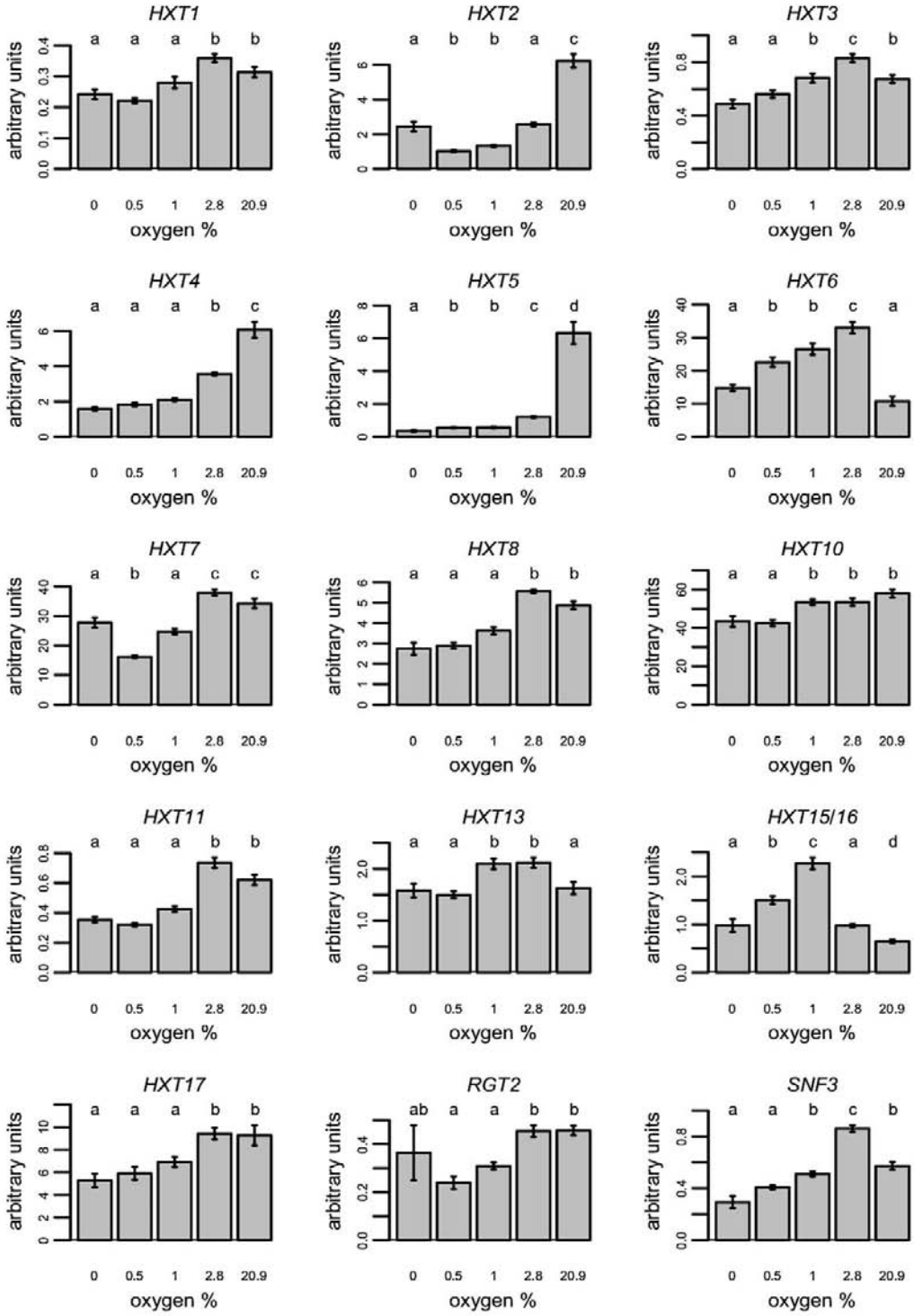


Figure 1

The expression of *HXT* genes at different steady state oxygen levels. The expression of genes encoding hexose transporters and glucose sensors in steady state oxygen concentrations of 0, 0.5, 1.0, 2.8 or 20.9% O_2 in glucose-limited chemostats (D = 0.10 h^{-1}, pH 5.0, 30°C, and 1.5 vvm gas flow). Error bars indicate ± sem for 4 to 8 samples taken during steady states in 2 to 4 cultivations. Values with the same letter (a to e) for the same gene did not differ significantly ($p > 0.05$, Dunnett's T3 multiple range test) from data points showing the same letter.

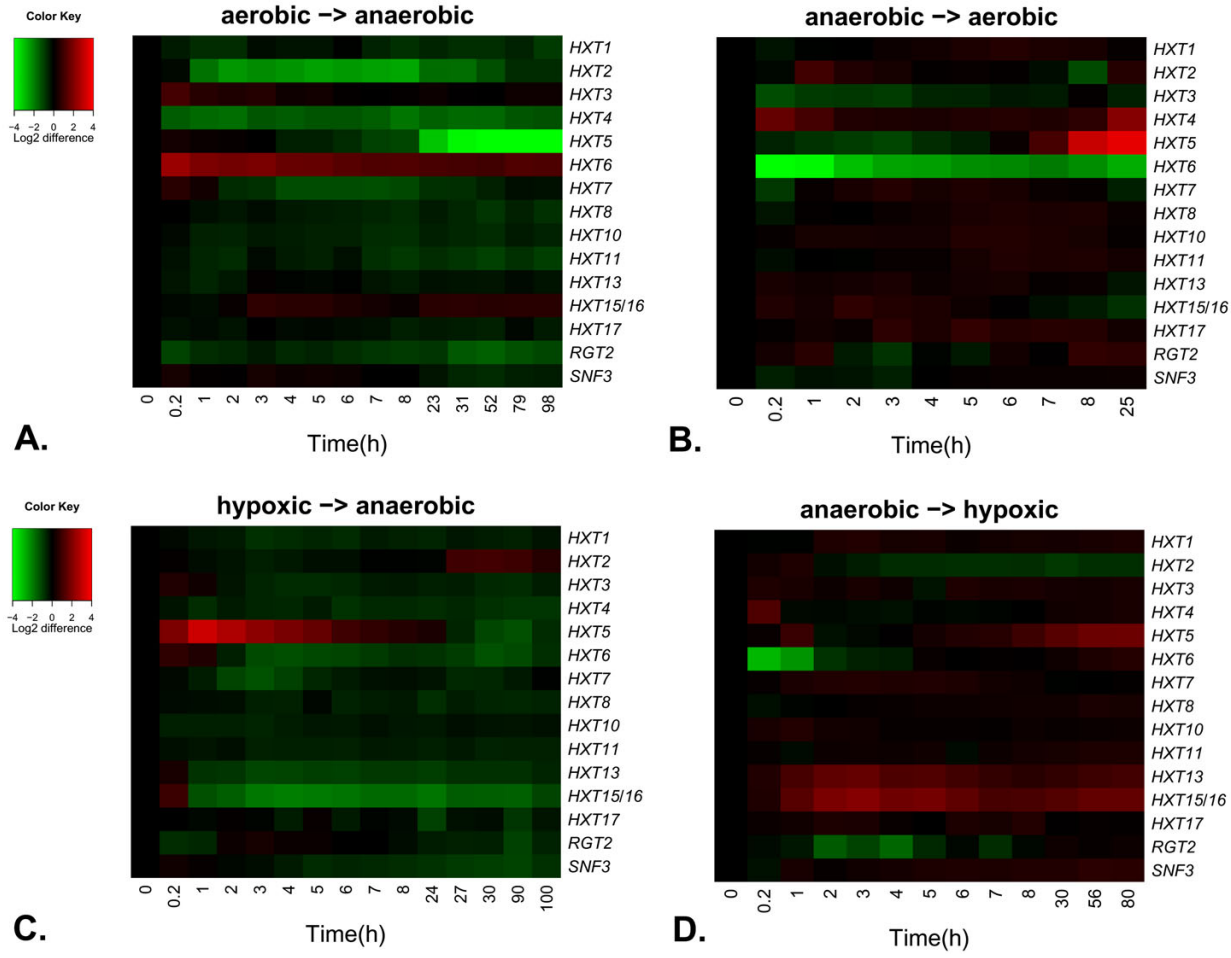


Figure 2
Relative expression levels of *HXT* genes after a change in oxygen provision. The heatmap of the relative expression levels of genes encoding hexose transporters and glucose sensors after change in oxygen provision. A. Shift from aerobic to anaerobic conditions. B. Shift from anaerobic to aerobic conditions. C. Shift from hypoxic (1% O_2) to anaerobic conditions. D. Shift from anaerobic to hypoxic (1% O_2) conditions.

Expression of transporter encoding genes after a change between hypoxic and anaerobic conditions

The response in expression of *HXT* and glucose sensor genes was also assessed following a shift from hypoxic (1% O_2) to anaerobic and from anaerobic to hypoxic conditions. Samples were taken until a new steady state was achieved.

The response in the expression of the hexose transporter encoding genes to oxygen depletion was different when the starting point was hypoxic and not fully aerobic (Figures 2C–D and 3). When oxygen was replaced with nitrogen in the cultures which had been hypoxic, most of the *HXT* genes were downregulated (Figure 2C). The expression of *HXT3*, *HXT4*, *HXT6*, *HXT13* and *HXT15/16* was transiently upregulated in cells after 10 min in anaerobic conditions, but downregulated already after 60 min. Only *HXT5* was transiently upregulated for a longer period (the first 7 hours). *HXT2* was upregulated towards the end of the adaptation to the anaerobic conditions, around 30 hours after oxygen was depleted.

When hypoxic conditions were introduced to anaerobic cultivations (Figure 2D) the immediate (10 min) responses were similar to those observed when fully aerobic conditions were introduced (Figure 2B), but the subsequent responses differed. Only the expression of *HXT2*, *HXT5*, *HXT13* and *HXT15/16* changed significantly as the cells approached the hypoxic steady state. *HXT5*, *HXT13* and *HXT15/16* were upregulated whereas *HXT2* was downregulated. *HXT6* and *RGT2* were transiently down-

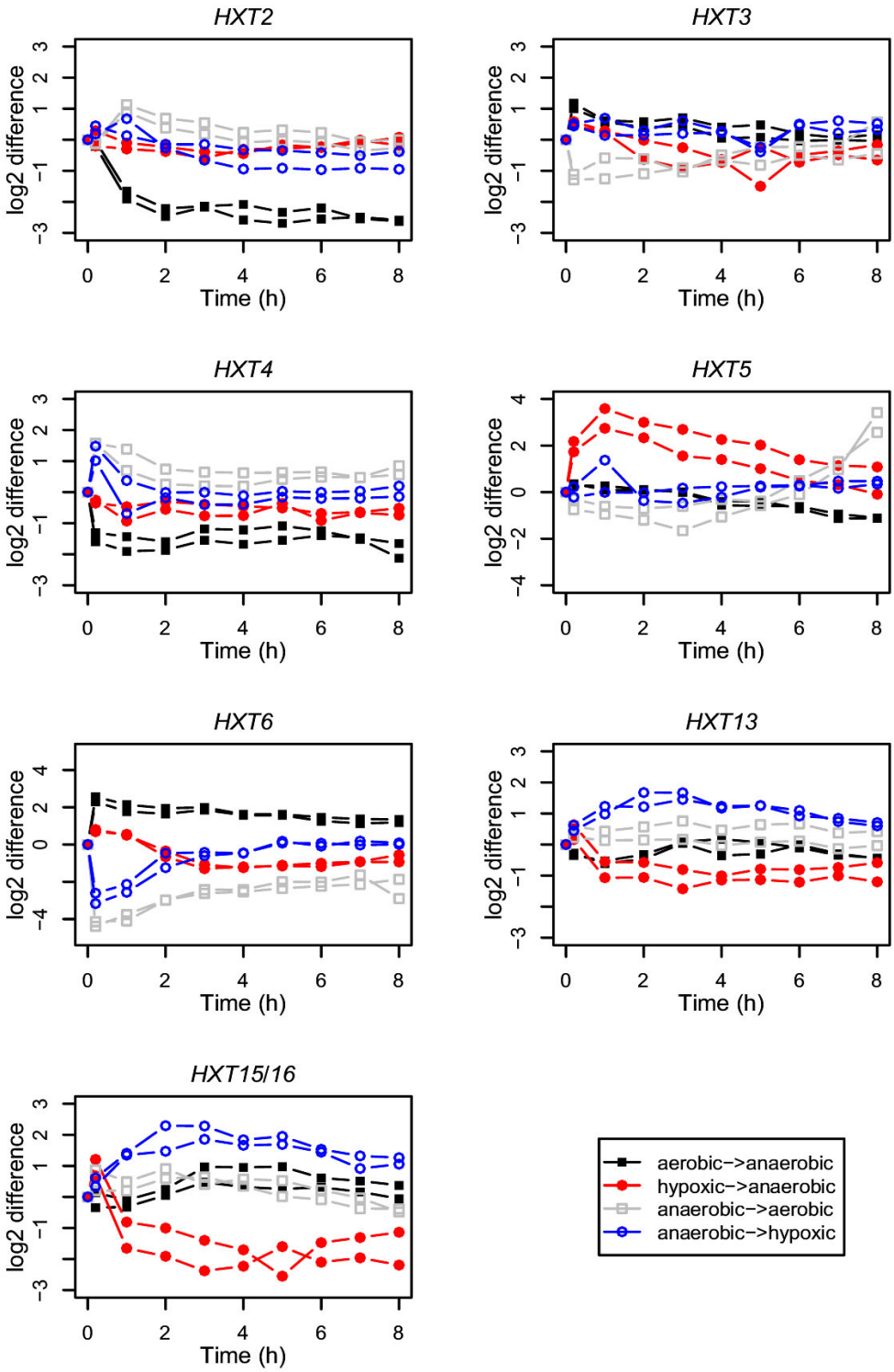


Figure 3
Initial responses of specific *HXT* genes to the change in oxygen concentration. The relative expression of *HXT2*-*HXT6*, *HXT13* and *HXT15/16* during the first eight hours after the switch in oxygen concentration. Symbols with the same colours represent replicate cultivations carried out in the same conditions.

regulated during the first 2 and 2–5 hours, respectively, following the switch to hypoxic conditions.

Promoter analysis of the **HXT** *genes induced in hypoxic conditions*

The promoter regions (950 bp upstream from the start codon) of the genes encoding Hxt3p, Hxt6p, Hxt13p, and Hxt15/16p transporters were sequenced in the CEN.PK113-1A strain. The upstream sequences of *HXT3*, *HXT6* and *HXT15/16* were identical to those of strain S288C, whose genomic sequence is found in the data banks. In the promoter region of *HXT13* there were five separate nucleotides in CEN.PK113-1A that differed from the published sequence of strain S288C. The DNA Pattern [42] web-based tool was used to search the promoters of *HXT3*, *HXT6*, *HXT13* and *HXT15/16* for binding sites of transcription factors known to be involved in hypoxic control. These were LORE (low oxygen response element); 5' ACTCAACAA 3' [43] and HRE (hypoxic response element); 5' BRCGTGVBBB 3' [44]. Two LORE sequences were found in the *HXT3* promoter and one in the *HXT15/16* promoter (one mismatch allowed), whereas one HRE site was found in the *HXT13* promoter of both S288C and CEN.PK113-1A strains.

Discussion

Most of the published data indicate that the expression of the hexose transporter encoding genes of *S. cerevisiae* is mainly regulated by extracellular glucose concentration. In the present paper, the expression of these genes was studied under various oxygen concentrations in glucose-limited chemostats with an extracellular glucose concentration below detection limit and thus not affecting the transcription of *HXT* genes [20]. When oxygen becomes limiting in a glucose-limited chemostat cultivation, the glucose uptake rate per cell increases. We addressed the question of how the expression level of hexose transporter encoding genes is modified as a result of increased specific glucose consumption rate under oxygen restricted conditions.

In aerobic glucose-limited chemostat cultures grown at a dilution rate of 0.1 h^{-1}, both high and moderately low affinity glucose uptake is detected [20]. A similar result was observed here; expression of the moderately low affinity transporter genes *HXT2*, *HXT4* and *HXT5* and of the high affinity transporter genes *HXT6* and *HXT7* was seen. Expression of *HXT1* and *HXT3*, encoding low affinity transporters was also detected, but on a very low level. In their chemostat study, Diderich and co-workers [20] only detected the expression of *HXT2*, *HXT5* and *HXT7*. This is most probably due to differences in the sensitivity of the techniques used. We were also able to detect the expression of all other hexose transporter genes except *HXT9*, *HXT14* and *GAL2*, although some were only expressed at a very low level.

In anaerobic conditions, the expression of *HXT2*, *HXT4* and *HXT5*, encoding moderately low affinity transporters was significantly reduced compared to aerobic conditions (Figure 1). This was also seen during transition from aerobic or hypoxic growth to anaerobic growth and agrees with earlier studies which detected only high affinity transport activity in anaerobic glucose-limited chemostat cultivations [20]. In addition to the decreased expression levels of the moderately low affinity transporter encoding genes, the activity of Hxt2p may be modulated towards higher affinity, as both high and moderately low affinity components have been observed in a strain expressing only this transporter gene [4].

Interestingly, the expression of none of the hexose transporter encoding genes was increased in anaerobic compared to aerobic conditions. However, the decrease in the moderately low affinity uptake may result in a relative increase in high affinity uptake, which may allow the higher specific glucose consumption rate to occur in anaerobic conditions. In contrast, *Trhxt1*, encoding a glucose transporter, is induced by anoxia but not by hypoxia in the filamentous fungus *Trichoderma reesei* [45]. This may represent a difference between facultative anaerobic and strictly aerobic fungi.

Earlier studies have shown that *HXT5* is expressed at a higher level in aerobic than in anaerobic conditions [20,46]. Our studies show that the transcription of *HXT5* was significantly reduced not only in anaerobic, but also in hypoxic, compared to aerobic conditions. In addition, the regulation of *HXT5* following a change in oxygen provision differed from that of other *HXT* genes. When hypoxic condition became anaerobic, there was a long (7 h), transient upregulation of expression of this gene, that was opposite to the final response of downregulation. It is known that the transcription of *HXT5* is not regulated by extracellular glucose concentration, but by growth rate, and that the expression is under the control of STRE and HAP elements in its promoter [17,19]. The reduction in specific growth rate for up to 15 h following a shift from hypoxic to anaerobic conditions may have contributed to the transient upregulation of *HXT5*, but it should be noted that transient upregulation was not observed following the change from aerobic to anaerobic conditions even though the specific growth rate was similarly reduced. HAP2/3/4/5p elements are needed for the induction of transcription of respiratory genes, like the ones encoding the subunits of cytochrome *c* oxidase (*COX4*, *COX5a*, *COX6*) and enzymes of the tricarboxylic acid cycle (*KGD1*, *CIT1*) [47-51]. These elements are also likely to be

involved in the aerobic induction of *HXT5* seen in this study.

The change in specific growth rate after a change in oxygen provision may also have affected the expression of other hexose transporter encoding genes, directly or inderectly. However, responses to increased oxygen provision (20.9 or 1.0%) which occurred within less than 2 hours were independent of specific growth rate, which only increased after this time. Further, specific growth rate increased 2 hours after a change to either 20.9 or 1.0% oxygen, but for those genes whose transcription was affected by the change between 2 and 8 hours after it occurred, during which time the specific growth rate was increased, the response was generally not the same with 20.9 as with 1.0% oxygen. Thus it is unlikely that the increase in specific growth rate contributed as much as the increase in oxygen availability in regulation of the hexose transport genes. The decrease in specific growth rate which occurred following a change to anaerobic conditions was also unlikely to be as important as the reduction in oxygen availability in affecting transcription of the hexose transport genes, since a similar reduction in specific growth rate was observed regardless of the initial concentration of oxygen provided, but changes in gene transcription were dependent on the initial oxygen provision.

In hypoxic (0.5–2.8% oxygen) conditions, the expression of four of the *HXT* genes was higher than in aerobic conditions. Even with 2.8% oxygen, compared to aerobic conditions, the expression of *HXT3*, *HXT6*, *HXT13* and *HXT15/16* was higher, whereas the expression of *HXT2*, *HXT4* and *HXT5* was lower. In our earlier study we observed that the expression levels of 50% of the 69 genes of central carbon metabolism which were studied were different under 2.8% oxygen provision compared to full aeration [32], suggesting that the cell is able to recognise the reduced oxygen level, even though the cultures still maintain a high biomass concentration and a high oxygen uptake rate. Reduced transcript levels of *HXT2* and *HXT5* and increased transcript levels of *HXT3* have previously been observed during respiro-fermentative metabolism at high dilution rates in chemostat cultures [20].

The expression of the high affinity transporter encoding gene *HXT6* was relatively strong in all the hypoxic oxygen levels studied compared to aerobic or anaerobic conditions. This was seen in both steady state and non-steady state cultures. In contrast, the expression of the other high affinity transporter encoding gene, *HXT7* was very similar in aerobic cultivations and in those receiving 2.8% oxygen and was low in cultures with less or no oxygen, especially in the culture receiving 0.5% oxygen. This observation agrees with the earlier observation that even though *HXT6* and *HXT7* are 99% identical in their coding regions, they are not co-regulated [20] Indeed, their promoter regions are only 51% identical.

HXT13 and *HXT15/16* exhibited their highest relative expression in hypoxic steady states and also showed clear responses to a shift in oxygen concentration. These genes were induced when hypoxia was introduced after anaerobic conditions and repressed when hypoxic cultivations became anaerobic. The transient responses following shifts between fully aerobic and anaerobic conditions were smaller, reflecting the comparable expression level of these two genes under the two conditions. *HXT13* and *HXT15* have previously been shown to be slightly induced by non-fermentable carbon sources [24]. In the promoter regions of *HXT15* and *HXT16* there are LORE (low oxygen response element) [43] sequences with one mismatch to the consensus. These elements could be responsible for the induction in the hypoxic conditions, although the site is located -900 bp from the start codon of the genes. The promoter of *HXT13*, on the other hand, contains an HRE (hypoxia response element) sequence, which is responsible for the regulation of hypoxic genes in mammalian cells (*e.g. GLUT1* glucose transporter in human) [52-55]. It has recently been speculated that this element is also present in the yeast genome [44].

Only small differences in the expression levels of *HXT8*, *HXT10*, *HXT11* and *HXT17* were seen, either between different steady states or as a response to a change in oxygen concentration. In addition to regulation by glucose concentration (excluding *HXT11)*, the expression levels are known to be affected by change in pH (*HXT8*, *HXT9*, *HXT11* and *HXT17*), and *HXT11* has been indicated to be involved in multidrug resistance [7,24,56].

Most of the *HXT* genes had different expression in different oxygen concentrations, either during the steady states or following a change between anaerobic and hypoxic or aerobic conditions. Transcription of *HXT2*, *HXT4* and *HXT5* was high in aerobic and that of *HXT6*, *HXT13* and *HXT15/16* was high in hypoxic conditions. Additionally, the three former transporter encoding genes clearly had lower expression levels in oxygen-restricted conditions. However, questions concerning the significance of the hypoxic induction, and why the moderately low affinity transporters are expressed in glucose-limited chemostats with very low extracellular glucose concentration, remain to be addressed in future studies. It is tempting to speculate, that the hypoxic induction of expression, in particular that of *HXT6*, encoding a known high affinity transporter, indicates a response to enhance sugar uptake upon oxygen limitation. The fact that the relative transcript levels of this gene are lower in fully anaerobic compared to hypoxic cultures may not represent the situation at the protein level on the plasma membrane. The

decreased amount of moderately low affinity transporters, achieved by both regulating the expression of these genes and by degradation of the respective protein(s), under conditions of low oxygen may provide additional membrane space for the high affinity transporters to occupy the membrane and thus increase the glucose uptake rate.

Conclusion

The expression of hexose transporter encoding genes was affected by change in oxygen provision. The expression of genes encoding moderately low affinity transporters was lower in anaerobic than aerobic conditions. As the expression of none of the hexose transporter encoding genes was increased in anaerobic compared to aerobic conditions it seems that the decrease in the moderately low affinity uptake and consequently the relative increase of high affinity uptake may itself allow a higher specific glucose consumption rate to occur in oxygen restricted and anaerobic conditions. Further, the gene encoding the high affinity transporter Hxt6p and the genes encoding Hxt13p and Hxt15/16p were upregulated in hypoxic conditions, and the expression of moderately low affinity transporters, particularly that of *HXT5*, was reduced with only 2.8% oxygen compared to fully aerobic conditions. The regulation of hexose transporter encoding genes is thus different in oxygen restricted conditions compared to fully anaerobic conditions.

Methods

Yeast strain and culture conditions

Saccharomyces cerevisiae CEN.PK113-1A (*MATα*, *URA3*, *HIS3*, *LEU2*, *TRP1*, *MAL2-8c*, *SUC2)* was grown in 0.8 to 1 L medium in Braun Biotech International (Sartorius) Biostat® CT (2.5 L working volume) bioreactors in the defined minimal medium described by Verduyn *et al*. [57], with 10 g glucose l^{-1} as carbon source, and supplemented with 10 mg ergosterol l^{-1} and 420 mg Tween 80 l^{-1}. BDH silicone antifoam (0.5 mL l^{-1}) was used to prevent foam production in the cultures. Chemostat cultures were maintained at D = 0.10 ± 0.02 h^{-1}, pH 5.0, 30°C, with 1.5 volume gas [volume culture]$^{-1}$ min^{-1} (vvm). For cultures which received less than 20.9% O_2 in the gas stream, air was replaced with the equivalent volume of N_2, so that total gas flow was maintained constant for all experiments. Cultures which were fed 2.8 or 20.9% O_2 were subject to oscillations. To prevent these, approximately 5% of the total cell concentration in the bioreactor was added to the culture as cells in mid to late exponential phase at the time when continuous medium feed was started [58]. The cultivations and the culture conditions, biomass determination and metabolite analyses are described in more detail by Wiebe and coworkers [32].

In some cultures, the steady state was disrupted by replacing air (20.9 or 1.0% O_2) with 100% N_2 (0% O_2) or by introducing air (20.9 or 1.0% O_2) to anaerobic (100% N_2) cultures. When 20.9% O_2 was introduced to an anaerobic culture, dissolved oxygen was present in the culture within less than 15 seconds and was >70% within less than 2 minutes. When 1.0% O_2 was introduced to an anaerobic culture, dissolved oxygen was measurable in the culture within less than 2 minutes and for more than 2 h before returning to 0. When 20.9% O_2 was replaced by N_2, the dissolved oxygen decreased from ~80% to 0 within less than 2 minutes. No change in dissolved oxygen, which was 0, occurred when 1% O_2 was replaced by N_2.

The dilution rate and other conditions were maintained constant following a shift in conditions, but the differences in biomass yield in 0% (0.12 Cmol biomass [Cmol glucose]$^{-1}$), 1.0% (0.36 Cmol biomass [Cmol glucose]$^{-1}$) and 20.9% (0.60 Cmol biomass [Cmol glucose]$^{-1}$) O_2 [32] resulted in changes in specific growth rate for up to approximately 15 h following the shift. In cultures provided with either 20.9 or 1.0% O_2, no increase in specific growth rate was observed during the first 2 h following the addition of O_2 . Between 2 and approximately 10 h, cells in cultures receiving 20.9% O_2 grew at 0.32 h^{-1}, while those receiving 1.0% O_2 grew at 0.21 h^{-1}. After 10 h, growth continued at 0.10 h^{-1}. In cultures which were made anaerobic, the specific growth rate decreased to 0.06 h^{-1} almost immediately after O_2 was replaced with N_2 and returned to 0.10 h^{-1} after approximately 15 h [32]. During these time intervals the cultures also experienced changes in extracellular metabolite concentrations, with ethanol and glycerol concentrations increasing in cultures which became anaerobic [32] and decreasing in cultures which became aerobic or hypoxic. Glycerol consumption did not occur, but ethanol consumption was observed in aerobic cultures. Ethanol production continued in the hypoxic cultures.

Oscillations occurred in cultures which were maintained in steady state anaerobic conditions and then provided with 20.9% O_2 approximately 25 h after O_2 was provided. Fresh, exponentially growing cells were not added to prevent oscillations since the transcript levels in the added cells may have affected the overall results disproportionately.

Transcriptional analysis

Transcriptional analysis was performed with the TRanscript analysis with aid of Affinity Capture (TRAC) assay described by Rautio *et al*. [59]. Total mRNA was extracted from 10 mL cell culture samples (10 – 50 mg DW) which had been rapidly frozen in liquid N_2 and stored at -80°C. GeneScan-120LIZ size standard (Applied Biosystems, USA) was added to each sample to calibrate the separation of the detection probes by size. In addition, *in vitro* synthe-

sized mRNA (MEGAscript transcription kit; Ambion, USA) of the *Escherichia coli traT* gene was added to each sample (1.5 fmol [100 μl]$^{-1}$) so that the results for each probe in any analysis could be correlated to this internal standard, eliminating experimental variation in different hybridizations and samples. Probes were divided into 2 probe pools with 8 and 9 probes per pool. The identity of the probes was determined by the migration behaviour and the quantity by the peak area. Total polyA RNA was quantified from the cell lysates, after eluting the polyA RNA in dimethyl pyrocarbonate (DMPC) treated H_2O, using the RiboGreen RNA quantification kit (Molecular Probes, the Netherlands). Individual mRNA expression levels are given as the standardised (using *traT* internal standard) amount per total polyA RNA.

Probes for TRAC analysis

The probes used in the TRAC analyses are listed in Table 1. They were designed using the mathematical algorithms presented in Kivoja *et al*. [60]. The probes of *HXT1-5*, *HXT8-10*, *GAL2*, *RGT2* and *SNF3* were designed to hybridise to the 3' end of the coding regions. The coding regions of *HXT13* and *HXT17* as well as *HXT9* and *HXT11* have sequence similarity of 97%. Only the 5' ends of the coding region of these genes differ enough to enable the design of specific probes. *HXT6* and *HXT7* are 99% identical within the coding region, but differences can be found in the 3' flank of the genes. The flanking region of *HXT7* is AT rich which leads to a melting temperature of the probe (50°C) that is lower than that of the other probes (62–71°C). *HXT15* and *HXT16* are identical within their coding regions and within 1 kb in both directions from the coding region, except one nucleotide. Therefore only one probe was designed which detected both of these genes.

Sequencing of promoter regions

The following oligonucleotides were used in PCR of promoter fragments from the genomic DNA of CEN.PK113-1A: *HXT3* promoter forward1 5'ACCGGTATATCAAATGGCGGTGTA 3', *HXT3* promoter reverse 5'TCAGGCATGTTCATTACCTGAGAG 3', *HXT6* promoter forward1 5'TGGCATCAAATTTGGGAA 3', *HXT6* promoter reverse 5'GAGAGATGCTCCACAGGA 3', *HXT13* promoter forward1 5'TGCTGCAATTTGCTATTT 3', *HXT13* promoter reverse 5'CATCTCCATCGCTATCAA 3', *HXT15* promoter forward1 5'CCATTTTTTCAGAATCCT 3', *HXT15* promoter reverse 5'CTGTTTAGATTATCTGCA 3'. Three parallel PCR-reactions for each of the *HXT* promoters sequenced were carried out using Dynazyme Ext or Phusion high fidelity polymerases (Finnzymes, Finland), and purified PCR-fragments were sequenced using the same oligonucleotides that were used in the PCR-reactions. In addition, the following oligonucleotides were used in the sequencing: *HXT3* promoter forward2 5'GGAACATTCTAGCTCGTT 3', *HXT6* promoter forward2 5'TACTTGGAAATTAATGTA 3', *HXT13* promoter forward2 5'ATCATTTGTCGTGTTCCT 3' and *HXT15* promoter forward2 5'CCAAATATCTTATACGTT 3'.

Data analysis and graphs

Statistical analysis of the data was carried out using SPSS software (version 14.0, SPSS Inc, USA,). Analysis of variance (ANOVA) and Dunnett's T3 multiple comparison test were used for the comparative analysis of the data. All graphs were prepared using the R environment [61,62].

Table 1: Probes used in the TRAC analysis

Gene	Probe sequence	Position[a]
HXT1	ATGGTCAGGTGGGCATTTGTTAACTTTAGCTAA	826
HXT2	TAACTCACCCCAAGAAGCGTTACCG	891
HXT3	ATACCAATGGCACCATATAACAAACAGTTACGACGTC	1145
HXT4	CTGGATCGTCTGCGCTGACCTTATTTGAAAGAGCAATAG	842
HXT5	ATCATACCCATTAGTGTACGTCTGAAC	1026
HXT6	CATCTTGCCATACAATATAAATCGTAAGGGTTCAT	+78
HXT7	GTATATATTAAAAACGTATTTACTTTTCAAGATATCATTAAAA	+83
HXT8	AGAAGGGTTTCTCGTCATGCTGTAATTTTTCGT	1661
HXT9	ATGCTCAGTTTTTACAGATGGTGCATTTGCTACTGAG	60
HXT10	TGGCCAAAGACCTTCTAGCTTCTTCATACTTACCTTTTTCTAC	742
HXT11	GGCTCATTGGCATCTAGGTTAAGGGAATT	109
HXT13	ATCTCGAACATCTCCATCGCTATCAATAGAGGATT	14
HXT14	TATGGCCCTCGTTCTTAGAGGGAACAATTCG	1386
HXT15/16	GCCCATGTCGTTGCAAAGCAGAATATATAGAAGCATGTG	1284
HXT17	GACCATCCTGAATATCTCTATCACT	19
SNF3	TCTGTACTAGTAGGAATATCAACACGTTCTG	1892
RGT2	TTCACTTGTTTTGAAACAATCTAAAAGTGTTGACGGGCCGA	1007
GAL2	AGAGGCCAACGCCATACATTTCGACTTGA	1397

[a] The start position of the probe inside the coding region. + indicates that the probe sequence is on the 3' side of the stop codon and the position is given relative to the stop codon.

Authors' contributions

ER, LR and MP conceived the study. ER carried out the transcriptional, promoter and statistical analyses, participated in the fermentations and drafted the manuscript. AT and MGW carried out the fermentations and MGW revised the manuscript. LR supervised the work and revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank Pirjo Tähtinen, Outi Könönen, Eila Leino and Tarja Hakkarainen for excellent technical assistance. The financial support of Tekes, Finnish Funding Agency for Technology and Innovation (Project numbers 40135/04 and 40537/05) and Academy of Finland, (Centre of Excellence, Industrial Biotechnology 2000–2005; project number 214568, and SYSBIO programme; project number 207435) is gratefully acknowledged.

References

1. Kruckeberg AL: **The hexose transporter family of *Saccharomyces cerevisiae*.** *Arch Microbiol* 1996, **166:**283-292.
2. Özcan S, Dover J, Rosenwald AG, Wolfl S, Johnston M: **Two glucose transporters in *Saccharomyces cerevisiae* are glucose sensors that generate a signal for induction of gene expression.** *Proc Natl Acad Sci USA* 1996, **93:**12428-12432.
3. Reifenberger E, Freidel K, Ciriacy M: **Identification of novel *HXT* genes in *Saccharomyces cerevisiae* reveals the impact of individual hexose transporters on glycolytic flux.** *Mol Microbiol* 1995, **16:**157-167.
4. Reifenberger E, Boles E, Ciriacy M: **Kinetic characterization of individual hexose transporters of *Saccharomyces cerevisiae* and their relation to the triggering mechanisms of glucose repression.** *Eur J Biochem* 1997, **245:**324-333.
5. Platt A, Reece RJ: **The yeast galactose genetic switch is mediated by the formation of a Gal4p-Gal80p-Gal3p complex.** *EMBO J* 1998, **17:**4086-4091.
6. Wieczorke R, Krampe S, Weierstall T, Freidel K, Hollenberg CP, Boles E: **Concurrent knock-out of at least 20 transporter genes is required to block uptake of hexoses in *Saccharomyces cerevisiae*.** *FEBS Lett* 1999, **464:**123-128.
7. Özcan S, Johnston M: **Function and regulation of yeast hexose transporters.** *Microbiol Mol Biol Rev* 1999, **63:**554-569.
8. Özcan S, Johnston M: **Three different regulatory mechanisms enable yeast hexose transporter (*HXT*) genes to be induced by different levels of glucose.** *Mol Cell Biol* 1995, **15:**1564-1572.
9. Lakshmanan J, Mosley AL, Özcan S: **Repression of transcription by Rgt1 in the absence of glucose requires Std1 and Mth1.** *Curr Genet* 2003, **44:**19-25.
10. Polish JA, Kim JH, Johnston M: **How the Rgt1 transcription factor of *Saccharomyces cerevisiae* is regulated by glucose.** *Genetics* 2005, **169:**583-594.
11. Schmidt MC, McCartney RR, Zhang X, Tillman TS, Solimeo H, Wolfl S, Almonte C, Watkins SC: **Std1 and Mth1 proteins interact with the glucose sensors to control glucose-regulated gene expression in *Saccharomyces cerevisiae*.** *Mol Cell Biol* 1999, **19:**4561-4571.
12. Kim JH, Brachet V, Moriya H, Johnston M: **Integration of transcriptional and posttranslational regulation in a glucose signal transduction pathway in *Saccharomyces cerevisiae*.** *Eukaryot Cell* 2006, **5:**167-173.
13. Kim JH, Johnston M: **Two glucose-sensing pathways converge on Rgt1 to regulate expression of glucose transporter genes in *Saccharomyces cerevisiae*.** *J Biol Chem* 2006, **281:**26144-26149.
14. Palomino A, Herrero P, Moreno F: **Tpk3 and Snf1 protein kinases regulate Rgt1 association with *Saccharomyces cerevisiae HXK2* promoter.** *Nucleic Acids Res* 2006, **34:**1427-1438.
15. Özcan S, Leong T, Johnston M: **Rgt1p of *Saccharomyces cerevisiae*, a key regulator of glucose-induced genes, is both an activator and a repressor of transcription.** *Mol Cell Biol* 1996, **16:**6419-6426.
16. Kaniak A, Xue Z, Macool D, Kim JH, Johnston M: **Regulatory network connecting two glucose signal transduction pathways in *Saccharomyces cerevisiae*.** *Eukaryot Cell* 2004, **3:**221-231.
17. Verwaal R, Arako M, Kapur R, Verkleij AJ, Verrips CT, Boonstra J: ***HXT5* expression is under control of STRE and HAP elements in the *HXT5* promoter.** *Yeast* 2004, **21:**747-757.
18. Verwaal R, Paalman JW, Hogenkamp A, Verkleij AJ, Verrips CT, Boonstra J: ***HXT5* expression is determined by growth rates in *Saccharomyces cerevisiae*.** *Yeast* 2002, **19:**1029-1038.
19. Diderich JA, Schuurmans JM, Van Gaalen MC, Kruckeberg AL, Van Dam K: **Functional analysis of the hexose transporter homologue *HXT5* in *Saccharomyces cerevisiae*.** *Yeast* 2001, **18:**1515-1524.
20. Diderich JA, Schepper M, van Hoek P, Luttik MA, van Dijken JP, Pronk JT, Klaassen P, Boelens HF, de Mattos MJ, van Dam K, Kruckeberg AL: **Glucose uptake kinetics and transcription of *HXT* genes in chemostat cultures of *Saccharomyces cerevisiae*.** *J Biol Chem* 1999, **274:**15350-15359.
21. Nourani A, Wesolowski-Louvel M, Delaveau T, Jacq C, Delahodde A: **Multiple-drug-resistance phenomenon in the yeast *Saccharomyces cerevisiae*: involvement of two hexose transporters.** *Mol Cell Biol* 1997, **17:**5453-5460.
22. Gross C, Kelleher M, Iyer VR, Brown PO, Winge DR: **Identification of the copper regulon in *Saccharomyces cerevisiae* by DNA microarrays.** *J Biol Chem* 2000, **275:**32310-32316.
23. Jungmann J, Reins HA, Lee J, Romeo A, Hassett R, Kosman D, Jentsch S: **MAC1, a nuclear regulatory protein related to Cu-dependent transcription factors is involved in Cu/Fe utilization and stress resistance in yeast.** *EMBO J* 1993, **12:**5051-5056.
24. Greatrix BW, van Vuuren HJ: **Expression of the *HXT13, HXT15* and *HXT17* genes in *Saccharomyces cerevisiae* and stabilization of the *HXT1* gene transcript by sugar-induced osmotic stress.** *Curr Genet* 2006, **49:**205-217.
25. Liu Z, Boles E, Rosen BP: **Arsenic trioxide uptake by hexose permeases in *Saccharomyces cerevisiae*.** *J Biol Chem* 2004, **279:**17312-17318.
26. Kruckeberg AL, Ye L, Berden JA, van Dam K: **Functional expression, quantification and cellular localization of the Hxt2 hexose transporter of *Saccharomyces cerevisiae* tagged with the green fluorescent protein.** *Biochem J* 1999, **339(Pt 2):**299-307.
27. Krampe S, Boles E: **Starvation-induced degradation of yeast hexose transporter Hxt7p is dependent on endocytosis, autophagy and the terminal sequences of the permease.** *FEBS Lett* 2002, **513:**193-196.
28. Ye L, Berden JA, van Dam K, Kruckeberg AL: **Expression and activity of the Hxt7 high-affinity hexose transporter of *Saccharomyces cerevisiae*.** *Yeast* 2001, **18:**1257-1267.
29. Krampe S, Stamm O, Hollenberg CP, Boles E: **Catabolite inactivation of the high-affinity hexose transporters Hxt6 and Hxt7 of *Saccharomyces cerevisiae* occurs in the vacuole after internalization by endocytosis.** *FEBS Lett* 1998, **441:**343-347.
30. van Suylekom D, van Donselaar E, Blanchetot C, Nguyen Do Ngoc L, Humbel BM, Boonstra J: **Degradation of the hexose transporter Hxt5p in *Saccharomyces cerevisiae*.** *Biol Cell* 2007, **99:**13-23.
31. Rogers PJ, Stewart PR: **Energetic efficiency and maintenance. Energy characteristics of *Saccharomyces cerevisiae* (wild type and petite) and *Candida parapsilosis* grown aerobically and micro-aerobically in continuous culture.** *Arch Microbiol* 1974, **99:**25-46.
32. Wiebe MG, Rintala E, Tamminen A, Simolin H, Salusjärvi L, Toivari M, Kokkonen JT, Kiuru J, Ketola RA, Jouhten P, Huuskonen A, Maaheimo H, Ruohonen L, Penttilä M: **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 Res* 2008, **8(1):**140-54.
33. Davies SE, Brindle KM: **Effects of overexpression of phosphofructokinase on glycolysis in the yeast *Saccharomyces cerevisiae*.** *Biochemistry* 1992, **31:**4729-4735.
34. Ernandes JR, De Meirsman C, Rolland F, Winderickx J, de Winde J, Brandao RL, Thevelein JM: **During the initiation of fermentation overexpression of hexokinase PII in yeast transiently causes a similar deregulation of glycolysis as deletion of Tps1.** *Yeast* 1998, **14:**255-269.
35. Blazquez MA, Lagunas R, Gancedo C, Gancedo JM: **Trehalose-6-phosphate, a new regulator of yeast glycolysis that inhibits hexokinases.** *FEBS Lett* 1993, **329:**51-54.

36. Schaaff I, Heinisch J, Zimmermann FK: **Overproduction of glycolytic enzymes in yeast.** *Yeast* 1989, **5:**285-290.
37. Fell DA, Thomas S: **Physiological control of metabolic flux: the requirement for multisite modulation.** *Biochem J* 1995, **311(Pt 1):**35-39.
38. Reijenga KA, Snoep JL, Diderich JA, van Verseveld HW, Westerhoff HV, Teusink B: **Control of glycolytic dynamics by hexose transport in *Saccharomyces cerevisiae*.** *Biophys J* 2001, **80:**626-634.
39. Diderich JA, Teusink B, Valkier J, Anjos J, Spencer-Martins I, van Dam K, Walsh MC: **Strategies to determine the extent of control exerted by glucose transport on glycolytic flux in the yeast *Saccharomyces bayanus*.** *Microbiology* 1999, **145(Pt 12):**3447-3454.
40. Ye L, Kruckeberg AL, Berden JA, van Dam K: **Growth and glucose repression are controlled by glucose transport in *Saccharomyces cerevisiae* cells containing only one glucose transporter.** *J Bacteriol* 1999, **181:**4673-4675.
41. Elbing K, Larsson C, Bill RM, Albers E, Snoep JL, Boles E, Hohmann S, Gustafsson L: **Role of hexose transport in control of glycolytic flux in *Saccharomyces cerevisiae*.** *Appl Environ Microbiol* 2004, **70:**5323-5330.
42. van Helden J, Andre B, Collado-Vides J: **Extracting regulatory sites from the upstream region of yeast genes by computational analysis of oligonucleotide frequencies.** *J Mol Biol* 1998, **281:**827-842.
43. Vasconcelles MJ, Jiang Y, McDaid K, Gilooly L, Wretzel S, Porter DL, Martin CE, Goldberg MA: **Identification and characterization of a low oxygen response element involved in the hypoxic induction of a family of *Saccharomyces cerevisiae* genes. Implications for the conservation of oxygen sensing in eukaryotes.** *J Biol Chem* 2001, **276:**14374-14384.
44. Ferreira TC, Hertzberg L, Gassmann M, Campos EG: **The yeast genome may harbor hypoxia response elements (HRE).** *Comp Biochem Physiol C Toxicol Pharmacol* 2007, **146:**255-263.
45. Ramos AS, Chambergo FS, Bonaccorsi ED, Ferreira AJ, Cella N, Gombert AK, Tonso A, El-Dorry H: **Oxygen- and glucose-dependent expression of *Trhxt1*, a putative glucose transporter gene of *Trichoderma reesei*.** *Biochemistry* 2006, **45(26):**8184-92.
46. ter Linde JJ, Liang H, Davis RW, Steensma HY, van Dijken JP, Pronk JT: **Genome-wide transcriptional analysis of aerobic and anaerobic chemostat cultures of *Saccharomyces cerevisiae*.** *J Bacteriol* 1999, **181:**7409-7413.
47. Forsburg SL, Guarente L: **Communication between mitochondria and the nucleus in regulation of cytochrome genes in the yeast *Saccharomyces cerevisiae*.** *Annu Rev Cell Biol* 1989, **5:**153-180.
48. Hodge MR, Kim G, Singh K, Cumsky MG: **Inverse regulation of the yeast *COX5* genes by oxygen and heme.** *Mol Cell Biol* 1989, **9:**1958-1964.
49. Repetto B, Tzagoloff A: **Structure and regulation of *KGD1*, the structural gene for yeast alpha-ketoglutarate dehydrogenase.** *Mol Cell Biol* 1989, **9:**2695-2705.
50. Rosenkrantz M, Kell CS, Pennell EA, Devenish LJ: **The HAP2,3,4 transcriptional activator is required for derepression of the yeast citrate synthase gene, *CIT1*.** *Mol Microbiol* 1994, **13:**119-131.
51. Trawick JD, Wright RM, Poyton RO: **Transcription of yeast *COX6*, the gene for cytochrome c oxidase subunit VI, is dependent on heme and on the *HAP2* gene.** *J Biol Chem* 1989, **264:**7005-7008.
52. Takagi H, King GL, Aiello LP: **Hypoxia upregulates glucose transport activity through an adenosine-mediated increase of GLUT1 expression in retinal capillary endothelial cells.** *Diabetes* 1998, **47:**1480-1488.
53. Ouiddir A, Planes C, Fernandes I, VanHesse A, Clerici C: **Hypoxia upregulates activity and expression of the glucose transporter GLUT1 in alveolar epithelial cells.** *Am J Respir Cell Mol Biol* 1999, **21:**710-718.
54. Royer C, Lachuer J, Crouzoulon G, Roux J, Peyronnet J, Mamet J, Pequignot J, Dalmaz Y: **Effects of gestational hypoxia on mRNA levels of Glut3 and Glut4 transporters, hypoxia inducible factor-1 and thyroid hormone receptors in developing rat brain.** *Brain Res* 2000, **856:**119-128.
55. Hayashi M, Sakata M, Takeda T, Yamamoto T, Okamoto Y, Sawada K, Kimura A, Minekawa R, Tahara M, Tasaka K, Murata Y: **Induction of glucose transporter 1 expression through hypoxia-inducible factor 1alpha under hypoxic conditions in trophoblast-derived cells.** *J Endocrinol* 2004, **183:**145-154.
56. Serrano R, Ruiz A, Bernal D, Chambers JR, Arino J: **The transcriptional response to alkaline pH in *Saccharomyces cerevisiae*: evidence for calcium-mediated signalling.** *Mol Microbiol* 2002, **46:**1319-1333.
57. Verduyn C, Postma E, Scheffers WA, Van Dijken JP: **Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation.** *Yeast* 1992, **8:**501-517.
58. Zamamiri AQ, Birol G, Hjortso MA: **Multiple stable states and hysteresis in continuous, oscillating cultures of budding yeast.** *Biotechnol Bioeng* 2001, **75:**305-312.
59. Rautio JJ, Kataja K, Satokari R, Penttilä M, Söderlund H, Saloheimo M: **Rapid and multiplexed transcript analysis of microbial cultures using capillary electrophoresis-detectable oligonucleotide probe pools.** *J Microbiol Methods* 2006, **65:**404-416.
60. Kivioja T, Arvas M, Kataja K, Penttilä M, Söderlund H, Ukkonen E: **Assigning probes into a small number of pools separable by electrophoresis.** *Bioinformatics* 2002, **18(Suppl 1):**S199-206.
61. R Development Core Team: **R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing.** In *Version 2.3.0* Vienna, Austria; 2005.
62. Warnes GR: **gplots: Various R programming tools for plotting data. Included R source code and/or documentation contributed by Ben Bolker and Thomas Lumley.** *R package version 2.3.0* 2006.

Publication III

Low oxygen levels as a trigger for enhancement of respiratory metabolism in *Saccharomyces cerevisiae*

In: BMC Genomics 2009, 10:461.

.

BMC Genomics

BioMed Central

Research article

Open Access

Low oxygen levels as a trigger for enhancement of respiratory metabolism in *Saccharomyces cerevisiae*

Eija Rintala*, Mervi Toivari, Juha-Pekka Pitkänen, Marilyn G Wiebe, Laura Ruohonen and Merja Penttilä

Address: VTT Technical Research Centre of Finland, P.O. Box 1000, FI-02044 VTT, Finland

Email: Eija Rintala* - eija.rintala@vtt.fi; Mervi Toivari - mervi.toivari@vtt.fi; Juha-Pekka Pitkänen - juha-pekka.pitkanen@vtt.fi; Marilyn G Wiebe - marilyn.wiebe@vtt.fi; Laura Ruohonen - laura.ruohonen@vtt.fi; Merja Penttilä - merja.penttila@vtt.fi

* Corresponding author

Published: 5 October 2009

BMC Genomics 2009, **10**:461 doi:10.1186/1471-2164-10-461

Received: 11 November 2008

Accepted: 5 October 2009

This article is available from: http://www.biomedcentral.com/1471-2164/10/461

Abstract

Background: The industrially important yeast *Saccharomyces cerevisiae* is able to grow both in the presence and absence of oxygen. However, the regulation of its metabolism in conditions of intermediate oxygen availability is not well characterised. We assessed the effect of oxygen provision on the transcriptome and proteome of *S. cerevisiae* in glucose-limited chemostat cultivations in anaerobic and aerobic conditions, and with three intermediate (0.5, 1.0 and 2.8% oxygen) levels of oxygen in the feed gas.

Results: The main differences in the transcriptome were observed in the comparison of fully aerobic, intermediate oxygen and anaerobic conditions, while the transcriptome was generally unchanged in conditions receiving different intermediate levels (0.5, 1.0 or 2.8% O_2) of oxygen in the feed gas. Comparison of the transcriptome and proteome data suggested post-transcriptional regulation was important, especially in 0.5% oxygen. In the conditions of intermediate oxygen, the genes encoding enzymes of the respiratory pathway were more highly expressed than in either aerobic or anaerobic conditions. A similar trend was also seen in the proteome and in enzyme activities of the TCA cycle. Further, genes encoding proteins of the mitochondrial translation machinery were present at higher levels in all oxygen-limited and anaerobic conditions, compared to fully aerobic conditions.

Conclusion: Global upregulation of genes encoding components of the respiratory pathway under conditions of intermediate oxygen suggested a regulatory mechanism to control these genes as a response to the need of more efficient energy production. Further, cells grown in three different intermediate oxygen levels were highly similar at the level of transcription, while they differed at the proteome level, suggesting post-transcriptional mechanisms leading to distinct physiological modes of respiro-fermentative metabolism.

Background

Oxygen is one of the basic determinants of cellular physiology. Oxygen is needed for energy metabolism and sterol, fatty acid and heme biosynthesis, but may also cause oxidative damage, especially when cells are exposed to oxygen after being in oxygen-restricted conditions [1]. Regulation of metabolism in response to oxygen availability is needed for rapid adaptation to changing environ-

ments both in nature and in industrial bioprocesses. *Saccharomyces cerevisiae*, a major industrial organism, is able to grow both in the presence and in the complete absence of oxygen by adjusting the mode of metabolism from respiratory to respirofermentative and fermentative. Among yeasts, *S. cerevisiae* and other *Saccharomyces* species are unique in being able to restrict respiration and increase fermentative metabolism on glucose, even in the presence of oxygen, by the repression of respiratory genes [2].

The concentration of heme plays a central role in the regulation of oxygen responsive genes in *S. cerevisiae*, through the biosynthetic pathway of heme which is not active in the absence of oxygen. However, 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 [3]. The transcription factor Hap1p acts as an activator or as a repressor of certain genes depending on the presence or absence of heme. In the presence of heme, Hap1p activates the expression of genes involved in respiration and oxidative stress [4,5]. Transcriptional activation by Hap1p increases dramatically between anaerobic and severely oxygen-restricted conditions, but only gradually between 1 μM O_2 and fully aerobic conditions [3]. Hap1p also induces the expression of *ROX1*, which encodes a repressor of genes needed during severe hypoxia or in anaerobic conditions [6,7]. In the absence of heme, Hap1p acts as a repressor of genes involved in ergosterol biosynthesis [8]. The transcription factor Hap2/3/4/5p is also suggested to be activated by heme and it induces the expression of many genes involved in respiratory metabolism in the presence of oxygen [9,10]. However, while the regulation of Hap1p by heme has been widely studied, the regulation of Hap2/3/4/5p by heme and oxygen is largely unknown [11].

In anaerobiosis, the cell wall and cell membrane of *S. cerevisiae* is remodelled, which enables import of sterols and fatty acids, which, like heme, are not synthesised in the absence of oxygen [9,12-16]. Transcription factors Upc2p, Ecm22p and Sut1p are known to play a role in the import of sterols, but the exact mechanisms are not known [17,18]. However, nearly one third of anaerobically upregulated genes contain Upc2p/Ecm22p binding sites in their promoters [19,20]. Upc2p and Ecm22p bind the same sequence and the binding is dependent on sterol concentration [21]. In addition, Mox1p and Mox2p have been suggested to be repressors interacting with Upc2p [22]. The target genes of Sut1p are not known, but the overexpression of *SUT1* has been shown to enable uptake of sterols in aerobic conditions [23,24].

Genome wide studies have revealed that a large part of the *S. cerevisiae* transcriptome reacts to the presence or absence of oxygen, partly depending on the carbon source and nutrient limitation [12-14,25,26]. While Piper and co-workers identified 877 transcripts differentially expressed between aerobic and anaerobic glucose-limited conditions, Tai and co-workers found that only 155 of these genes responded consistently to anaerobiosis under four different macronutrient limitations [25,26]. Lai and co-workers monitored the transcriptome of *S. cerevisiae* during the transition from aerobic to anaerobic conditions in batch cultivations on glucose and galactose [13,14]. These studies revealed an initial response of stress-activated genes only on galactose, while later responses of downregulation of mitochondrial functions, upregulation of carbohydrate metabolism and redox regulation and activation of networks involved in sterol and cell wall homeostasis were similar on both carbon sources. In addition to transcriptome analyses, a recent comparison of the transcriptome and proteome revealed post-transcriptional regulation of glycolysis and of the aminoacyl-tRNA, purine and amino acid biosynthetic pathways, in respect to oxygen availability [27].

To our knowledge, there is no published data of the transcriptome or proteome in steady state conditions with intermediate oxygen levels. Studies of yeast provided with different oxygen levels could reveal regulation that is dependent not only on the presence or absence oxygen, but also on oxygen concentration. Severe hypoxia is known to modulate gene expression of some gene pairs in a Hap1p, Hap2/3/4/5p and Rox1p dependent manner and it is thought to enable more efficient oxygen utilisation. *COX5a*/*COX5b*, *CYC1*/*CYC7*, *AAC2*/*AAC3* and *TIF51a*/*ANB1* are pairs of interchangeable genes, of which one member of the pair is used under aerobic conditions and the other under severe oxygen restriction [9]. This switch occurs only in very low oxygen concentrations [28] and nothing is known about the expression of these gene pairs under conditions of moderately low oxygen.

Under steady state glucose-limited conditions, glucose repression of respiratory functions does not occur and it is possible to study the effect of oxygen on metabolism without interfering effects of using different carbon sources or changes in the specific growth rate. We cultivated *S. cerevisiae* in highly controlled glucose-limited chemostat cultures with 0, 0.5, 1.0, 2.8 and 20.9% oxygen in the feed gas and studied the levels of selected transcripts, metabolites and fluxes of central carbon metabolism [29,30]. Our studies revealed that cells grown with 2.8% oxygen in the feed gas were very similar to those grown with 20.9% oxygen (fully aerobic conditions) in terms of oxygen uptake rate, carbon evolution rate, and biomass production, while only minor changes in fluxes were seen. However, the metabolism was already respiro-fermentative with 2.8% oxygen and a large fraction of measured transcripts

levels differed from those observed in cells grown with 20.9% oxygen [29,30]. Furthermore, even though the biomass yield and the respirative carbon flux through the TCA cycle were significantly reduced when cells were fed 1.0% or 0.5% oxygen, compared to fully aerobic conditions, 36% and 25% of the ATP was still generated through respiration with 1.0% and 0.5% oxygen, respectively [29,30]. In order to get a global view on the metabolism of *S. cerevisiae* under various conditions of oxygen provision, we have performed whole transcriptome and partial proteome analysis of *S. cerevisiae* cells grown in glucose-limited chemostat cultures with 0, 0.5, 1.0, 2.8 or 20.9% oxygen in the feed gas and used both well established and recently published computational tools for a thorough analysis of the data.

Results

The effect of oxygen provision on gene transcription in steady state glucose-limited chemostats

Microarray analysis of yeast from glucose-limited chemostat cultivations with 0, 0.5, 1.0, 2.8 and 20.9% oxygen in the feed gas was performed. Statistical analysis of the steady state data revealed that 3435 genes responded significantly ($p < 0.01$) to oxygen availability under the five conditions studied. While the highest number of responsive genes (2900) was observed between the anaerobic and fully aerobic conditions, the number of genes expressed differently in conditions of intermediate oxygen (0.5-2.8%) was relatively small (Figure 1A and 1B). The transcriptome from cultures with 0.5% and 1.0% oxygen was particularly similar: only 10 genes had statistical differences ($p < 0.01$) in their expression. When the anaerobic or fully aerobic conditions were compared to conditions of intermediate oxygen, significant differences were found in 2000-2400 and 1500-1600 genes, respectively.

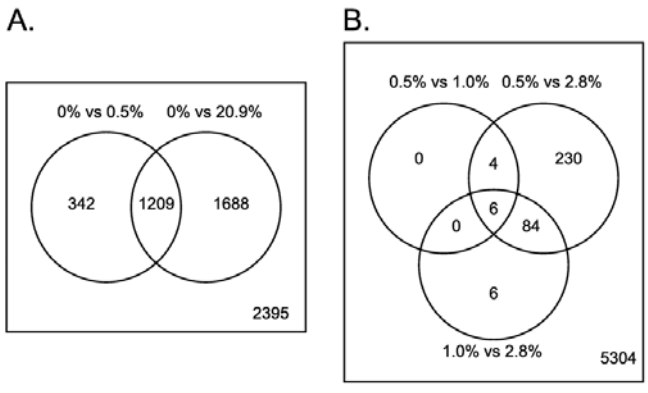


Figure 1
Venn diagrams of the genes which differ significantly ($p < 0.01$) in conditions of different oxygen provision in the feed gas. A. Anaerobic and either 0.5 or 20.9% oxygen in the feed gas and B. 0.5% and 1.0%, 0.5% and 2.8%, and 1.0% and 2.8% oxygen in the feed gas. The number in the lower right corner of the figures A and B represents the number of genes that were not differentially expressed.

To obtain an overall picture of metabolic pathways responding to oxygen availability, gene set enrichment analysis was performed. This analysis allows the identification of defined sets of genes with differential expression between two classes of samples [31,32]. Parametric gene set enrichment analysis (PAGE) uses fold changes between experimental groups to calculate Z scores for predefined gene sets and uses normal distribution to infer the statistical significance of the gene sets [33]. This approach was used in the present study to identify KEGG pathways and GO categories (containing 10 or more genes) which contained genes that were differentially expressed in conditions of different oxygen provision. Pair wise comparisons of successive oxygen levels and of the anaerobic and fully aerobic conditions are shown in Table 1. Comparison of intermediate oxygen levels showed that few pathways were differentially expressed when cells were provided with 0.5, 1.0 or 2.8% oxygen. In particular, comparison of 0.5% and 1.0% oxygen found no statistically significant differences, even at a p-value of 0.05 (data not shown).

Most of the genes (78%) which were differentially expressed between anaerobic and 0.5% provided oxygen were likewise differentially expressed between anaerobic and fully aerobic conditions (Figure 1A). PAGE analysis revealed that the pathways that were differentially expressed between anaerobic and 0.5 or 1.0% provided oxygen, but not between anaerobic and fully aerobic conditions were those of oxidative phosphorylation, pheromone signalling, arginine, proline and glutathione metabolism and exocytosis (Table 1). Pathways unique to the comparison of 2.8% and 20.9% provided oxygen were protein folding, iron ion homeostasis, protein targeting to membrane and metabolism of phenylamine and amino sugars.

Clustering of transcription data and promoter analysis of the clusters

Cluster analysis of the transcriptional data was carried out using fuzzy c-means clustering, which enabled clustering without prefiltering of the genes and thus included potentially interesting genes that did not differ strongly in the different conditions and which would otherwise have been discarded from the analysis [34]. Fuzzy c-means clustering is a soft clustering method that assigns genes to clusters with gradual membership values between zero and one. Not all genes are forced into clusters, as is often the case in traditional clustering of predetermined, significantly changing genes. Moreover, the membership values

Table 1: Parametric gene set enrichment analysis of GO classes and KEGG pathways

GO category	p-value	KEGG pathway	p-value
0% vs. 0.5 or 1.0%		**0% vs. 0.5 or 1.0%**	
Sterol transport	2.83E-08	Oxidative phosphorylation	6.63E-12
Pheromone-dependent signal transduction during conjugation with cellular fusion	5.20E-06	Arginine and proline metabolism	1.59E-09
Aerobic respiration	0.0009	Glutathione metabolism	1.26E-05
Glutamate biosynthetic process	0.0015	Citrate cycle (TCA cycle)	1.74E-05
Tricarboxylic acid cycle	0.0025	MAPK signalling pathway	2.87E-05
Exocytosis	0.0065		
0% vs. 2.8%		**0% vs. 2.8%**	
Sterol transport	1.67E-07	Arginine and proline metabolism	2.50E-13
Pheromone-dependent signal transduction during conjugation with cellular fusion	8.38E-06	Citrate cycle (TCA cycle)	4.65E-07
Glutamate biosynthetic process	7.23E-05	MAPK signalling pathway	0.0002
Tricarboxylic acid cycle	0.0006	Porphyrin metabolism	0.0009
Aerobic respiration	0.0032	Sulphur metabolism	0.0039
Exocytosis	0.0056		
0% vs. 20.9%		**0% vs. 20.9%**	
Response to stress	1.54E-06	Glyoxylate and dicarboxylate metabolism	2.77E-17
Sterol transport	2.27E-05	Citrate cycle (TCA cycle)	3.16E-12
Meiosis	0.0008	Bile acid biosynthesis	3.05E-08
NADH oxidation	0.0025	Ascorbate and aldarate metabolism	5.83E-07
Sporulation	0.0063	Nucleotide sugars metabolism	3.18E-05
Sphingolipid biosynthetic process	0.0075	Propanoate metabolism	7.84E-05
		Fatty acid metabolism	0.0002
		Pentose phosphate pathway	0.0004
0.5% vs. 1.0%		**0.5% vs. 1.0%**	
0.5 or 1.0% vs. 2.8%		**0.5 or 1.0% vs. 2.8%**	
		Glyoxylate and dicarboxylate metabolism	6.15E-20
		Fatty acid metabolism	0.0001
		Porphyrin metabolism	0.0002
0.5 or 1.0% vs. 20.9%		**0.5 or 1.0% vs. 20.9%**	
Sporulation (sensu Fungi)	0.0015		
Pheromone-dependent signal transduction during conjugation with cellular fusion	0.0027		
Meiosis	0.0048		
2.8% vs. 20.9%		**2.8% vs. 20.9%**	
Response to stress	1.1E-12	Glyoxylate and dicarboxylate metabolism	3.93E-06
Sporulation (sensu Fungi)	0.0003	Phenylalanine metabolism	0.0064
Protein folding	0.0013	Amino sugars metabolism	
Pheromone-dependent signal transduction during conjugation with cellular fusion	0.0017		
SRP-dependent co-translational protein targeting to membrane, translocation	0.0019		
Hexose transport	0.0032		
Iron ion homeostasis	0.0077		

Pair wise comparison of transcriptome data from cells grown in glucose-limited chemostats receiving 0, 0.5, 1.0, 2.8 or 20.9% oxygen (p-values < 0.01). For 0.5 and 1.0% oxygen, the data has been combined and the p-values are averaged p-values.

for the clusters can be used to determine the level of coregulation under consideration. The fuzzy c-means clustering of gene expression data from *S. cerevisiae* cultures grown with different amounts of oxygen and the most significant over-represented GO-categories and KEGG-pathways in these clusters are presented in Figure 2 and Additional file 1), respectively. Analysis of the gene expression data revealed 22 clusters containing 37-267 genes with alpha values higher than 0.5, i.e. the genes belonged with highest probability to the respective cluster.

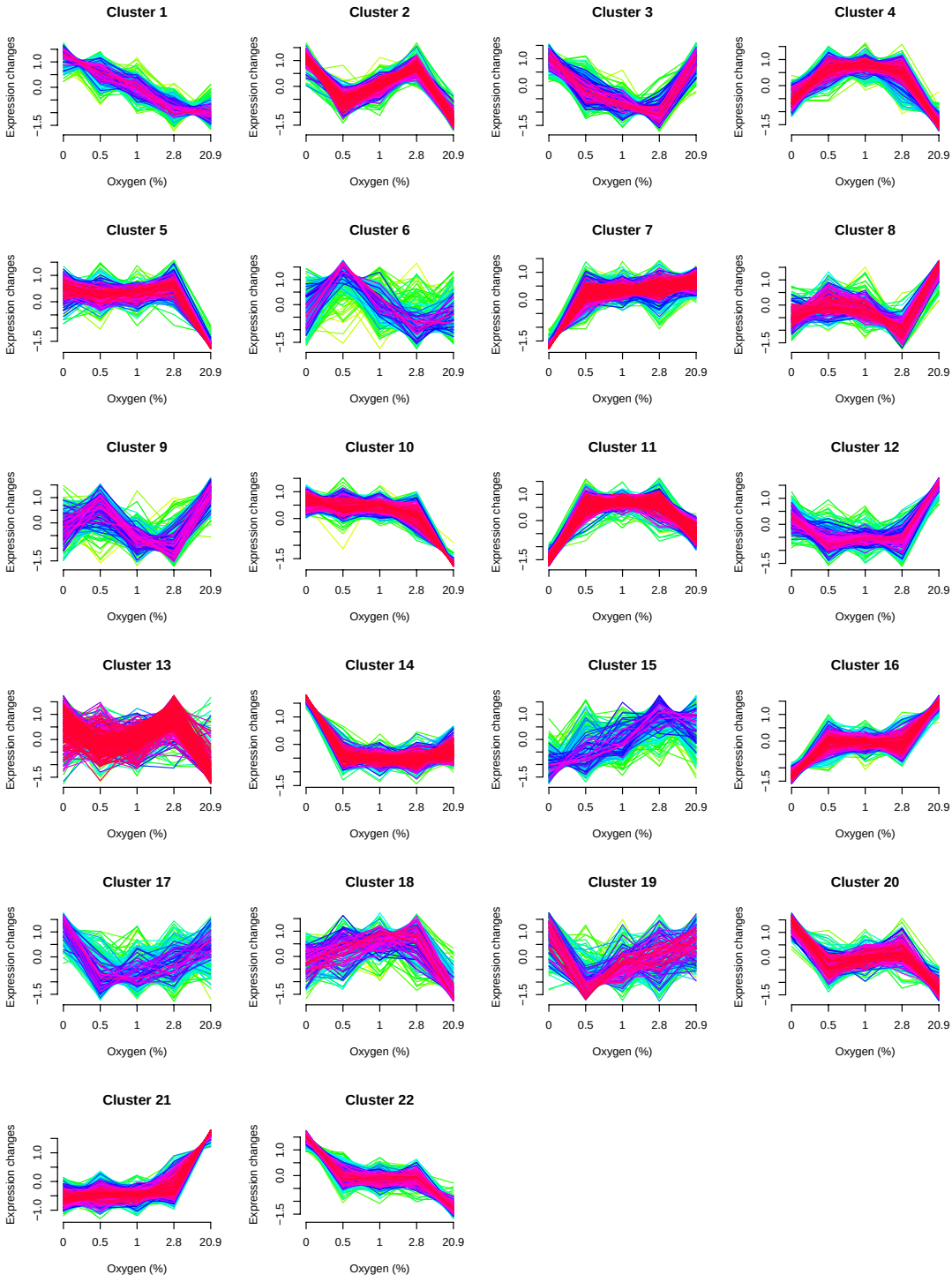


Figure 2

Fuzzy c-means clustering of gene expression patterns in cells grown with 0, 0.5, 1.0, 2.8 and 20.9% oxygen in the feed gas. The clustering was performed with individual samples, but average values for each condition are shown in the graphs. The expression values are centred and scaled around a mean of zero and standard deviation of 1, for all the genes. Red and purple represent genes that have membership values higher than 0.5 while green and yellow represent genes that have membership values below 0.5.

The promoter and 3'UTR sequences of genes in the clusters identified using fuzzy c-means clustering were analysed using FIRE software [35] and the results of the analysis are shown in Figure 3. The analysis revealed 17 transcription factor binding site motifs and 7 3'UTR motifs, of which some had significant co-occurrence and/or co-localisation patterns. A more detailed description of the results of clustering and promoter analysis is provided below.

Genes of the respiratory pathway and TCA-cycle have enhanced expression in intermediate compared to fully aerobic conditions

Two steady state clusters (cluster 4 and cluster 11) contained genes that had higher expression in all intermediate oxygen conditions compared to either anaerobic or fully aerobic conditions. The transcription levels of genes in cluster 4 were higher in anaerobic than aerobic conditions, while the opposite was observed in cluster 11. Cluster 4 was enriched in genes of KEGG pathways for the cell cycle and glycerophospholipid metabolism, while cluster 11 was enriched in genes related to oxidative phosphorylation, the TCA cycle, the MAPK signalling pathway and pyruvate metabolism. FIRE analysis revealed that different motifs were enriched in the promoters and 3'UTR sequences of the genes of these two clusters. In genes of cluster 4, motifs for Puf3p 3'UTR sites were found, while genes in cluster 11 were enriched in binding sites of the Hap2/3/4/5p transcription factor and two previously undescribed 3'UTR motifs (WHATATTC and HTTTAWTTH). All three motifs found in cluster 11 had significant co-occurrence amongst the genes.

Nearly all of the genes encoding nuclear-encoded subunits of respiratory chain complexes were located in cluster 11 (30 out of 37) and cluster 4 (4 out of 37), thus having their highest expression levels in the intermediate oxygen conditions. Cluster 11 and 4 also contained genes encoding several TCA cycle enzymes: Cit1p, Aco1p, Idh1p, Kgd1p, Kgd2p, Lpd1p, Mdh1p (cluster 11) and Idh2p (cluster 4). The increase in the expression was mainly less than 2-fold, suggesting a subtle change of the components of these pathways. Of the genes encoding the main enzymes of the TCA cycle, only *FUM1*, *LSC1* and *LSC2* did not have their highest expression level in the intermediate oxygen conditions, but in the fully aerobic conditions. Further, genes encoding isoenzymes of the enzymes of the TCA cycle had their highest expression either in fully aerobic (*IDP2*, *IDP3*, *MDH2*, *MDH3*, *CIT3*, *YLR164W*, *YJL045W*, *YMR118C*) or anaerobic (*CIT2*) conditions.

Many respiratory enzymes contain metals and accordingly, many genes involved in metal transport and homeostasis were found in clusters 4 and 11. Genes encoding vacuolar iron transporters Fth1p and Fet5p, plasma membrane copper transporters Ccc2p and Ctr1p, the metal ion transporter Smf1p and iron and copper reductase Fre1p were found in cluster 11. Additionally, genes encoding metallopeptidases/proteases Yta12p, Axl1p, Qri7p, and the copper deprivation induced ORF *YOR296W* were amongst the members of this cluster. Cluster 4 contained genes encoding plasma membrane siderophore-iron transporter Arn1p, oxidoreductase Fet3p, vacuolar zinc transporter Zrc1p and Ggc1p involved in mitochondrial iron homeostasis. Comparing gene expression in 2.8% oxygen and the fully aerobic conditions, 9 out of 16 genes known to be involved in transport of iron from the extracellular medium to the cytosol [36] had 2-16 fold higher expression and only two genes had lower expression in 2.8% oxygen than in the fully aerobic conditions.

Cluster 4 was enriched in genes related to mitochondrial organisation and biogenesis (*RPM2*, *POR1*, *UTH1*, *PNT1*, *CLU1*, *DNM1*, *MGM1*, *MBA1*). In addition, genes encoding mitochondrial translation elongation factors (*TUF1*, *MEF1*), mitochondrial translational activators (*CBS2*, *PET309*), mitochondrial ribosome recycling factor (*RRF1*) and subunits of mitochondrial ribosomes (10 genes) were found in this cluster. Cluster 10, in which the lowest level of expression occurred in the fully aerobic conditions and similar, higher expression levels occurred in the oxygen-limited and anaerobic conditions, also contained genes related to mitochondrial protein synthesis. 57 genes encoding components of mitochondrial ribosomes and 10 genes of mitochondrial protein import machinery were found in cluster 10. The 3' UTR motif for binding of Puf3p, which promotes degradation of mRNAs of nuclear-encoded mitochondrial proteins, was over-represented both in clusters 4 and 10. The expression of *PUF3* itself was low and remained constant under all the conditions of different oxygen provision studied.

Effect of oxygen on transcription of genes involved in lipid metabolism

Clusters 16 and 21 were enriched in genes related to fatty acid oxidation and peroxisomal biogenesis. Cluster 16 showed highest expression in fully aerobic conditions, lowest expression in anaerobic conditions and a similar, intermediate level of expression in all the intermediate oxygen conditions. Genes encoding activities of fatty acid β-oxidation (*TES1*, *POX1*, *CTA1*, *PXA1*, *SPS19*, *DCI1*, *ANT1*, *FOX2*, *POT1*, *PEX11*, *PXA2*), the oleate responding transcription factor *OAF1* and 4 genes related to peroxisomal biogenesis (*PEX15*, *PEX2*, *PEX8*, *PEX18*) were located in this cluster. Gene expression in cluster 21 was at its highest in fully aerobic conditions, and at a lower, comparable level in the oxygen-limited and anaerobic conditions. This cluster contained 6 genes (*PCD1*, *YOR084W*, *CAT2*, *IDP3*, *ECI1*, *AAT2*) related to fatty acid metabolism, and 7 genes related to peroxisomal biogenesis

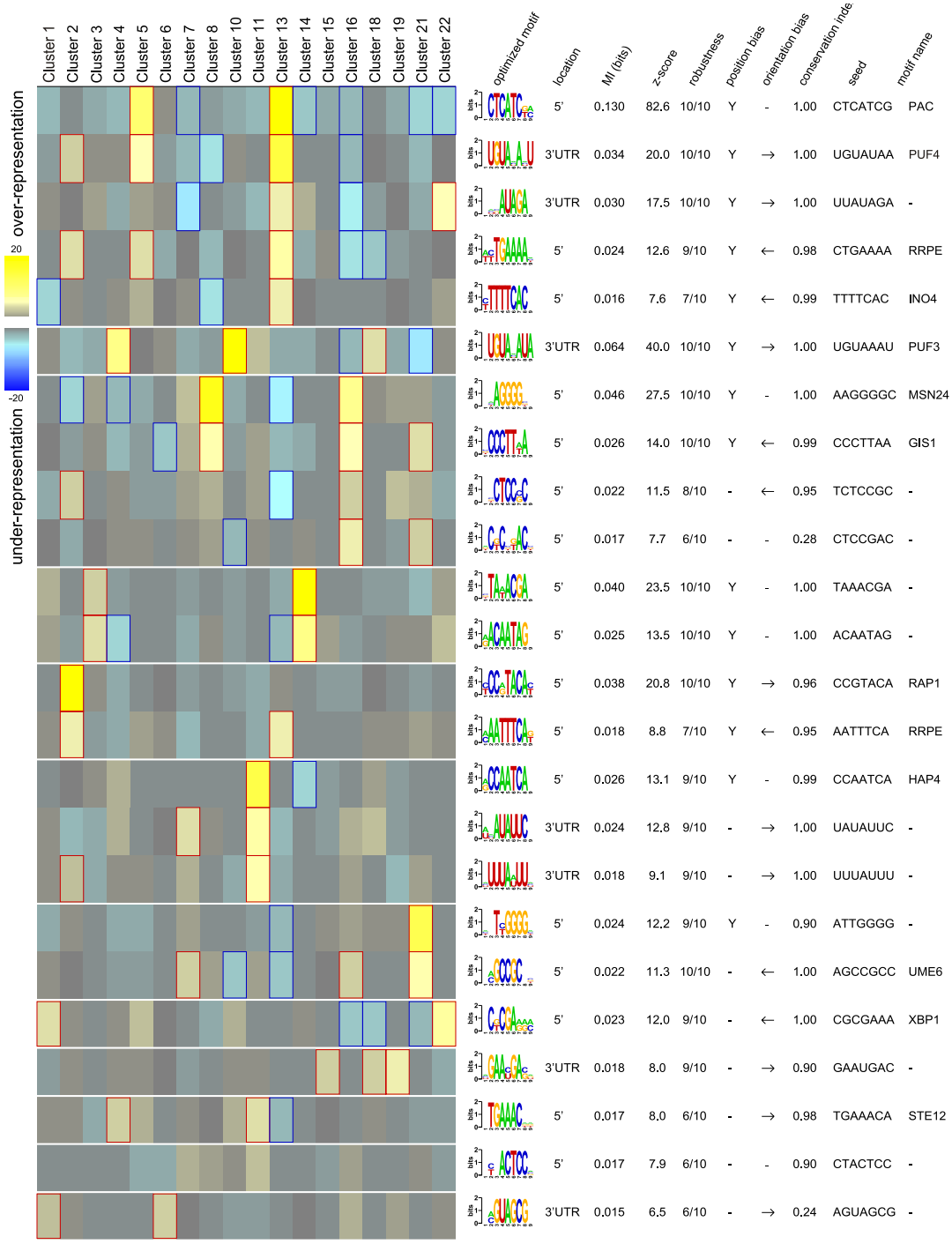


Figure 3

FIRE analysis for transcriptional regulatory motifs occurring in the clusters presented in figure 2. For each cluster, the most significant GO enrichments are shown at the top. Yellow indicates over-representation of a motif in a given cluster and significant ($p < 0.05$) overrepresentation is highlighted with red frames. Similarly, blue blocks and blue frames indicate significant ($p < 0.05$) under-representation. For each motif, the location (either 5' upstream or 3' UTR), mutual information (MI) value, Z score associated with the MI value, a robustness score ranging from 1/10 to 10/10, a position bias indicator ("Y" indicates position bias is observed), orientation bias indicator, conservation index, the seed that gave rise to the motif and name of the closest known motif are presented. For more details, see Elemento and co-workers 2007 [35].

(*PEX14*, *PEX5*, *PEX19*, *PEX30*, *PEX28*, *PEX1*, *PEX3*, *YMR018W*). The oleate responding transcription factor *PIP2* was also located in this cluster.

Clusters 3 and 14 were enriched in genes related to sterol metabolism. Genes of cluster 3 were transcribed at lower levels in intermediate oxygen conditions, compared to fully aerobic or anaerobic conditions. The cluster contained genes encoding activities of ergosterol biosynthesis (*ERG6*, *ERG11*, *HMG2*, *ERG25*, *DAP1*), sterol transport (*SUT2*, *OSH2*), sterol homeostasis (*TGL1*) and synthesis of membrane sterols (*ATG26*). Genes in cluster 14 were transcribed at a lower level in all oxygen containing conditions, compared to anaerobic conditions. The cluster was enriched in genes encoding proteins involved in ergosterol biosynthesis (*ERG26*, *ERG7*, *ERG2*, *ERG3*, *ERG1*, *ERG10*, *NCP1*, *ERG9*, *ERG27*, *ERG24*, *ERG28*, *HES1*), sterol esterification (*ARE1*), sterol transport (*AUS1*, *SWH1*) and regulation of sterol transport and biosynthesis (*UPC2*, *ECM22*). Also *DAN/TIR* genes, encoding cell wall mannoproteins, and *PAU* genes of unknown function were accumulated in cluster 14 (*DAN1-4*, *TIR1-4*, *PAU2,3,5,9*). When a less strict α-value of 0.1 was used to define the genes belonging to this cluster, three additional *PAU* genes were found in it (*PAU7,17,18*).

Promoters of the genes in clusters 3 and 14 were enriched in two putative transcription factor binding sites that had strong, positive co-occurrence. The motif BTAWACGA was found in all the sterol metabolism-related genes of cluster 14, except in *SWH1*, and in all the three *ERG* genes of cluster 3. The motif RACAATAG was found in the promoters of 11 out of the 29 genes related to sterol metabolism of cluster 14, and in 2 out of 9 of those in cluster 3.

Oxygen dependent stress responses

Three clusters (clusters 3, 8 and 16), with distinct expression profiles, showed enrichment in genes in the GO category of stress response, and binding sites of stress-related transcription factors Msn2/4p and Gis1p were over-represented among the promoters of the genes in two of these clusters (clusters 8 and 16). In the promoters of the genes in cluster 16, binding sites of Ume6p and two unknown transcription factors were also over-represented while, binding sites for a stress-activated transcriptional repressor Xbp1p were under-represented. Further, the gene encoding Xbp1p was a member of cluster 16. The expression level of *XBP1* was induced 3-fold in the intermediate oxygen (0.5-2.8%) and 8-fold in the fully aerobic conditions compared to the anaerobic conditions. Promoter analysis revealed enrichment of the binding site for Xbp1p in clusters 1 and 22. These clusters had an average correlation of -0.81 and -0.97, respectively, to the expression level of *XBP1*. 72% and 68% of the genes in clusters 1 and 22, respectively, contained the central core bases (CTCGA) of the Xbp1p binding site. Many of these genes are related to the regulation of cell division (*GIC1*, *BUD4*, *TOS4*, *KIP2*, *TOS1*, *KIN4*, *TUB4*, *CIN8*, *TUB3*, *VIK1*, *SMC2*, *UNG1*, *PIN4*, *FKH1*) and cell wall organisation (*EXG2*, ORF YFL052W, *TOS1*, *BUD7*, *MHP1*, *DSE1*, *SUN4*).

The MAPK signalling pathway for pheromone response and filamentous growth is affected by oxygen availability

Clusters 4, 7 and 11, of which clusters 4 and 11 have been discussed above with reference to genes involved in the TCA cycle and respiration, and which contain those genes which were more highly expressed in the conditions of intermediate oxygen availability, were enriched in genes involved in mating and filamentous growth. These clusters contained genes which showed a low level of expression in anaerobic, compared to intermediate oxygen conditions. However, they differed in the fully aerobic conditions, genes of clusters 4 and 11 had lower expression levels in the aerobic than in the intermediate oxygen conditions, but in cluster 7 the expression levels were comparable in all conditions provided with oxygen.

Genes in cluster 11 included some encoding proteins of the MAPK signalling pathways for pheromone response and filamentous growth (Ste3p, Gpa1p, Fus3p, Sst2p, Kss1p), genes regulated by these signalling pathways (*FUS2*, *FUS1*, *FIG1*, *SAG1*, *FIG2*, *PRM6*, *AGA1*, *PRM1*, *CLN1*, *BUD8*, *MSB2*, *CWP1*, *GFA1*, *KTR2*, *SVS1*) and the transcription factors (Ste12p, Tec1p) that are activated by these pathways. According to FIRE analysis, this cluster as well as cluster 4, which contained a set of genes related to mating (*FAR1*, *STE4*, *CLN2*, *MSG5*, *STE23*, *KAR5*, *ASH1*, *HO*, *CCW12*), were enriched in genes whose promoters contain the transcription factor binding site for Ste12p. Cluster 7 contained genes regulated by the MAPK signalling pathway for mating (*PRM5*, *PRM10*, *AGA2*, *MDG1*, *AFR1*, *PRR2*, *PRM8*, *CHS1*). While promoters of genes in cluster 7 were overall enriched with a binding site of Ume6p transcription factor, Ume6p binding site was not enriched in the promoters of the genes related to pheromone signalling.

Comparison with previous data and oxygen dependence of genes of pentose phosphate pathway

We previously published transcription data for 72 selected genes related to central carbon metabolism, measured with the TRAC method [29]. Of those genes analysed with both Affymetrix ($p < 0.01$) and with TRAC ($p < 0.05$) methods, 61 showed statistically significant differences in their expression levels with both methods. Sixteen of the significantly changing genes showed >3-fold difference in expression and had an average correlation of 0.8 between the TRAC and the Affymetrix analysis. Thirteen of the significantly changing genes showed 2 to 3-fold difference in

expression and had an average correlation of 0.6. Twenty-four of the significantly changing genes had <2-fold difference in their expression and had an average correlation of only 0.2. However, five of these genes which had <2-fold difference had correlations > 0.7. The genes that showed poor correlation between the TRAC and the Affymetrix data, and that showed ≥ 2-fold differences in the Affymetrix were *GPD2*, *CIT2*, *ACS1*, *HAP1*, *MAE1* and *PCK1*, the signals of the three latter genes being very close to the detection limit using the TRAC method.

Large changes in the expression of *SOL4*, *GND2*, *TKL2* and the ORF *YGR043C*, from the pentose phosphate pathway, were observed in Affymetrix data. These genes had their highest levels of expression in the aerobic and lowest levels of expression in the anaerobic conditions (cluster 16). The fold differences were 2-15 between the anaerobic and intermediate oxygen and 16 to 40-fold between the anaerobic and fully aerobic conditions. In addition, *SOL3* was slightly (1.5-fold) upregulated in the 2.8% oxygen and fully aerobic conditions compared to lower oxygen levels. Of these genes, the expression of *GND2*, *TKL2* and ORF *YGR043C* had also been measured with the TRAC method and the correlation between the Affymetrix and TRAC measurements was > 0.7.

ZWF1 was also measured with both Affymetrix and TRAC. With both methods *ZWF1* expression was shown to increase 1.3-fold, compared to expression in fully aerobic cells, however, this increase was seen in cells provided with 0, 0.5 and 1.0% oxygen in the Affymetrix analysis, but only in cells provided with 2.8% oxygen in the TRAC analysis. Of the other genes from the pentose phosphate pathway, *GND1*, *TKL1* and *TAL1* did not show significant differences in their expression levels in different oxygen conditions when measured with Affymetrix.

Effect of oxygen on the proteome and enzyme activities, correlated with transcriptome changes

2D-gel analysis of 2-4 independent cultures from each level of oxygen provision resulted in a proteome of 484 protein spots in total that were included in the statistical analysis. After quantile normalisation, a similar analysis for statistically significant changes in quantity with linear modelling was performed as with the gene expression data. This analysis revealed 145 spots that differed significantly ($p < 0.01$) when the cells were provided different levels of oxygen. Of the 484 spots, 209 were identified. The data is presented in additional data file 2.

Enzymes of the TCA cycle and those involved in respiration showed either a slight increase in quantity (1.5 to 2-fold) in the intermediate oxygen conditions, compared to other conditions (Idh2p, Mdh2p, Sdh1p, Atp3p, Atp5, Atp7p, Qcr2p, Rip1), a strong increase (3 to 64-fold) in fully aerobic conditions (Cit1p, Fum1p, Lsc1p, Idp2, Atp1, Cyb2p) or did not differ in different levels of oxygen provision (Aco1p, Idh2p, Atp2, Atp7p, Idp1p, Lsc2p). Many of the proteins involved in glucose fermentation 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 Hxk1 (2).

Enzyme activities were measured from crude cell extracts, providing a measure of the combined activity of all isoforms of the respective enzymes in the cell (Figure 4). The activities were expressed as units (U) per total soluble protein. It has previously been shown that there are only small differences in the protein content of the cells grown in aerobic and anaerobic glucose-limited chemostats at the growth rate of 0.1 h^{-1}·[27]. In comparison of enzyme activities we assumed that the protein content of cells grown in oxygen limited conditions would be similar to those of cells grown anaerobically and aerobically. The activities of citrate synthase (CS), aconitase (ACO), isocitrate dehydrogenase (IDH) and malate dehydrogenase (MDH), from the TCA cycle, strongly correlated (correlation > 0.89) with the transcriptome data for the corresponding genes of the TCA cycle (*CIT1*, *ACO1*, *IDH1,2* and *MDH1*, respectively). Of the enzymes of the pentose phosphate pathway, the activity of glucose-6-phosphate dehydrogenase (G6PDH) had a correlation of 0.7 with the corresponding gene, *ZWF1*. The activities of 6-phosphogluconate dehydrogenase (6PGDH), transketolase (TKL) and transaldolase (TAL) had a correlation of 0.5 to *GND1*, *TKL1* and *TAL1*, respectively, and no correlation to *GND2*, *TKL2* and ORF *YGR043C*, respectively.

In all the aeration conditions studied, the Pearson's correlation between proteins identified in the 2D gels and the mRNA levels of the corresponding genes in the transcriptome was similar, with an r-value between 0.41 and 0.55. For a more detailed comparison, the 107 significantly changing protein spots (from the 2D-gels) and the corresponding transcripts were hierarchically clustered (Figure 5). In the case of multiple protein isoforms, the corresponding transcript was assigned to each isoform separately. Of the eight groups formed by the cluster analysis, the protein and transcript quantities in groups 1 and 6 showed a high correlation (average 0.80 and 0.77, respectively). Members of group 1, related to metabolism of ethanol (ADH2), the glyoxylate cycle (ICL1, MLS1), fatty acid metabolism (FAA2), acetyl CoA synthesis (ACS1, ALD6, ALD4), and glycolysis (FBA1), were at high levels in fully aerobic conditions and both the expression of the genes and the quantity of the proteins decreased with decreasing oxygen availability. Members of group 6, involved in translation (DED1, PAB1, DYS1, HTS1) and amino acid

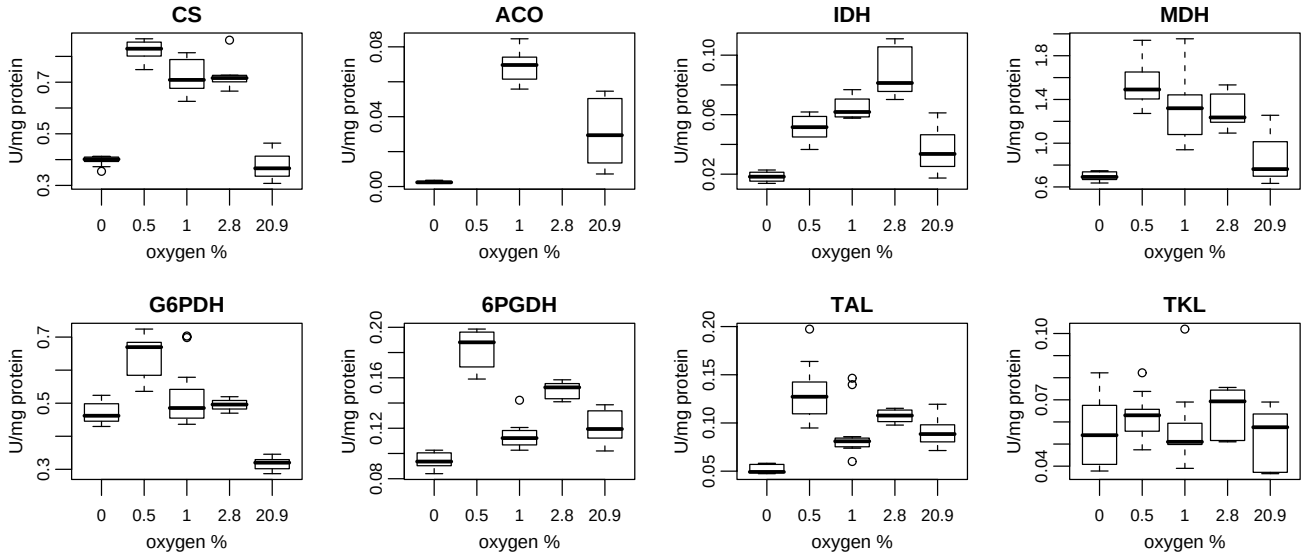


Figure 4

Enzyme activity levels in 0, 0.5, 1.0, 2.8 and 20.9% oxygen. Activity of TCA cycle enzymes citrate synthase (CS), aconitase (ACO), isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH) and of the PPP enzymes glucose-6- phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH), transketolase (TKL) and transaldolase (TAL). The data was obtained from 2 to 4 samples taken during steady states in 2 to 4 parallel cultivations. In the boxplots the box corresponds to the IQR (inter-quartile range) and the midpoint corresponds to the sample median. The whiskers extend to extreme values of the data (within 1.5 times the IQR from the upper or lower quartile). Open circles correspond to outliers.

metabolism (MET17, SER1, SAM2), glycolysis and ethanol fermentation (HXK1, ADH1), were at high levels in anaerobic conditions and on low levels in fully aerobic conditions. In groups 2, 4 and 5 the transcript and protein levels differed significantly only in cells provided with 0.5% oxygen. Group 2 contained genes and proteins involved in oxidative stress (SOD2, TSA1), redox balance (GCY1, CYB2), fatty acid metabolism (ETR1) and the TCA cycle (FUM1, LSC1). The protein levels in group 2 were high with 1.0 to 20.9% provided oxygen, while the transcript levels were already high with 0.5% provided oxygen. In group 4, related to the TCA cycle (ACO1, IDH2, SDH1), oxidative phosphorylation (ATP1, QCR2, RIP1, ATP7, ATP3) and other mitochondrial reactions (ILV2, MCR1, TUF1, POR1), the protein levels were highest with 1.0 and 2.8% provided oxygen and the transcript levels were again high already with 0.5% provided oxygen. In group 5, containing genes and proteins related to redox balancing (TRR1, RHR2, DLD3, YEL047C), the highest protein levels were observed in anaerobic conditions and when 0.5% oxygen was provided, while gene expression levels were highest under anaerobic conditions. Members of group 3, involved in various different functions, had their highest protein and gene expression levels in fully aerobic conditions, but in oxygen-restricted conditions the levels did not correlate. Group 7 contained genes and proteins, the expression and quantity of which correlated in some levels of provided oxygen. Group 8 contained genes and proteins that did not show any correlation.

Discussion

Our results demonstrate that the oxygen limitation, not only the presence or absence of oxygen, strongly affects both the transcriptome and proteome of the yeast *S. cerevisiae*. Genes related to the respiratory pathway, the TCA cycle, metal ion homeostasis and the MAPK signalling pathways of mating and filamentous growth responded specifically to intermediate oxygen availability, a response not seen when focusing only on anaerobic and aerobic growth conditions. In addition, comparison of array and proteome data indicated post-transcriptional regulation, especially with 0.5% oxygen in the feed gas.

Respiratory functions inevitably have the highest oxygen-demand of cellular reactions. Interestingly, analysis of the transcriptome revealed an upregulation of nearly all genes encoding subunits of respiratory complexes and the main enzymes of the TCA cycle in conditions of intermediate oxygen. The differences at the transcriptional level were less than 2-fold and would have been neglected in clustering analyses involving a pre-selection of genes. The same trend was observed in the proteome as an increase in the concentration of some of the proteins of the TCA cycle and respiratory chain, and further confirmed by increased

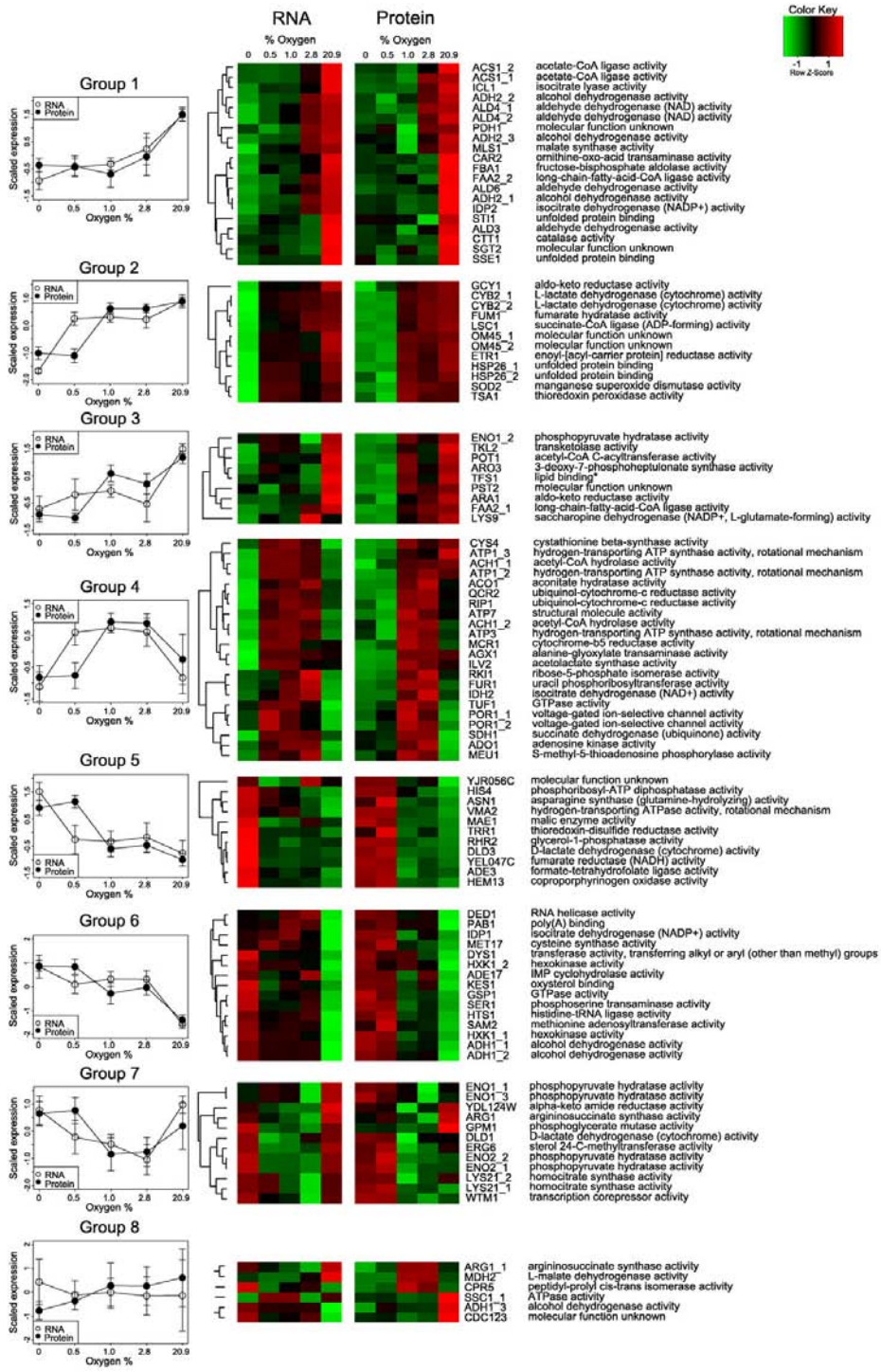


Figure 5

Comparison of protein and transcript level data. Clustering of protein spots which differed significantly in the cultures receiving different oxygen levels with their corresponding gene expression profiles. The expression values are centred and scaled around a mean of zero and standard deviation of 1. In heatmaps, green indicates low expression and red indicates high expression. For each group, the mean profile of the gene and protein expression is plotted with corresponding standard deviations. Different pI forms of the proteins are differentiated with numbers 1, 2 and 3.

enzyme activities of the enzymes of the TCA cycle in the intermediate oxygen levels. This may indicate that the cell senses that oxygen is restricted and tries to enhance respiration by global upregulation of the genes related to these functions. Supporting this hypothesis, several genes encoding enzymes that function in the transport of iron and zinc also had their highest expression in the intermediate oxygen conditions. Proteins functioning in oxygen binding and oxygen-dependent metabolism contain a large proportion of the cellular iron, copper and other metals [37] and additional metals are thus needed to enhance the synthesis of oxygen binding proteins. To our knowledge, the global upregulation of respiratory pathways observed in this study as a response to intermediate oxygen availability has not previously been described.

The Hap2/3/4/5p transcription factor, the binding site of which was enriched among the promoters of the respiratory genes which were upregulated in the intermediate oxygen conditions, is known to act as an activator of many genes encoding subunits of the respiratory chain complexes. It may have a role in gene regulation induced by oxygen restriction, possibly in combination with regulation mediated by the two 3'UTR motifs found to be enriched in the genes that had their highest expression level in the intermediate oxygen conditions. One of these motifs (WHATATTC) resembles the motif AATATTCTT found in a comparative genomics study of three yeast species [38] and a similar motif found by the matrixREDUCE algorithm [39]. Both of these studies identified the motif as over-represented among genes related to energy metabolism, the latter being particularly over-represented among the genes of the electron transport chain.

Hap2/3/4/5p is suggested to play a role in the activation of respiration during growth rates above 0.08 h^{-1} providing excess respiratory capacity that allows for respiratory metabolism at higher glucose fluxes [10,11]. Both high specific growth rates and intermediate oxygen provision in glucose-limited chemostats lead to onset of respiro-fermentative metabolism [29,40]. In fully aerobic conditions at growth rates below 0.3 h^{-1}, the cell is able to maintain fully respiratory metabolism. In conditions with only 0.5 to 2.8% oxygen provided, however, the restricted oxygen provided is not sufficient for purely respiratory metabolism even though the cell responds by upregulation of Hap2/3/4/5p controlled networks.

Hap2/3/4/5p has also been suggested to control other mitochondrial processes than respiration [10] to coordinate functions of both nucleus- and mitochondrion-encoded mitochondrial proteins. However, at the transcriptional level, our data indicates that mitochondrial translation and import machineries are not co-ordinately regulated with respiratory functions, as the genes encoding the former were more highly transcribed in anaerobic rather than in fully aerobic conditions while the genes of the latter were not. In addition, the 3'UTR sites found in these groups were different. The results suggest that mitochondrial import and translational machineries have an important role also during anaerobic conditions, in which mitochondria are known to exist in the form of pro-mitochondria, the role of which is not yet very well understood [41].

Utilisation of fatty acids is an oxygen-dependent process [42]. Accordingly, the genes encoding activities of fatty acid β-oxidation and peroxisomal biogenesis were more highly expressed in the fully aerobic compared to oxygen-limited conditions. Interestingly, there was a clear difference between cells grown with 20.9% and 2.8% oxygen in the feed gas, even though the oxygen uptake rate under these two conditions was very similar. *CTA1* and *POX1* have previously been shown to be regulated by oxygen [12,43], via a heme-dependent pathway that does not involve Hap1p, and via another mechanism that does not rely on heme, but the exact pathways are not known [43]. It is possible that the other genes of the fatty acid β-oxidation pathway in the same cluster (*TES1*, *CRC1*, *ANT1* and *FOX2*) are regulated by oxygen via the same, still unknown pathway as *CTA1* and *POX1*.

Sterol biosynthesis, which is also an oxygen-requiring process, is regulated differently than fatty acid oxidation. In contrast to fatty acid oxidation, which is mainly related to energy production, sterol biosynthesis is essential for the cell [15,44,45]. Our promoter analysis revealed two putative transcription factor binding site motifs that are possibly involved in sterol metabolism. One of these (BTAWACGA) was found in 18 out of 20 promoters of the genes of ergosterol biosynthesis, in all promoters of DAN/TIR genes encoding cell wall mannoproteins, and interestingly also in *UPC2* and *ECM22*, genes which encode transcription factors known to be involved in the regulation of sterol uptake and biosynthesis [20,46-48]. A closer look at this motif revealed that it corresponds to the AR1 (TCGTATA) and SRE (TCGTTYAG) motifs which are involved in Upc2p/Ecm22p mediated transcriptional regulation of anaerobically induced genes and *ERG* genes, respectively [20,49]. The other motif found by the FIRE programme, RACAATAG, has previously been observed through phylogenetic footprinting analysis to be enriched in genes of lipid metabolism [50] and in genes which where upregulated on galactose medium in anaerobic conditions [13]. The motif was found in the promoters of 6 *ERG* genes, *UPC2* and *ECM22*. Thus this motif may be involved in regulation of genes of ergosterol biosynthesis directly or through Upc2p and Emc22p.

Even though the ergosterol biosynthesis pathway is not active in the absence of oxygen [51-53], some of the genes of this pathway (*ERG* genes) had higher expression in

anaerobic cultures compared to the cultures receiving oxygen. This has previously been observed in cultures which were anaerobic or severely oxygen-restricted [13,21,27], but the opposite has also been reported. It was recently shown that some of the *ERG* genes are repressed by Hap1p in severely oxygen restricted conditions [8]. Anaerobic upregulation of *ERG* genes may reflect the fact that sterols are essential, by maintaining high levels of transcription for pathways of sterol and unsaturated fatty acid synthesis, priority may be given to these pathways in conditions where oxygen becomes available after a period of anaerobicity to facilitate mitochondrial membrane biogenesis [54].

Gene set enrichment analysis of our data indicated that the genes of GO category of stress response were affected by oxygen provision. The GO category of stress response contains a wide set of genes responding to different stress conditions and the cluster analysis showed that these genes were indeed enriched in three clusters with distinct expression profiles. However, all these clusters showed a higher expression level of stress-related genes in the fully aerobic conditions, compared to 2.8% provided oxygen. *XBP1*, encoding a stress-induced transcriptional repressor, was found to be regulated in an oxygen-dependent manner: the level of transcripts of this gene was lowest in anaerobic, highest in the fully aerobic and on a similar, intermediate level in the intermediate oxygen conditions. The DNA-binding domain of Xbp1 is homologous to the DNA-binding domains of cell cycle regulators Swi4p and Mbp1 and binds a related sequence [55,56]. Xbp1p has been indicated to act as a sporulation specific regulator in diploid cells, but the role of this transcription factor in haploid cells is not clear [55]. The transcription of *XBP1* is induced by various stress situations, including glucose starvation and oxidative stress [55,56]. The enrichment of its binding sites in the promoters of the genes whose transcription negatively correlated to the transcription of this repressor, indicated a role related to cell division and cell wall organisation. However, it is not clear why these functions would be regulated in conditions of constant growth rate.

Interestingly, several genes of the MAPK signalling pathway of mating and filamentous growth were considerably upregulated in the conditions of intermediate oxygen availability. Glucose limitation is known to provoke filamentous growth in haploid cells with concomitant action of MAPK, 5'-AMP-dependent and 5'-cyclic AMP-dependent kinase pathways (reviewed in [57,58]). The MAPK signalling pathway of mating and filamentous growth may be activated by low glycosylation of the exo-cellular domain of Msb2p in low glucose conditions. However, since the glucose was below detection limit in all cultures studied, oxygen limitation in addition to glucose limitation, contributed to the induction of this signalling pathway in the conditions of intermediate oxygen availability. No clear filamentation of the cells was observed under these conditions, possibly due to lack of activity of the other kinase pathways.

In conditions of restricted respiration, the carbon flux through glycolysis is increased while the flux through the pentose phosphate pathway (PPP) remains constant [30,59-62]. The regulation of glycolysis has been shown to be predominantly post-transcriptional [27,29,63], but less is known about the regulation of the PPP. Expression of the genes encoding the main isoenzymes of the PPP did not differ significantly in conditions of different oxygen provision, while the expression of the genes encoding the minor isoenzymes of PPP, *GND2*, *TKL2*, *SOL4* and ORF YGR043C (*NQM1)* was strongly affected by oxygen provision. The expression of the latter genes was not dependent on the absolute level of oxygen in the feed gas, but rather on the absence, limited provision or excess oxygen. Genes encoding these isoenzymes are also induced after the diauxic shift [64]. It thus seems that they are important for respiratory metabolism and that the downregulation of their expression in fermentative conditions is not dependent only on glucose repression.

We previously published transcription data for 72 selected genes related to central carbon metabolism, measured with the TRAC method [29]. Comparison of the results of TRAC analysis and the present data revealed good correlation for most of the transcripts showing more than 2-fold difference in their expression when measured with Affymetrix. This is similar to the correlation observed in comparison of transcription data for 1375 human genes using microarray or real-time PCR analysis [65]. The proportion of genes that showed similar significant differences with both arrays and real-time PCR was dramatically decreased when changes less than 2-fold were considered [65]. The differences between the TRAC and the Affymetrix measurements may be due to differences in sample preparation and the need for cDNA synthesis: in TRAC crude cell lysates are used and no cDNA synthesis is performed while in Affymetrix purified RNA is used to synthesise cDNA for hybridisation [66]. In addition, normalisation and probe design may affect the results [67].

Our results on the correlation of the proteome and transcriptome (Pearson correlation 0.41 to 0.55) are consistent with previous studies in yeast [68], although both higher [69] and lower [70] correlation has been reported. The 35 proteins spots and the corresponding genes of oxygen dependent reactions and respiration, and of acetyl-CoA synthesis, amino acid metabolism, translation and glycolysis in groups 1 and 6 were very similarly affected by

the oxygen availability, suggesting regulation at the transcriptional level. However, the glycolytic enzymes in particular, as well as aldehyde dehydrogenases, were found as multiple pI isoforms and the responses of the isoforms of glycolytic enzymes differed from each other, indicating regulation by phosphorylation [71].

Interestingly, for several transcript-protein pairs the expression correlated when oxygen availability was high, but varied in low oxygen conditions, especially when only 0.5% oxygen was provided (members of groups 2, 4 and 5). Proteins of the TCA cycle and the electron transport chain, and those related to oxidative stress and additional mitochondrial functions, which clustered in groups 2 and 4, were present at relatively low levels when 0.5% oxygen was provided, although the gene expression levels were already high, suggesting post-transcriptional regulation of these proteins. In group 5, related to redox status of the cell, the opposite was observed, i.e. protein levels were high, even though gene expression was down-regulated, again indicating a post-transcriptional level of regulation. Thus, both physiological characteristics and the fluxes [29] demonstrate that provision of 0.5, 1.0 or 2.8% oxygen in the feed gas results in distinct modes of respiro-fermentative metabolism which are clearly not achieved through transcriptional regulation alone.

Conclusion

The level of oxygen provision affected a significant part of the transcriptome of *S. cerevisiae*. However, there were only a few genes, the expression of which strictly correlated with oxygen concentration in the feed gas. Rather, the differences were observed in comparison of anaerobic, oxygen-limited and fully aerobic conditions, which require different modes of metabolism in *S. cerevisiae*. An overview of the interactions of the genes and transcription factors highlighted in this study is given in the Additional file 3 in which the relative gene expression in the different conditions is indicated along with the relevant transcription factors. Further, comparison of transcriptome and proteome level data indicated post-transcriptional regulation, especially when 0.5% oxygen was provided.

In the oxygen-limited conditions, the genes encoding respiratory pathways were more highly expressed and the quantities of proteins were higher than in either anaerobic or aerobic conditions. Regulation was possibly achieved at the transcriptional level through the action of the Hap2/3/4/5p transcription factor and the two previously undescribed 3'UTR elements. While the expression levels of these genes were high in all three oxygen-limited conditions studied, the protein quantities were high only when 1.0 or 2.8% oxygen was provided, suggesting a post-transcriptional level of regulation. In addition, according to these results, the transcriptional responses of respiratory and mitochondrial translational machineries were not coordinated, since the transcription of the latter was at a higher level in oxygen-limited and anaerobic conditions, compared to fully aerobic conditions, whereas the transcription of the former was higher in oxygen-limited conditions, compared to either anaerobic or fully aerobic conditions. Further, the regulatory elements enriched in the genes of mitochondrial translation machinery were different to those enriched in genes related to respiratory pathways.

Methods

Strain and culture conditions

The cultivations were described and the fermentation data published by Wiebe and co-workers [29]. Briefly, *Saccharomyces cerevisiae* CEN.PK113-1A (*MAT*, *URA3*, *HIS3*, *LEU2*, *TRP1*, *MAL2-8c*, *SUC2)* was grown in 0.8 to 1 L medium in B. Braun Biotech International (Sartorius) Biostat® CT (2.5 L working volume) bioreactors in the defined minimal medium described by Verduyn *et al*. [72], with 10 g glucose l^{-1} as carbon source, and supplemented with 10 mg ergosterol l^{-1} and 420 mg Tween 80 l^{-1}. Silicone antifoam (BDH 331512K, VWR International, UK; 0.5 mL l^{-1}) was used to prevent foam production in the cultures. Chemostat cultures were maintained at D = 0.10 ± 0.02 h^{-1}, pH 5.0, 30°C, with 1.5 volume gas [volume culture]$^{-1}$ min^{-1} (vvm). For cultures which received less than 20.9% O_2 (vol/vol) in the gas stream, air was replaced with the equivalent volume of N_2, so that total gas flow was maintained constant for all experiments. Cultures which were fed 2.8 or 20.9% O_2 were subject to oscillations. To prevent these, approximately 5% of the total cell concentration in the bioreactor was added to the culture as cells in mid to late exponential phase at the time when continuous medium feed was started [73].

Transcriptome analysis

Affymetrix microarray analysis of two (0.5%, 2.8% O_2) or four (0%, 1.0%, 20.9% O_2) parallel cultivations was performed. From cultures which received 0.5% and 2.8% O_2, two separate steady state samples were also analysed. In addition, from one of the cultivations with 1.0% O_2 in the feed gas, four separate steady state samples were analysed. The cells were collected in cold (+4°C) 10 mM Na-phosphate buffer, pH 7. After centrifugation (3500 rpm, 5 min, +4°C), the cell pellet was frozen in liquid nitrogen. For anaerobic samples, the buffer was saturated with nitrogen in advance. For RNA extraction, 5-20 mg dry mass of cells were suspended in 400 μl cold (+4°C) disruption buffer (20 mM Tris-HCl, pH 7.4, 100 mM KCl, 2 mM $MgCl_2$, 2 mM DTT). 400 μl phenol-chlorophorm (50:50), 5 μl 20% (w/v) SDS and 400 μl glass beads (0.5 mm diameter, Biospec Products) were added. The cells were disrupted with a Fastprep machine (Q-Biogene), 2 × 20 s, at speed 6. After centrifugation (14000 rpm, 15 min, +4°C), superna-

tant was used for total RNA extraction. The total RNA extraction was done with an RNeasy kit (Qiagen) according to manufacturer's instructions.

Hybridisations were carried out at the Finnish DNA Microarray Centre at Turku Centre for Biotechnology. 2 μg of total RNA was used as starting material for sample preparation. Samples were processed according to the One-Cycle Target Labelling protocol in the GeneChip Expression Analysis Manual (Affymetrix). Both before and after the amplifications the total RNA/cRNA concentrations were assessed with Nanodrop ND-1000 and total RNA/cRNA quality was assessed by BioRad's Experion electrophoresis station. Each sample was hybridised to the GeneChip Yeast Genome 2.0 Array at +45°C overnight (16 h) according to the GeneChip Expression Analysis Technical Manual (Affymetrix). A GeneChip Fluidics Station 450 was used to wash and stain the arrays, and a GeneChip Scanner 3000 with AutoLoader was used to scan the arrays. CEL-files were extracted with GCOS Manager 1.4.

All data analysis was done using R/Bioconductor, version 2.5.1 [74,75]. The raw data was normalised with Robust Multichip Average (RMA) normalisation [76]. Statistical differences in the expression were analysed using linear modelling with the tools of limma package [77]. For each gene, a linear model was fitted by the least squares method and differential expression within pairs of experimental conditions was computed using the empirical Bayesian approach [78]. For correction of multiple testing errors, the Benjamini & Hochberg -method controlling false discovery rate (FDR) was used [79]. The microarray data can be accessed through GEO accession number GSE12442.

The Gene Ontology (GO) classes and KEGG pathways differentiating the conditions studied were computed using parametric gene set enrichment analysis [33,80]. Pair wise fold changes between conditions of different oxygen provision were calculated and the fold changes were used to calculate Z scores for the gene sets. Statistical significances of Z scores were determined against normal distribution.

The clustering analysis of gene expression data was performed using fuzzy c-means clustering [34,81]. The clustering method assigns genes to clusters with gradual membership (values between 0 and 1). For the clustering, the expression values were scaled and centred to have a mean of zero and standard deviation of one. The parameter m, which controls the sensitivity of the clustering process to noise, was adjusted to 1.25 to prevent the detection of clusters in randomised data. The number of clusters was selected, such that no clusters were formed where all the genes would have membership values below 0.5. The enriched GO classes and KEGG metabolic pathways in the clusters were computed with the GOstats package [82]. Transcriptional regulatory motifs in the clusters were analysed with the FIRE method [35].

Proteome analysis

Cells for proteome analysis were collected in cold Na-phosphate buffer, pH 7 as described for the microarray analysis. 5-10 mg dry mass of cells was re-suspended in 150 μl of 10% (v/v) trichloro acetic acid (TCA, Merck) in 1.5 ml micro centrifuge tubes. 500 μl glass beads (0.5 mm diameter, Biospec Products) were added and the tube inserted into a MiniBeadbeater 8 (Biospec Products) and shaken at homogenisation speed, three times for 30 seconds. The tubes were cooled on ice between each homogenisation step. The suspensions were withdrawn and proteins were precipitated by adding 600 μl of -20°C acetone and incubating 30 min. on ice. Precipitated proteins were collected by centrifugation for 30 min., 13 000 rpm, at 4°C, rinsed once with 600 μl of -20°C acetone and re-suspended in 450 μl of 7 M urea (Promega, USA), 2 M thiourea (Fluka, USA), 4% (w/v) CHAPS (Fluka), 1% (w/v) Pharmalytes 3-10 (Pharmacia, Sweden) and 1% (w/v) DTT (Sigma) by gently shaking for 20 min. at room temperature. Supernatants were collected by centrifugation for 5 min. 13 000 rpm (Eppendorf bench centrifuge). The protein concentration of supernatants was determined by the Non-Interfering Protein Assay (Geno Technology, Inc.) and the samples were stored at -70°C before isoelectric focusing.

Isoelectric focusing and second dimension 11% (w/v) SDS-PAGE were carried out as described earlier [83]. After electrophoresis the gels were fixed for one and a half hours in 30% (v/v) ethanol and 0.5% (v/v) acetic acid and stained with Sypro Ruby (Molecular Probes), according to manufacturers' instructions. The stained gels were scanned with a resolution of 100 microns on a Typhoon instrument (GE Healthcare). The gel images were analysed using the Progenesis software (Nonlinear Dynamics). The gel patterns from different gels were automatically matched, with some additional manual editing, and the quantities of matching spots in different gels were compared. From each condition, samples from 2-4 independent cultivations were used and for each sample 4 gels were analysed. After background correction, the data was transferred to the R environment for normalisation and data analysis.

Background corrected proteome data of 500 spots was obtained from Progenesis software. For each condition, the spots that had zero values in more than 50% of the gels were treated as real zeroes and set to the lowest value of each gel [84]. The rest of the missing values were estimated using the k- nearest neighbour-method [85]. The data was log transformed and quantile normalised as

described in [86]. The statistical analysis of differences was done using linear modelling [77].

Protein identifications were carried out in the Protein Chemistry Unit, Institute of Biomedicine, Anatomy, Biomedicum, Helsinki. For protein identification, excised gel spots were washed and dehydrated with acetonitrile (Rathburn, Scotland, HPLC grade S). Proteins were reduced with 20 mM DTT and incubated at 56°C for 30 min before alkylation with 55 mM Iodoacetamide (Sigma, USA)/100 mM ammonium hydrogen carbonate (NH_4HCO_3) in the dark at room temperature for 15 min. After washing with 100 mM NH_4HCO_3 and dehydration with acetonitrile the gel pieces were rehydrated in 10 to 15 μl sequencing grade trypsin (Promega, USA) in 100 mM NH_4HCO_3, to a final concentration of 0.01 μg/μl trypsin and incubated for trypsin digestion overnight at 37°C. Tryptic peptides were eluted from the gel pieces by incubating for 15 min at room temperature successively in 25 mM NH_4HCO_3 and then twice in 5% formic acid. The tryptic peptides were desalted using Zip Tip μC-18 reverse phase (Millipore, USA) and directly eluted with 50% v/v acetonitrile/0.1% v/v trifluoroacetic acid (TFA) onto a MALDI target plate. Then, a saturated matrix solution α-cyano-4-hydroxy cinnamic acid (CHCA) (Sigma, USA) in 33% ACN/0.1% TFA was added. MALDI-TOF analyses were carried out with an Autoflex (Bruker Daltonics, Bremen Germany) equipped with a nitrogen pulsed laser (337 nm) and operating in positive mode. Typically, mass spectra were acquired by accumulating spectra of 240 laser shots. External calibration was performed for molecular assignments using a peptide calibration standard (Bruker Daltonics GmbH, Leipzig, Germany). Trypsin autolytic peptide masses were used to check or correct the calibration.

Protein identifications were performed by searching the peptide masses against the National Center for Biotechnology Information (NCBI) non-redundant database using Matrix Science's Mascot - Peptide Mass Fingerprint http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=PMF. Protein identifications by peptide mass fingerprinting were further evaluated by comparing the calculated and observed molecular mass and pI, as well as the number of peptides matched and percent sequence coverage.

Comparison of proteome and transcriptome data was done according to Gallardo and co-workers [87]. Significantly ($p < 0.01$) changing protein spots (107) and the corresponding genes were clustered using a hierarchical clustering with average linkage method and correlation as distance metric. Visualisation was done in R/Bioconductor and Inkscape version 0.46 [88].

Enzyme activity assays

Enzyme activities were measured as units (U) per mg of total soluble protein. One U was defined as the activity which converts one μmol substrate per min. Enzyme activities were measured from cell extracts prepared by disrupting the yeast cells with glass beads in 100 mM Hepes-buffer, pH 7.6, 1 mM DTT, supplemented with Complete protease inhibitor cocktail (Roche Applied Science, USA). The protein concentration of the cell extracts was determined with the Bio-Rad Protein Assay (Bio-Rad Laboratories, USA), using bovine serum albumin as the standard. All enzyme activities were measured as triplicates using the Konelab Arena 20XT automated analyser (Thermo Scientific, Finland) at 30°C and 340 nm in 79 mM TEA, pH 7.6, except citrate synthase activity which was measured at 420 nm. The isocitrate dehydrogenase (EC 1.1.1.41) assay mixture contained 1.2 mM $MnCl_2$ and 0.48 mM NAD. The reaction was started by adding DL-isocitrate trisodium salt to a final concentration of 4 mM. The citrate synthase (EC 4.1.3.7) assay mixture contained 0.38 mM Acetyl-CoA and 0.1 mM DTNB. The reaction was started by addition of oxaloacetic acid to a final concentration of 0.5 mM. The aconitase (EC 4.2.1.3) assay mixture contained 1.2 mM $MnCl_2$, 0.48 mM NADP and 0.8 U ml^{-1} isocitrate dehydrogenase (NADP dependent). The reaction was started by addition of sodium citrate to a final concentration of 2 mM. The malate dehydrogenase (EC 1.1.1.37) assay mixture contained 0.2 mM NADH. The reaction was started by addition of oxaloacetic acid to a final concentration of 0.56 mM. The transketolase (EC 2.2.1.1) and transaldolase (EC 2.2.1.2) assay mixture contained 0.2 mM NADH, 20 U ml^{-1} triosephosphate isomerase and 20 U ml^{-1} glycerolphosphate isomerase. In addition, transketolase mixtures contained 0.3 mM thiamine pyrophosphate and 4 mM $MgCl_2$. The transketolase assay was started by addition of ribulose-5P and ribose-5P to final concentrations of 5 mM and ribulose epimerase to 25 U ml^{-1}. The transaldolase assay was started by addition of erythrose-4 phosphate and fructose-6 phosphate to final concentrations of 0.7 mM and 5.4 mM, respectively. Glucose-6 phosphate dehydrogenase and 6-phosphogluconate dehydrogenase assay mixtures contained 0.2 mM NADP and 8 mM $MgCl_2$. The reactions were started by addition of glucose-6 phosphate and 6-phoshphogluconate, respectively, to final concentrations of 0.3 mg ml^{-1}.

Authors' contributions

ER, MT, LR and MP conceived the study. ER and MT carried out the transcriptome and proteome analyses and drafted the manuscript. MGW, ER and MT carried out the fermentations and MGW revised the manuscript. ER performed the enzyme activity measurements. J-PP participated in the statistical analysis and preparation of Figure S1. LR supervised the work and revised the manuscript. All authors read and approved the final manuscript.

Additional material

Additional file 1

Over-represented GO- and KEGG-classes in clusters as determined by fuzzy c-means clustering of gene expression in cells receiving 0, 0.5, 1.0, 2.8 or 20.9% oxygen, and illustrated in Figure2.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-10-461-S1.PDF]

Additional file 2

Total soluble protein data. Normalised spot intensities and corresponding standard deviations in cells receiving 0, 0.5, 1.0, 2.8 or 20.9% oxygen.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-10-461-S2.XLS]

Additional file 3

Overview of gene expression data in cells receiving 0, 0.5, 1.0, 2.8 or 20.9% oxygen of genes encoding the main metabolic pathways and oxidative phosphorylation of*Saccharomyces cerevisiae *and the transcription factors known to regulate these genes.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-10-461-S3.PDF]

Acknowledgements

We thank Pirjo Tähtinen, Eila Leino and Tarja Hakkarainen for excellent technical assistance and Dr. Mikko Arvas for fruitful discussions and setting up the bioinformatics tools. Rabah Soliymani and Dr. Marc Baumann, from the Protein Chemistry Unit, Institute of Biomedicine, Anatomy Biomedicum-Helsinki, Finland, are gratefully thanked for the protein identifications of the proteome studies. The microarray analyses were carried out at the Finnish DNA Microarray Centre at Turku Centre for Biotechnology. The financial support of Tekes, The Finnish Funding Agency for Technology and Innovation (Project numbers 40135/04 and 40537/05) and Academy of Finland (Centre of Excellence, Industrial Biotechnology 2000-2005, project number 214568, and SYSBIO programme, project number 207435) are gratefully acknowledged.

References

1. Bunn HF, Poyton RO: **Oxygen sensing and molecular adaptation to hypoxia.** *Physiol Rev* 1996, **76:**839-885.
2. Gancedo JM: **Yeast carbon catabolite repression.** *Microbiol Mol Biol Rev* 1998, **62:**334-361.
3. Hon T, Dodd A, Dirmeier R, Gorman N, Sinclair PR, Zhang L, Poyton RO: **A mechanism of oxygen sensing in yeast. Multiple oxygen-responsive steps in the heme biosynthetic pathway affect Hap1 activity.** *J Biol Chem* 2003, **278:**50771-50780.
4. Becerra M, Lombardia-Ferreira LJ, Hauser NC, Hoheisel JD, Tizon B, Cerdan ME: **The yeast transcriptome in aerobic and hypoxic conditions: effects of hap1, rox1, rox3 and srb10 deletions.** *Mol Microbiol* 2002, **43:**545-555.
5. Zhang L, Guarente L: ***HAP1* is nuclear but is bound to a cellular factor in the absence of heme.** *J Biol Chem* 1994, **269:**14643-14647.
6. Lowry CV, Zitomer RS: **Oxygen regulation of anaerobic and aerobic genes mediated by a common factor in yeast.** *Proc Natl Acad Sci USA* 1984, **81:**6129-6133.
7. Ter Linde JJ, Steensma HY: **A microarray-assisted screen for potential Hap1 and Rox1 target genes in *Saccharomyces cerevisiae*.** *Yeast* 2002, **19:**825-840.
8. Hickman MJ, Winston F: **Heme levels switch the function of Hap1 of *Saccharomyces cerevisiae* between transcriptional activator and transcriptional repressor.** *Mol Cell Biol* 2007, **27(21):**7414-24.
9. Kwast KE, Burke PV, Poyton RO: **Oxygen sensing and the transcriptional regulation of oxygen-responsive genes in yeast.** *J Exp Biol* 1998, **201:**1177-1195.
10. Buschlen S, Amillet JM, Guiard B, Fournier A, Marcireau C, Bolotin-Fukuhara M: **The *S. cerevisiae* HAP complex, a key regulator of mitochondrial function, coordinates nuclear and mitochondrial gene expression.** *Comp Funct Genomics* 2003, **4:**37-46.
11. Raghevendran V, Patil KR, Olsson L, Nielsen J: **Hap4 is not essential for activation of respiration at low specific growth rates in *Saccharomyces cerevisiae*.** *J Biol Chem* 2006, **281:**12308-12314.
12. ter Linde JJ, Liang H, Davis RW, Steensma HY, van Dijken JP, Pronk JT: **Genome-wide transcriptional analysis of aerobic and anaerobic chemostat cultures of *Saccharomyces cerevisiae*.** *J Bacteriol* 1999, **181:**7409-7413.
13. Lai LC, Kosorukoff AL, Burke PV, Kwast KE: **Metabolic-state-dependent remodeling of the transcriptome in response to anoxia and subsequent reoxygenation in *Saccharomyces cerevisiae*.** *Eukaryot Cell* 2006, **5:**1468-1489.
14. Lai LC, Kosorukoff AL, Burke PV, Kwast KE: **Dynamical remodeling of the transcriptome during short-term anaerobiosis in *Saccharomyces cerevisiae*: differential response and role of Msn2 and/or Msn4 and other factors in galactose and glucose media.** *Mol Cell Biol* 2005, **25:**4075-4091.
15. Schneiter R: **Intracellular sterol transport in eukaryotes, a connection to mitochondrial function?** *Biochimi* 2007, **89:**255-259.
16. Lorenz RT, Rodriguez RJ, Lewis TA, Parks LW: **Characteristics of sterol uptake in *Saccharomyces cerevisiae*.** *J Bacteriol* 1986, **167:**981-985.
17. Snoek I, Steensma H: **Factors involved in anaerobic growth of *Saccharomyces cerevisiae*.** *Yeast* 2007, **24:**1-10.
18. Alimardani P, Regnacq M, Moreau-Vauzelle C, Ferreira T, Rossignol T, Blondin B, Berges T: ***SUT1*-promoted sterol uptake involves the ABC transporter Aus1 and the mannoprotein Dan1 whose synergistic action is sufficient for this process.** *Biochem J* 2004, **381:**195-202.
19. Kwast KE, Lai LC, Menda N, James DT 3rd, Aref S, Burke PV: **Genomic analyses of anaerobically induced genes in *Saccharomyces cerevisiae*: functional roles of Rox1 and other factors in mediating the anoxic response.** *J Bacteriol* 2002, **184:**250-265.
20. Vik A, Rine J: **Upc2p and Ecm22p, dual regulators of sterol biosynthesis in *Saccharomyces cerevisiae*.** *Mol Cell Biol* 2001, **21:**6395-6405.
21. Davies BS, Rine J: **A role for sterol levels in oxygen sensing in *Saccharomyces cerevisiae*.** *Genetics* 2006, **174:**191-201.
22. Abramova NE, Cohen BD, Sertil O, Kapoor R, Davies KJ, Lowry CV: **Regulatory mechanisms controlling expression of the DAN/TIR mannoprotein genes during anaerobic remodeling of the cell wall in *Saccharomyces cerevisiae*.** *Genetics* 2001, **157:**1169-1177.
23. Bourot S, Karst F: **Isolation and characterization of the *Saccharomyces cerevisiae SUT1* gene involved in sterol uptake.** *Gene* 1995, **165:**97-102.
24. Ness F, Bourot S, Regnacq M, Spagnoli R, Berges T, Karst F: ***SUT1* is a putative Zn[II]2Cys6-transcription factor whose upregulation enhances both sterol uptake and synthesis in aerobically growing *Saccharomyces cerevisiae* cells.** *Eur J Biochem* 2001, **268:**1585-1595.
25. Piper MD, Daran-Lapujade P, Bro C, Regenberg B, Knudsen S, Nielsen J, Pronk JT: **Reproducibility of oligonucleotide microarray transcriptome analyses. An interlaboratory comparison using chemostat cultures of *Saccharomyces cerevisiae*.** *J Biol Chem* 2002, **277:**37001-37008.
26. Tai SL, Boer VM, Daran-Lapujade P, Walsh MC, de Winde JH, Daran JM, Pronk JT: **Two-dimensional transcriptome analysis in chemostat cultures. Combinatorial effects of oxygen availability and macronutrient limitation in *Saccharomyces cerevisiae*.** *J Biol Chem* 2005, **280:**437-447.
27. de Groot MJ, Daran-Lapujade P, van Breukelen B, Knijnenburg TA, de Hulster EA, Reinders MJ, Pronk JT, Heck AJ, Slijper M: **Quantitative proteomics and transcriptomics of anaerobic and aerobic**

yeast cultures reveals post-transcriptional regulation of key cellular processes. *Microbiology* 2007, **153:**3864-3878.
28. Burke PV, Raitt DC, Allen LA, Kellogg EA, Poyton RO: **Effects of oxygen concentration on the expression of cytochrome c and cytochrome c oxidase genes in yeast.** *J Biol Chem* 1997, **272:**14705-14712.
29. Wiebe MG, Rintala E, Tamminen A, Simolin H, Salusjärvi L, Toivari M, Kokkonen JT, Kiuru J, Ketola RA, Jouhten P, Huuskonen A, Maaheimo H, Ruohonen L, Penttilä M: **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 Res* 2008, **8:**140-154.
30. Jouhten P, Rintala E, Huuskonen A, Tamminen A, Toivari M, Wiebe MG, Ruohonen L, Penttilä M, Maaheimo H: **Oxygen dependence of metabolic fluxes and energy generation of *Saccharomyces cerevisiae* CEN.PK113-1A.** *BMC Syst Biol* 2008, **2:**60.
31. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP: **Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles.** *Proc Natl Acad Sci USA* 2005, **102:**15545-15550.
32. Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, Puigserver P, Carlsson E, Ridderstrale M, Laurila E, Houstis N, Daly MJ, Patterson N, Mesirov JP, Golub TR, Tamayo P, Spiegelman B, Lander ES, Hirschhorn JN, Altshuler D, Groop LC: **PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes.** *Nat Genet* 2003, **34:**267-273.
33. Kim SY, Volsky DJ: **PAGE: parametric analysis of gene set enrichment.** *BMC Bioinformatics* 2005, **6:**144.
34. Futschik ME, Carlisle B: **Noise-robust soft clustering of gene expression time-course data.** *J Bioinform Comput Biol* 2005, **3:**965-988.
35. Elemento O, Slonim N, Tavazoie S: **A universal framework for regulatory element discovery across all genomes and data types.** *Mol Cell* 2007, **28:**337-350.
36. Philpott CC, Protchenko O: **The Response to Iron Deprivation in *Saccharomyces cerevisiae*.** *Eukaryot Cell* 2007, **7:**20-7.
37. Kaplan J, McVey Ward D, Crisp RJ, Philpott CC: **Iron-dependent metabolic remodeling in *S. cerevisiae*.** *Biochim Biophys Acta* 2006, **1763:**646-651.
38. Kellis M, Patterson N, Endrizzi M, Birren B, Lander ES: **Sequencing and comparison of yeast species to identify genes and regulatory elements.** *Nature* 2003, **423:**241-254.
39. Foat BC, Houshmandi SS, Olivas WM, Bussemaker HJ: **Profiling condition-specific, genome-wide regulation of mRNA stability in yeast.** *Proc Natl Acad Sci USA* 2005, **102:**17675-17680.
40. Van Hoek P, Van Dijken JP, Pronk JT: **Effect of specific growth rate on fermentative capacity of baker's yeast.** *Appl Environ Microbiol* 1998, **64:**4226-4233.
41. Rosenfeld E, Schaeffer J, Beauvoit B, Salmon JM: **Isolation and properties of promitochondria from anaerobic stationary-phase yeast cells.** *Antonie Van Leeuwenhoek* 2004, **85:**9-21.
42. Gurvitz A, Rottensteiner H: **The biochemistry of oleate induction: transcriptional upregulation and peroxisome proliferation.** *Biochim Biophys Acta* 2006, **1763:**1392-1402.
43. Skoneczny M, Rytka J: **Oxygen and haem regulate the synthesis of peroxisomal proteins: catalase A, acyl-CoA oxidase and Pex1p in the yeast *Saccharomyces cerevisiae*, the regulation of these proteins by oxygen is not mediated by haem.** *Biochem J* 2000, **350(Pt 1):**313-319.
44. Poirier Y, Antonenkov VD, Glumoff T, Hiltunen JK: **Peroxisomal beta-oxidation--a metabolic pathway with multiple functions.** *Biochim Biophys Acta* 2006, **1763:**1413-1426.
45. Greijer AE, Groep P van der, Kemming D, Shvarts A, Semenza GL, Meijer GA, Wiel MA van de, Belien JA, van Diest PJ, Wall E van der: **Up-regulation of gene expression by hypoxia is mediated predominantly by hypoxia-inducible factor 1 (HIF-1).** *J Pathol* 2005, **206:**291-304.
46. Germann M, Gallo C, Donahue T, Shirzadi R, Stukey J, Lang S, Ruckenstuhl C, Oliaro-Bosso S, McDonough V, Turnowsky F, Balliano G, Nickels JT Jr: **Characterizing sterol defect suppressors uncovers a novel transcriptional signaling pathway regulating zymosterol biosynthesis.** *J Biol Chem* 2005, **280:**35904-35913.
47. Crowley JH, Leak FW Jr, Shianna KV, Tove S, Parks LW: **A mutation in a purported regulatory gene affects control of sterol uptake in *Saccharomyces cerevisiae*.** *J Bacteriol* 1998, **180:**4177-4183.
48. Shianna KV, Dotson WD, Tove S, Parks LW: **Identification of a UPC2 homolog in *Saccharomyces cerevisiae* and its involvement in aerobic sterol uptake.** *J Bacteriol* 2001, **183:**830-834.
49. Cohen BD, Sertil O, Abramova NE, Davies KJ, Lowry CV: **Induction and repression of *DAN1* and the family of anaerobic mannoprotein genes in *Saccharomyces cerevisiae* occurs through a complex array of regulatory sites.** *Nucleic Acids Res* 2001, **29:**799-808.
50. Cliften P, Sudarsanam P, Desikan A, Fulton L, Fulton B, Majors J, Waterston R, Cohen BA, Johnston M: **Finding functional features in *Saccharomyces* genomes by phylogenetic footprinting.** *Science* 2003, **301:**71-76.
51. Arthington BA, Bennett LG, Skatrud PL, Guynn CJ, Barbuch RJ, Ulbright CE, Bard M: **Cloning, disruption and sequence of the gene encoding yeast C-5 sterol desaturase.** *Gene* 1991, **102:**39-44.
52. Skaggs BA, Alexander JF, Pierson CA, Schweitzer KS, Chun KT, Koegel C, Barbuch R, Bard M: **Cloning and characterization of the *Saccharomyces cerevisiae* C-22 sterol desaturase gene, encoding a second cytochrome P-450 involved in ergosterol biosynthesis.** *Gene* 1996, **169:**105-109.
53. Bard M, Bruner DA, Pierson CA, Lees ND, Biermann B, Frye L, Koegel C, Barbuch R: **Cloning and characterization of *ERG25*, the *Saccharomyces cerevisiae* gene encoding C-4 sterol methyl oxidase.** *Proc Natl Acad Sci USA* 1996, **93:**186-190.
54. Rosenfeld E, Beauvoit B, Blondin B, Salmon JM: **Oxygen consumption by anaerobic *Saccharomyces cerevisiae* under enological conditions: effect on fermentation kinetics.** *Appl Environ Microbiol* 2003, **69:**113-121.
55. Mai B, Breeden L: **CLN1 and its repression by Xbp1 are important for efficient sporulation in budding yeast.** *Mol Cell Biol* 2000, **20:**478-487.
56. Mai B, Breeden L: **Xbp1, a stress-induced transcriptional repressor of the *Saccharomyces cerevisiae* Swi4/Mbp1 family.** *Mol Cell Biol* 1997, **17:**6491-6501.
57. Gagiano M, Bauer FF, Pretorius IS: **The sensing of nutritional status and the relationship to filamentous growth in *Saccharomyces cerevisiae*.** *FEMS Yeast Res* 2002, **2:**433-470.
58. Chen RE, Thorner J: **Function and regulation in MAPK signaling pathways: lessons learned from the yeast *Saccharomyces cerevisiae*.** *Biochim Biophys Acta* 2007, **1773:**1311-1340.
59. Gombert AK, Moreira dos Santos M, Christensen B, Nielsen J: **Network identification and flux quantification in the central metabolism of *Saccharomyces cerevisiae* under different conditions of glucose repression.** *J Bacteriol* 2001, **183:**1441-1451.
60. Fiaux J, Cakar ZP, Sonderegger M, Wuthrich K, Szyperski T, Sauer U: **Metabolic-flux profiling of the yeasts *Saccharomyces cerevisiae* and *Pichia stipitis*.** *Eukaryot Cell* 2003, **2:**170-180.
61. Franzen CJ: **Metabolic flux analysis of RQ-controlled microaerobic ethanol production by *Saccharomyces cerevisiae*.** *Yeast* 2003, **20:**117-132.
62. van Winden WA, van Dam JC, Ras C, Kleijn RJ, Vinke JL, van Gulik WM, Heijnen JJ: **Metabolic-flux analysis of *Saccharomyces cerevisiae* CEN.PK113-7D based on mass isotopomer measurements of (13)C-labeled primary metabolites.** *FEMS Yeast Res* 2005, **5:**559-568.
63. Daran-Lapujade P, Rossell S, van Gulik WM, Luttik MA, de Groot MJ, Slijper M, Heck AJ, Daran JM, de Winde JH, Westerhoff HV, Pronk JT, Bakker BM: **The fluxes through glycolytic enzymes in *Saccharomyces cerevisiae* are predominantly regulated at posttranscriptional levels.** *Proc Natl Acad Sci USA* 2007, **104:**15753-15758.
64. DeRisi JL, Iyer VR, Brown PO: **Exploring the metabolic and genetic control of gene expression on a genomic scale.** *Science* 1997, **278:**680-686.
65. Wang Y, Barbacioru C, Hyland F, Xiao W, Hunkapiller KL, Blake J, Chan F, Gonzalez C, Zhang L, Samaha RR: **Large scale real-time PCR validation on gene expression measurements from two commercial long-oligonucleotide microarrays.** *BMC Genomics* 2006, **7:**59.
66. Rautio J, Barken KB, Lahdenpera J, Breitenstein A, Molin S, Neubauer P: **Sandwich hybridisation assay for quantitative detection of yeast RNAs in crude cell lysates.** *Microb Cell Fact* 2003, **2:**4.
67. Rautio JJ, Kataja K, Satokari R, Penttilä M, Söderlund H, Saloheimo M: **Rapid and multiplexed transcript analysis of microbial cul-**

tures using capillary electrophoresis-detectable oligonucleotide probe pools. *J Microbiol Methods* 2006, **65:**404-416.
68. Schmidt MW, Houseman A, Ivanov AR, Wolf DA: **Comparative proteomic and transcriptomic profiling of the fission yeast *Schizosaccharomyces pombe*.** *Mol Syst Biol* 2007, **3:**79.
69. Lu P, Vogel C, Wang R, Yao X, Marcotte EM: **Absolute protein expression profiling estimates the relative contributions of transcriptional and translational regulation.** *Nat Biotechnol* 2007, **25:**117-124.
70. Guzy RD, Mack MM, Schumacker PT: **Mitochondrial complex III is required for hypoxia-induced ROS production and gene transcription in yeast.** *Antioxid Redox Signal* 2007, **9:**1317-1328.
71. Salusjärvi L, Kankainen M, Soliymani R, Pitkänen JP, Penttilä M, Ruohonen L: **Regulation of xylose metabolism in recombinant *Saccharomyces cerevisiae*.** *Microb Cell Fact* 2008, **7:**18.
72. Verduyn C, Postma E, Scheffers WA, Van Dijken JP: **Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation.** *Yeast* 1992, **8:**501-517.
73. Zamamiri AQ, Birol G, Hjortso MA: **Multiple stable states and hysteresis in continuous, oscillating cultures of budding yeast.** *Biotechnol Bioeng* 2001, **75:**305-312.
74. R Development Core Team: **R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. Vienna, Austria. version 2.5.1.** 2008 [http://www.r-project.org].
75. Warnes GR: **gplots: Various R programming tools for plotting data. Included R source code and/or documentation contributed by Ben Bolker and Thomas Lumley.** *R package version 2.3.0* 2006.
76. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP: **Exploration, normalization, and summaries of high density oligonucleotide array probe level data.** *Biostatistics* 2003, **4:**249-264.
77. Smyth GK: **Limma: linear models for microarray data. In:Bioinformatics and Computational Biology Solutions using R and Bioconductor.** *R package version 2.12.0* 2005.
78. Smyth GK: **Linear models and empirical bayes methods for assessing differential expression in microarray experiments.** *Stat Appl Genet Mol Biol* 2004, **3:**Article3.
79. Benjamini Y, Hochberg Y: **Controlling the false discovery rate: a practical and powerful approach to multiple testing.** *Journal of the Royal Statistical Society Series_B* 1995, **57:**289-300.
80. Furge K, Dykema K: **PGSEA: Parametric Gene Set Enrichment Analysis.** *R package version 1.2.0* 2006.
81. Futschik M: **Mfuzz: Soft clustering of time series gene expression data.** *R package version 1.6.0* 2007.
82. Falcon S, Gentleman R: **Using GOstats to test gene lists for GO term association.** *Bioinformatics* 2007, **23:**257-258.
83. Salusjärvi L, Poutanen M, Pitkänen JP, Koivistoinen H, Aristidou A, Kalkkinen N, Ruohonen L, Penttilä M: **Proteome analysis of recombinant xylose-fermenting *Saccharomyces cerevisiae*.** *Yeast* 2003, **20:**295-314.
84. Almeida JS, Stanislaus R, Krug E, Arthur JM: **Normalization and analysis of residual variation in two-dimensional gel electrophoresis for quantitative differential proteomics.** *Proteomics* 2005, **5:**1242-1249.
85. Gottardo R: **EMV: Estimation of Missing Values for a Data Matrix.** *R package version 1.3.1* 2006.
86. Chang J, Van Remmen H, Ward WF, Regnier FE, Richardson A, Cornell J: **Processing of data generated by 2-dimensional gel electrophoresis for statistical analysis: missing data, normalization, and statistics.** *J Proteome Res* 2004, **3:**1210-1218.
87. Gallardo K, Firnhaber C, Zuber H, Hericher D, Belghazi M, Henry C, Kuster H, Thompson R: **A combined proteome and transcriptome analysis of developing Medicago truncatula seeds: evidence for metabolic specialization of maternal and filial tissues.** *Mol Cell Proteomics* 2007, **6:**2165-2179.
88. **Inkscape scalable vector graphics editor. Version 0.46** [http://www.inkscape.org]

Publication IV

Transcriptional responses of *Saccharomyces cerevisiae* to shift from respiratory and respire-fermentative to fully fermentative metabolism

In: OMICS: A Journal of Integrative Biology.
In press.

.

Transcriptional responses of *Saccharomyces cerevisiae* to shift from respiratory and respiro-fermentative to fully fermentative metabolism

Eija Rintala*, Paula Jouhten, Mervi Toivari, Marilyn G. Wiebe, Hannu Maaheimo, Merja Penttilä and Laura Ruohonen

*Corresponding author

Eija Rintala:
VTT Technical Research Centre of Finland, P.O. Box 1000, FI-02044 VTT, Finland

Phone +358 50 355 7361, Fax +358 20 722 7071, email: eija.rintala@vtt.fi

Paula Jouhten:

VTT Technical Research Centre of Finland, P.O. Box 1000, FI-02044 VTT, Finland

Phone +358 20 722 0722, Fax +358 20 722 7071, email: paula.jouhten@vtt.fi

Mervi Toivari:
VTT Technical Research Centre of Finland, P.O. Box 1000, FI-02044 VTT, Finland

Phone +358 20 722 7309, Fax +358 20 722 7071, email: mervi.toivari@vtt.fi
Marilyn G Wiebe:
VTT Technical Research Centre of Finland, P.O. Box 1000, FI-02044 VTT, Finland

Phone +358 20 722 5139, Fax +358 20 722 7071, email: marilyn.wiebe@vtt.fi
Hannu Maaheimo:
VTT Technical Research Centre of Finland, P.O.Box 65, FI-00014 HELSINKI, Finland

Phone +358 20 722 2856, Fax +358 20 722 2860, email: hannu.maaheimo@vtt.fi

Merja Penttilä:
VTT Technical Research Centre of Finland, P.O. Box 1000, FI-02044 VTT, Finland

Phone +358 20 722 4504, Fax +358 20 722 7071, email: merja.penttila@vtt.fi

Laura Ruohonen:
VTT Technical Research Centre of Finland, P.O. Box 1000, FI-02044 VTT, Finland

Phone +358 20 722 5337, Fax +358 20 722 7071, email: laura.ruohonen@vtt.fi

Key words: *Saccharomyces cerevisiae*, oxygen, transcriptome, time-course

Abstract

In industrial fermentations of *Saccharomyces cerevisiae*, transient changes in oxygen concentration commonly occur and it is important to understand the behaviour of cells during these changes. Glucose-limited chemostat cultivations were used to study the time-dependent effect of sudden oxygen depletion on the transcriptome of *S. cerevisiae* cells initially in fully aerobic or oxygen-limited conditions. The overall responses to anaerobic conditions of cells initially in different conditions were very similar. Independent of initial culture conditions, transient downregulation of genes related to growth and cell proliferation, mitochondrial translation and protein import, and sulphate assimilation was seen. In addition, transient or permanent upregulation of genes related to protein degradation, and phosphate and amino acid uptake was observed in all cultures. However, only in the initially oxygen-limited cultures was a transient upregulation of genes related to fatty acid oxidation, peroxisomal biogenesis, oxidative phosphorylation, TCA cycle, response to oxidative stress, and pentose phosphate pathway observed. Furthermore, from the initially oxygen-limited conditions, a rapid response around the metabolites of upper glycolysis and the pentose phosphate pathway was seen, while from the initially fully aerobic conditions, a slower response around the pathways for utilisation of respiratory carbon sources was observed.

Background

Adaptation to oxygen availability is crucial for all organisms. Some organisms survive only in oxygen concentrations normally present in the atmosphere while for some oxygen is toxic. *Saccharomyces cerevisiae* is a facultative anaerobe, able to grow in a wide range of oxygen concentrations, including complete anaerobiosis. In the presence of oxygen, *S. cerevisiae* utilises respiration for efficient energy production while in anaerobic conditions, *S. cerevisiae* has a high redox neutral carbon flux to the fermentative pathway and energy is produced by substrate level phosphorylation. *S. cerevisiae*, a Crabtree positive yeast, is able to ferment glucose even in fully aerobic conditions, allowing a high rate of sugar utilisation. The ability for mixed respiro-fermentative metabolism in aerobic conditions and the ability to adapt to different oxygen concentrations have facilitated wide industrial applications of *S. cerevisiae*. However, in large-scale fermentations, especially in those of high-cell-density, provision of uniform aeration is problematic and spatial and transient perturbations in oxygen availability may occur. Thus the analysis of dynamic, oxygen-dependent regulation in *S. cerevisiae* is important not only to understand general adaptation mechanisms of living cells to various effects of oxygen, but also to understand how variations in oxygen supply may affect production processes.

Expression levels of a large number of genes and proteins differ, depending on oxygen availability, in *S. cerevisiae* (de Groot *et al.* 2007; Lai *et al.* 2006; Lai *et al.* 2005; Kwast *et al.* 2002; ter Linde *et al.* 1999; Bruckmann *et al.* 2009). In batch cultures on galactose, exposure to anoxia leads to an acute and transient upregulation of Msn2p/Msn4p-regulated genes of reserve energy metabolism and catabolic pathways and to downregulation of genes involved in cell cycle and rRNA processing (Lai *et al.* 2006; Lai *et al.* 2005). In batch cultures on glucose, similar transient responses to lack of oxygen are not observed (Lai *et al.* 2006; Lai *et al.* 2005). The stress response in galactose-grown cells has been suggested to arise from cessation of respiration, which is less significant on glucose since respiratory activity is already low under glucose repression (Lai *et al.* 2005). In accordance with this hypothesis, it was recently shown that exposure of galactose-grown cells to antimycin A, an inhibitor of the respiratory chain leads to a similar transient transcriptomic response as anoxia (Lai *et al.* 2008). On galactose and glycerol (Guzy *et al.* 2007; Dirmeier *et al.* 2002), but not on glucose (Guzy *et al.* 2007), exposure to anaerobic conditions also leads to a transient oxidative stress response. This is seen as increased level 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* (Dirmeier *et al.* 2002) and elevated ROS (reactive oxygen species) levels (Guzy *et al.* 2007). When exposed to anoxia on acetate, a completely non-fermentable carbon source, *S. cerevisiae* enters a reversible state of suspended animation, in which sporulation and growth halt for the duration of anoxia but re-commence when oxygen availability is restored (Chan and Roth 2008).

Depletion of oxygen leads to transcriptional changes in *S. cerevisiae* that enable growth in severely oxygen-limited and anaerobic conditions. Some of these changes are mediated by depletion of heme and sterols, the syntheses of which are strictly aerobic processes (Kwast *et al.* 2002; Hon *et al.* 2003; Andreasen and Stier 1953; Smith *et al.* 1996). Heme and sterol levels decline by dilution during growth in the absence of oxygen and thus the changes mediated by them are slow to occur. As heme levels decline, heme-dependent Hap1p and Hap2/3/4/5p transcription factors, which activate many genes needed in aerobic conditions, become deactivated. The deactivation of Hap1p leads in turn to deactivation of Rox1p, which in aerobic conditions represses the genes required during severe hypoxia and anaerobic conditions (Becerra *et al.* 2002; Ter Linde and Steensma 2002; Ha *et al.* 1996; Kwast *et al.* 1998; Lowry and Zitomer 1984). Components of the respiratory chain have also been shown to be involved in the induction of specific hypoxic genes (Guzy *et al.* 2007; David and Poyton 2005; Kwast *et al.* 1999). Further, as a response to anoxia, the cell wall and plasma membrane of *S. cerevisiae* are remodelled for import of sterols and unsaturated fatty acids

(Kwast *et al.* 2002). Several genes encoding cell wall and plasma membrane proteins are differentially expressed in aerobic and anaerobic conditions and the transcription factors Upc2p, Ecm22p and Sut1p are known to play a role in the import of sterols, but the exact mechanism of the cell wall remodelling is not known (Snoek and Steensma 2007; Alimardani *et al.* 2004; Abramova *et al.* 2001; Bourot and Karst 1995; Shianna *et al.* 2001; Lewis *et al.* 1988).

To our knowledge there are no previous studies on genome-wide transcriptional adaptation of fully respiratory cultures of *S. cerevisiae* to purely fermentative growth under anaerobic conditions. Furthermore, the adaptation of respiro-fermentative *S. cerevisiae* cultures to anaerobic conditions has previously been studied only under carbon catabolite repression in batch cultures on glucose and galactose (Lai *et al.* 2006; Lai *et al.* 2005). We have previously shown that in glucose-limited chemostat cultivations at low growth rate (D = 0.1 h^{-1}) *S. cerevisiae* grows in respiratory, respiro-fermentative and fermentative metabolic modes, depending solely on the oxygen provision under the derepressed conditions (Wiebe *et al.* 2008). Providing 20.9% of oxygen in the chemostat inlet gas enabled fully respiratory growth, while providing 2.8, 1.0 or 0.5 % oxygen led to respiro-fermentative growth. In the current study we initially provided glucose-limited chemostat cultures (D = 0.1 h^{-1}) of *S. cerevisiae* with 20.9% or 1.0% oxygen in the inlet gas and monitored the time-dependent, genome-wide transcriptional adaptation to anaerobic conditions. The experimental set-up allowed the direct assessment of adaptation to anaerobicity, without the interference of carbon catabolite repression or use of different carbon sources. In other words, we were able to study the transcriptional adaptation of fully respiratory and respiro-fermentative cultures of *S. cerevisiae* to fully fermentative metabolism, and analyse whether the metabolic processes needed for the adaptation varied depending on the initial metabolic state.

Materials and methods

Strain and culture conditions

Saccharomyces cerevisiae CEN.PK113-1A (*MATα, URA3, HIS3, LEU2, TRP1, MAL2-8c, SUC2)* was grown in 0.8 to 1 L medium in B.Braun Biotech International (Sartorius) Biostat® CT (2.5 L working volume) bioreactors in the defined minimal medium described by Verduyn *et al*. (Verduyn *et al.* 1992), with 10 g glucose l^{-1} as carbon source, and supplemented with 10 mg ergosterol l^{-1} and 420 mg Tween 80 l^{-1}. BDH silicone antifoam (BDH 331512K, VWR International, UK; 0.5 mL l^{-1}) was used to prevent foam production in the cultures. Chemostat cultures were maintained at D = 0.10 ± 0.02 h^{-1}, pH 5.0, 30°C, with 1.5 volume gas [volume culture]$^{-1}$ min^{-1} (vvm).

Duplicate glucose-limited chemostat cultures were carried out with 20.9% (fully aerobic) or 1.0% (oxygen-limited) oxygen in the inlet gas. For cultures which received 1.0% O_2 in the gas stream, air was replaced with the equivalent volume of N_2, so that total gas flow was maintained constant. Cultures which were fed 20.9% O_2 were subject to oscillations. To prevent these, approximately 5% of the total cell concentration in the bioreactor was added to the culture as cells in mid to late exponential phase at the time when continuous medium feed was started (Zamamiri *et al.* 2001).

After steady states were established, the cultures were made anaerobic by replacing air (20.9 or 1.0% O_2) with 100% N_2. Samples were removed at intervals (0.2, 1, 3, 8, 24 and 79 (20.9% O_2) or 72 (1.0% O_2) h after the switch to 100% N_2) until a new steady state was achieved. These cultivations have been previously described in Wiebe *et al.* (2008) and biomass and metabolite analyses of the cultures were described in Wiebe *et al.* (2008), while the role of the glucose transporters during the transition was described in Rintala *et al.* (2008) and the transcriptomes of the initial steady states were described in Rintala *et al.* (2009).

Transcriptome analysis

Affymetrix microarray analysis of duplicate experiments for each of the two initial oxygen levels was performed. For the microarray analysis, the cells were collected by centrifugation (3500 rpm, 5 min, +4°C). After centrifugation, the cell pellet was frozen in liquid nitrogen. For RNA extraction, 5-20 mg dry mass of cells were suspended in 400 µl cold (+4°C) disruption buffer (20 mM Tris-HCl, pH 7.4, 100 mM KCl, 2 mM $MgCl_2$, 2 mM DTT). 400 µl phenol-chlorophorm (50:50), 5 µl 20% (w/v) SDS and 400 µl glass beads (0.5 mm diameter; Biospec Products) were added. The cells were disrupted with a Fastprep machine (Q-Biogene), 2 x 20 s, at speed 6. After centrifugation (14000 rpm, 15 min, +4°C), supernatant was used for total RNA extraction, using an RNeasy kit (Qiagen) according to the manufacturer's instructions.

Hybridisations were carried out at the Finnish DNA Microarray Centre at Turku Centre for Biotechnology. 2 µg total RNA was used as starting material for sample preparation. Samples were processed according to the One-Cycle Target Labelling protocol in the GeneChip Expression Analysis Manual (Affymetrix). Both before and after the amplifications the total RNA/ cRNA concentrations were assessed with Nanodrop ND-1000 and the total RNA/ cRNA quality was assessed using a BioRad Experion electrophoresis station. Each sample was hybridised to the GeneChip Yeast Genome 2.0 Array at +45°C overnight (16 h) according to the GeneChip

Expression Analysis Technical Manual (Affymetrix). A GeneChip Fluidics Station 450 was used to wash and stain the arrays, and a GeneChip Scanner 3000 with AutoLoader was used to scan the arrays. CEL-files were extracted with GCOS Manager 1.4.

All data analysis was done using R/Bioconductor, version 2.5.1 (R Development Core Team 2005; Warnes 2006). The raw data was normalised with Robust Multichip Average (RMA) normalisation (Irizarry *et al.* 2003). Statistical differences in the expression were analysed using linear modelling with the tools of limma package (Smyth 2005). For each gene, a linear model was fitted by the least squares method and differential expression within pairs of experimental conditions was computed using an empirical Bayesian approach (Smyth 2004). For correction of multiple testing errors, the Benjamini & Hochberg –method controlling false discovery rate (FDR) was used (Benjamini and Hochberg 1995). The microarray data can be accessed through GEO accession number GSE22832.

The clustering analysis of gene expression data was performed using fuzzy c-means clustering (Futschik and Carlisle 2005; Futschik 2007). The clustering method assigns genes to clusters with gradual membership (values between 0 and 1). For the clustering, the expression values were scaled and centred to have a mean of zero and standard deviation of one. The parameter m, which controls the sensitivity of the clustering process to noise, was adjusted to 1.25 to prevent the detection of clusters in randomised data. The number of clusters was selected, such that no clusters were formed where all the genes would have membership values below 0.5. The enriched GO classes and KEGG metabolic pathways in the clusters were computed with the GOstats package (Falcon and Gentleman 2007). Transcriptional regulatory motifs in the clusters were analysed with the FIRE method (Elemento *et al.* 2007).

Reporter Features and Reporter Metabolites, the transcriptional regulatory sites in biochemical interaction networks, were identified with the Reporter Features and Reporter Metabolites algorithms (Oliveira *et al.* 2008; Patil and Nielsen 2005). Reporter Features, here transcription factors and other regulatory proteins, were identified from the network of all known interactions between the regulators and genes by a multidimensional analysis. Absolute Pearson correlation coefficients were calculated for the time-series of the normalised expression levels of all the pairs of genes connected to a regulator. Scores for the regulators were derived from the correlations of the connected genes. To obtain p-values for identification of the Reporters, the scores were tested for the null hypothesis “the regulator score is observed by chance”. Reporter Metabolites were identified from a genome-wide metabolic network of *S. cerevisiae* by differential analysis of the

sequential time-points. The scores for the metabolites were derived from the p-values for statistical significances of the differential expression of the genes connected to the metabolites in a genome-wide metabolic network between the sequential time-points as explained in detail in Patil and Nielsen (2005). The p-values to identify the Reporter Metabolites were obtained from a statistical test similarly as above.

Results

Gene transcription after transition from fully aerobic or oxygen-limited to anaerobic conditions

Statistical analysis of microarray data obtained from samples taken 0.2 to 79/72 h after a switch from fully aerobic (20.9% inlet O_2) or oxygen-limited (1.0% inlet O_2) glucose-limited chemostat cultures of *S. cerevisiae* revealed 3811 (initially fully aerobic) and 3701 (initially oxygen-limited) genes which responded to the change in oxygen concentration ($p<0.01$). Of these genes, 3022 were common to both starting conditions.

In the cultures which were initially oxygen-limited, the switch to nitrogen feed resulted in a change in expression of 2287 genes within 0.2 h, while in the initially fully aerobic cultures, expression of only 1320 changed during 0.2 h, increasing to 2374 genes after 1 h (Figure 1A and 1B). In both experiments, most of the genes whose expression had changed in 3 to 8 h had already started to change before 3 h. The lowest number of responsive genes was observed at 8 h after the cultures became anaerobic, and after 8 h a new set of genes responded. After 24 h, there were 597 and 707 genes differentially expressed, compared to the initial steady state, that had not shown earlier changes in expression in the cultures initially oxygen-limited and fully aerobic, respectively. Gene expression in the final anaerobic steady state of cultures which had been either initially fully aerobic or oxygen-limited, differed for only 3 genes (p-value <0.05) and was similar to that observed previously (Rintala *et al.* 2009).

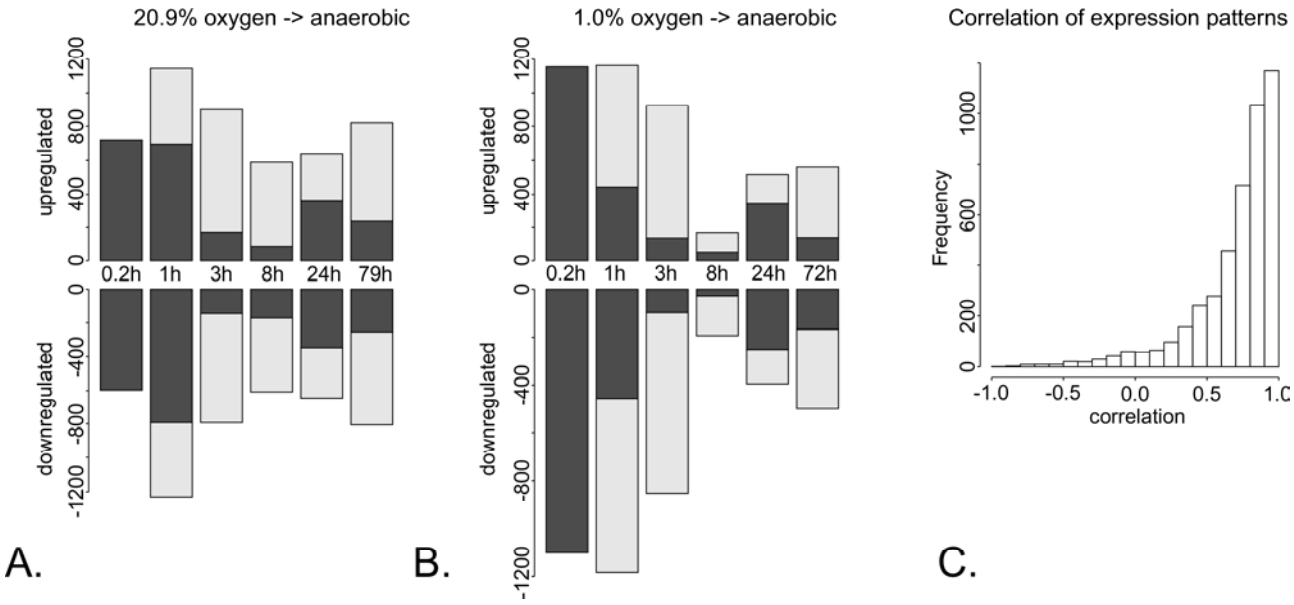


Figure 1. Significantly (p<0.01) changing genes. *A. Genes up- and downregulated after a change from fully aerobic to anaerobic conditions. Grey bars represent the genes that were differentially expressed at previous times, black bars represent genes that were differentially expressed for the first time at that time, compared to the initial steady state. B. Genes up- and downregulated after a change from oxygen-limited to anaerobic conditions. Colours as in A. C. Pearson correlation between the gene expression patterns during change from fully aerobic and oxygen-limited to anaerobic conditions.*

The expression patterns of the significantly changing genes in the two experiments were compared using Pearson correlation (Figure 1C). Although oxygen-limited cultures exhibited a greater initial response to anaerobic conditions than fully aerobic cultures, 1169 genes had a correlation of >0.9 in their expression patterns.

The expression patterns of genes which had previously been analysed with the TRAC method (Wiebe *et al.* 2008) were compared with the patterns observed in the Affymetrix analysis of the present study. Of the 67 genes analysed in both studies only 48 had significant changes in their expression levels. Of these, 41 showed good correlation (>0.6) between the two methods following the shift from fully aerobic to anaerobic conditions. Of the seven genes with correlation <0.6, five had less than 2 fold differences in their expression levels. The ones that had more than 2 fold differences in their expression, but, low correlation between the TRAC and the Affymetrix methods, were *CIT2* and *CIT3*.

Clustering and analysis of transcriptional regulation of the clusters

The data was clustered using fuzzy c-means clustering (Futschik and Carlisle 2005). This clustering method does not require prefiltering of genes and thus does not discard potentially interesting genes that do not respond strongly. In addition, all genes are assigned to clusters with a membership value between zero and one, which can be used to determine the level of co-regulation. The resulting clustering of gene expression data is presented in Figure 2 and 3 and the most significantly ($p < 0.01$) over-represented GO-categories and KEGG-pathways in the clusters are presented in additional data files: Table S1 and Table S2.

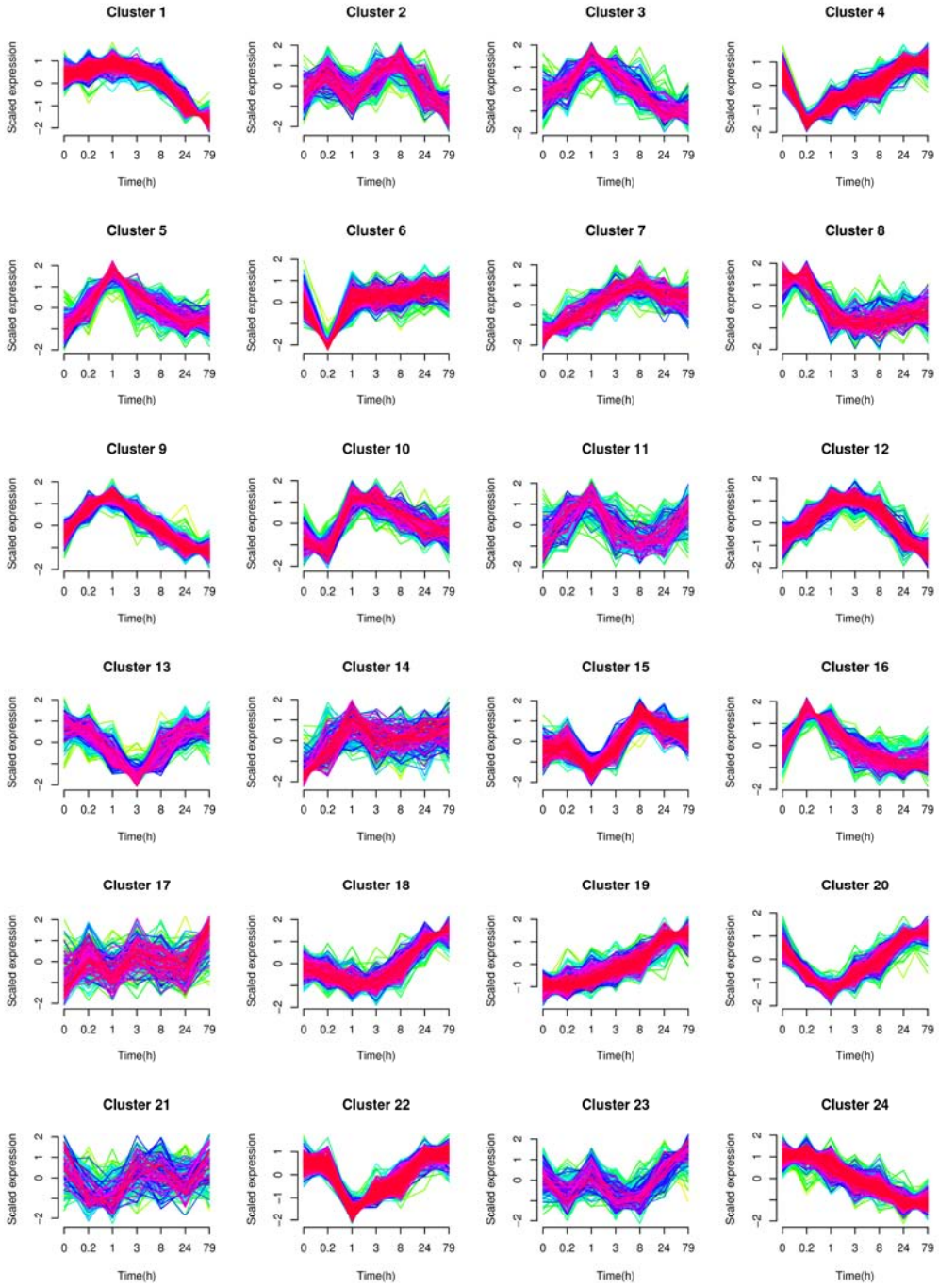


Figure 2. Fuzzy c-means clustering of gene expression patterns in cells initially grown in fully aerobic conditions and switched to anaerobic conditions.

The clustering was performed with individual samples, but average values for each condition are shown in the graphs. The expression values are centred and scaled around a mean of zero and standard deviation of 1, for all genes. Red and purple represent genes that have membership values higher than 0.5 while green and yellow represent genes that have membership values below 0.5.

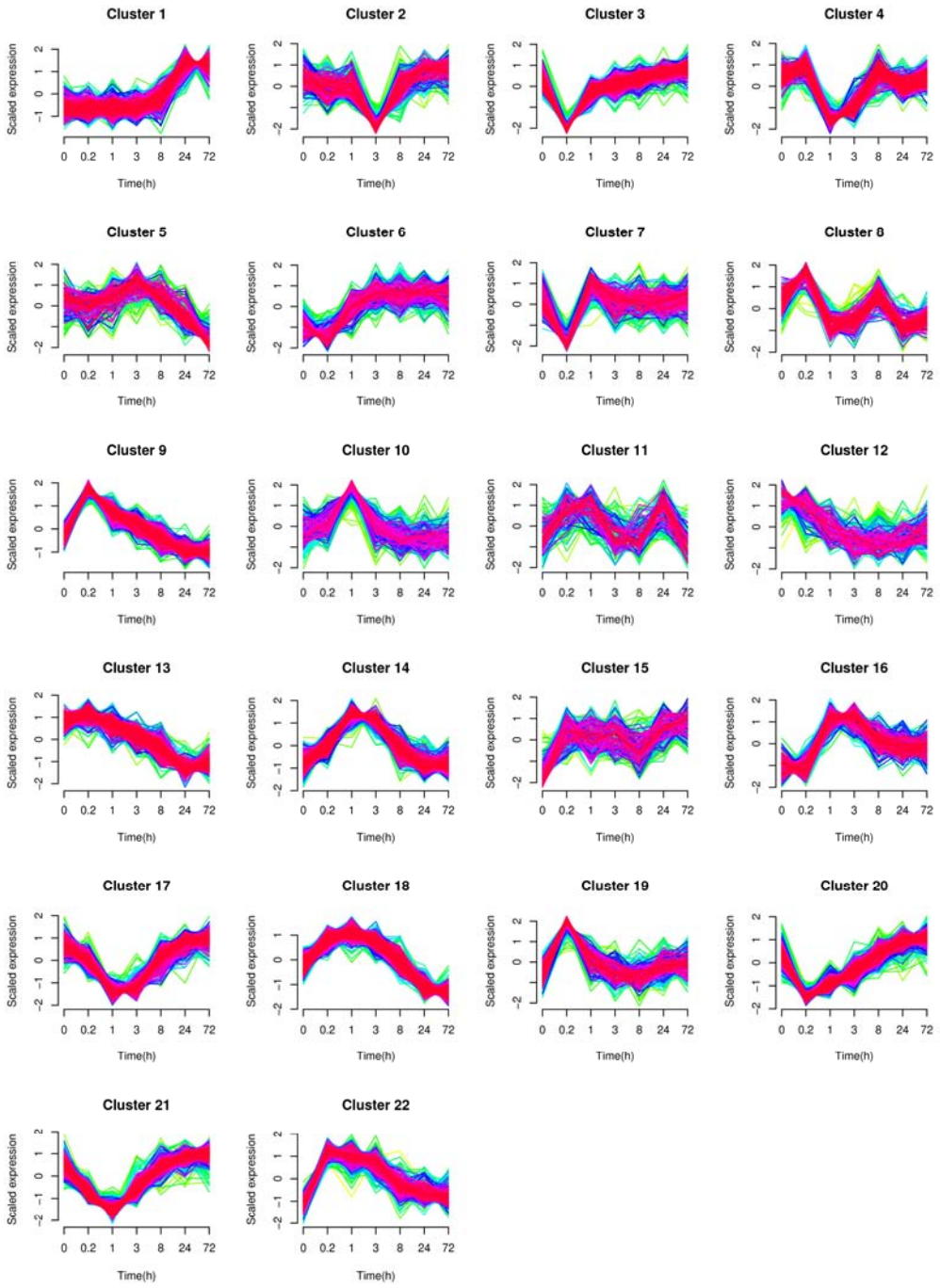


Figure 3. Fuzzy c-means clustering of gene expression patterns in cells initially grown in oxygen-limited conditions and switched to anaerobic conditions.
The clustering was performed with individual samples, but average values for each condition are shown in the graphs. The expression values are centred and scaled around a mean of zero and standard deviation of 1, for all genes. Red and purple represent genes that have membership values higher than 0.5 while green and yellow represent genes that have membership values below 0.5.

Analysis of the gene expression data of the experiment where 20.9 % oxygen in the feed gas was replaced with nitrogen revealed 24 clusters containing 70-294 genes with membership value higher than 0.5, *i.e.* belonging most strongly to the corresponding cluster (Figure 2). The promoter

sequences and 3'UTR's of the genes in these clusters were analysed using FIRE software (Elemento *et al.* 2007) (see additional data file: Figure S1). The analysis revealed 8 transcription factor binding site motifs and 4 3'UTR motifs, some of which had significant co-occurrence and/or spatial co-localisation patterns. The gene expression data of cultures in which 1.0 % oxygen in the feed gas was replaced with nitrogen revealed 22 clusters containing 71-372 genes with membership value higher than 0.5 (Figure 3). Analysis of the promoter sequences and 3'UTR's of genes in these clusters using FIRE software (see additional data file: Figure S2) revealed 14 transcription factor binding site motifs and 8 3'UTR motifs, some of which had significant co-occurrence and/or co-localisation patterns. A more detailed description of the clustering results for data obtained from these cultures, and of the analysis of the regulatory elements identified in the genes of these clusters, is provided below.

Transient downregulation upon oxygen depletion: processes related to growth (replication, transcription, translation) and mitochondrial function

In the initially fully aerobic cultures, genes in clusters 4, 13, 20 and 22 were transiently downregulated between 0.2 and 8 h (Figure 2). These clusters differed in the time at which the expression was at its lowest, which was 0.2 h (cluster 4), 1 h (clusters 20 and 22) or 3 h (cluster 13). GO categories enriched in clusters 4, 20 and 22 were largely overlapping and particularly included genes involved in amino acid and purine metabolism, ribosomal biogenesis, RNA processing, biogenesis of RNA polymerases and genes related to cell cycle and DNA replication and repair (Table S2). Genes encoding components of cytosolic ribosomes were statistically enriched in clusters 20 and 22, and 7 of these genes were found also in cluster 4. The transcription factor binding site motifs enriched in these clusters also overlapped (Figure S1). The PAC site, related to the regulation of the genes encoding components of ribosome biogenesis, was over-represented in clusters 4, 20 and 22, the XBP1 site, related to the regulation of genes encoding activities of stress response, in clusters 20 and 22 and the RAP1 site, related to the regulation of the genes encoding components of ribosome biogenesis, in cluster 22. In cluster 4, a putative 3'UTR motif [(A/U/G)(G/U)AUAGA], was enriched, however, this motif was not enriched in clusters 20 and 22, being in fact under-represented in cluster 22. Instead, genes in clusters 20 and 22 were enriched in another putative 3'UTR motif [UAUA(A/C)(G/U)A]. As with clusters 4, 13, 20 and 22, cluster 6, containing genes which were downregulated at 0.2 h but which had fully recovered at 1 h, contained genes involved in mRNA processing and splicing and in rRNA processing. 57% of the genes in cluster 6 contained the same 3'UTR motif [(A/U/G)(G/U)AUAGA] that was over-represented in

cluster 4. In the whole genome, this site is enriched in 3'UTRs of genes involved in RNA processing and ribosome biogenesis.

In the cultures which were initially oxygen-limited, clusters 3, 20 and 21 which were transiently downregulated between 0.2 and 3 h were enriched in genes related to ribosome biogenesis and assembly, rRNA processing, amino acid and purine metabolism, and DNA replication and repair (Figure 3 and Table S3). In addition, genes related to DNA replication and repair and cell cycle were enriched in cluster 17, which was also downregulated between 0.2 and 3 h. While these transient responses were similar to those seen when the fully aerobic cultures became anaerobic they started slightly earlier and had recovered earlier in the cultures which had been oxygen limited (in 1 to 3 h), than in those with initial oxygen of 20.9 %, in which most of these genes were still downregulated at 8 h. Genes in clusters 3 and 20 were enriched in transcription factor binding site motifs PAC and RRPE, the PUF4 3'UTR motif, involved in the regulation of genes encoding ribosomal proteins, and in a putative 3'UTR motif (AUAGA). Both of the 3'UTR motifs showed significant co-localisation. In addition, genes in cluster 21 were enriched in binding sites of Xbp1p, and Rap1p transcription factors, the RRPE motif, the PUF4 3'UTR and a putative 3'UTR (UCCGUAC) motif. Genes in cluster 17 were enriched in binding sites for Swi4p and Xbp1, transcription factors regulating genes encoding activities related to cell cycle and stress response. Similar to cluster 6 of the initially fully aerobic cultures, genes of cluster 7 in the initially oxygen-limited cultures were transiently downregulated at 0.2 h after the cultures became anaerobic and the level of transcription had fully recovered at 1 h. Cluster 7 contained genes related to amino acid metabolism, purine biosynthesis, transcription, chromatin remodelling and splicing. This cluster was enriched in binding sites of transcription factor Bas1p, regulating genes encoding activities related to purine and histidine biosynthesis, and the PUF5 3'UTR motif, associated with mRNAs encoding nuclear components. Genes in cluster 2 were transiently downregulated 3 h and were enriched in genes related to cell cycle, DNA packaging, organelle organisation and biogenesis, ribosomal protein and rRNA transport. Genes in cluster 2 were enriched in binding sites for Swi4p transcription factor and PUF5 3' UTR motif.

In the initially fully aerobic cultures, genes in cluster 15 were temporally downregulated but showed upregulation in the anaerobic steady state. The genes in this cluster were related to mitochondrial translation (57 genes) and protein targeting to mitochondria (*MAS1, TOM6, TOM20, TIM9, TIM13, POR1*). The 3'UTR site PUF3, which regulates the genes encoding mitochondrial proteins, was over-represented in this cluster. In the initially oxygen-limited cultures, cluster 4 had a

trend of downregulation during 1-3 h and a full recovery at 8 h. Among the genes of this cluster, 68 and 14 genes encoded activities involved in mitochondrial translation and mitochondrial protein import, respectively. This cluster also contained genes encoding additional mitochondrial membrane proteins (*SHE9, ADK2, ARH1, YGR012W, DPM1, OMS1, MSS51, YMR166C*) and genes related to respiration (*COQ10, DIA4, MAM33, YMR293C, COX23, ATP12, ATP11, COX17*). Genes in this cluster were also enriched in the PUF3 3'UTR motif and additionally two putative 3'UTR motifs, [CC(C/U)GUA(A/U)] and [(C/U)CT(A/U)GUA]. A closer evaluation of the two putative 3'UTR motifs revealed that they always preceded the PUF3 motif and represented the first part of it.

In the initially fully aerobic cultures, the genes involved in sulphate assimilation and methionine biosynthesis (*MET1, MET2, MET3, MET8, MET14, MET17, MET22, ECM17, YLL058W*) and sulphate uptake (*SUL2*) showed downregulation at 0.2 h and had fully recovered at 1 h (cluster 6). However, other genes related to sulphur amino acid metabolism (*MET32, MUP1, MET4, BDS1, MET28*) were upregulated during 1-72 h (cluster 10). In the initially oxygen-limited cultures, several genes related to methionine biosynthesis and sulphate assimilation (*MET10, MET17, SAM2, CYS3, MET14, MET1, OAC1, MET18*) also responded by transient downregulation, but with lowest level of transcription at 1 h and full recovery only at 8 h (cluster 21).

Transient upregulation upon oxygen depletion: processes of protein degradation

Protein degradation mechanisms were transiently upregulated as a response to lack of oxygen both in the initially fully aerobic and oxygen-limited cultures. In the initially fully aerobic cultures, genes of vacuolar transport (cluster 9), proteasome and autophagy (cluster 5) and protein catabolism (cluster 3) showed transient upregulation at 0.2 and 1 h (Figure 2 and Table S2).

When the initially oxygen-limited cultures became anaerobic, genes of vacuolar transport (cluster 18), protein targeting to vacuole (cluster 9), vacuolar protein catabolic process (cluster 22), autophagy (clusters 9 and 18), ubiquitin mediated proteolysis (cluster 10) and proteasome (cluster 14) showed transient upregulation during 0.2-3 h (Figure 3 and Table S3). In addition, clusters 15 and 16, which showed upregulation in the anaerobic steady state compared to the initial steady state, were enriched in genes of protein targeting to the vacuole and proteasome, respectively. Genes in clusters 9, 18 and 22 were enriched in binding sites of transcription factor binding sites Msn2/4p and Gis1p, regulating genes encoding activities related to stress response, while genes in

clusters 14 and 16 were enriched in binding sites of Rpn4p, regulating genes encoding components of the proteasome. Genes in cluster 15 were enriched in a putative transcription factor binding site [TA(A/T)ACGA].

Upregulation for anaerobic steady state: cell wall components, remodelling of cell wall, sterol and amino acid uptake, and iron/cation homeostasis

In the initially fully aerobic cultures, genes involved in sterol transport (*DAN1-3 TIR1-4, PAU2,3,4,5,8,9,14,18, OSH6, UPC2, PDR11, HES1, AUS1*) and genes related to cation homeostasis and iron transport (*FRE6, FET4, ENB1, CUP5, ATX2, COT1, TIS11, IZH3, SMF3, IRC7, SIT1, FIT2, ARN1*) were upregulated during 0.2-8 h after the change in oxygen provision and reached their new steady state values, with higher expression levels, within 24 h (cluster 19). In the initially oxygen-limited cultures, genes related to sterol uptake and biosynthesis (*DAN1-3, TIR1-4, PAU8,9,14,2 OSH6, UPC2, ERG8, ERG26, ERG7, ERG2, PDR11, DAP1, ARE1, SUT2, NCP1, ERG9, ERG27, ERG24, HES1, ERG28, AUS1, KES1*) and cation homeostasis (*IRC7, SMF3, IZH4, VMA2, SRO77, COT1, CUP5*) remained unchanged for the first 3 h after the change of oxygen provision, but were upregulated to their new anaerobic steady state values during 8 to 24 h (cluster 1).

Genes encoding plasma membrane phosphate transporters (*PHO86, PHO84, PHO89)* were transiently upregulated 0.2 h after the switch to nitrogen in the initially oxygen-limited cultures (cluster 19). *PHO84* was 30-fold, *PHO89* 4-fold and *PHO86* 1.5-fold upregulated at 0.2 h. While expression of *PHO84* and *PHO89* returned to their original levels within 3 h, and *PHO84* was 2-fold downregulated in the anaerobic steady state, *PHO86* remained 1.5-fold upregulated in the anaerobic steady state. Although not found in any of the clusters, *PHO84* and *PHO89* were also transiently upregulated during the first 3 h after the fully aerobic cultures became anaerobic and subsequently downregulated in the anaerobic steady state.

Genes encoding amino acid transporters (*BAP3, GNP1, DIP5, TAT1*) were highly upregulated during the adaptation to anaerobic conditions and in the anaerobic steady state in the initially fully aerobic cultures (cluster 7). A similar but weaker trend was observed in the initially oxygen-limited cultures. Several other genes involved in nitrogen transport showed transient upregulation, while the proline permease (encoded by *PUT4*) was downregulated during the adaptation and in the anaerobic steady state (additional data file: Figure S3).

Downregulation for anaerobic steady state: fatty acid oxidation, peroxisome biogenesis, oxidative phosphorylation, TCA cycle and PPP

In the initially fully aerobic cultures, genes related to fatty acid oxidation and peroxisomal biogenesis in cluster 1 were downregulated within 24 h (*PEX19, CRC1, PEX2, PEX8, PEX3, PEX18*), while genes related to fatty acid oxidation and peroxisomal biogenesis (*FOX2, POT1, PCD1, PXA2, PIP2, IDP3, PXA1, POX1, PEX11, SPS19, DCI1, ECI1, PXA2, PEX14, PEX5, PEX11, PCS60*) and response to oxidative stress (*SOD2, POS5, UBA, MCR1, CTT1, CTA1*) in cluster 24 showed downregulation already at 3 h. In the initially oxygen-limited cultures, genes in clusters 13 and 18 were downregulated as the cells approached the anaerobic steady state, but the genes in cluster 18 were transiently upregulated before the final downregulation. Cluster 18 contained genes of fatty acid oxidation and peroxisomal biogenesis (*POT1, PXA2, PIP2, CRC1, OAF1, PXA1, SPS19, MDH3, ECI1, PEX27, PEX15, PEX30, PEX2, PEX22, PEX3*), genes related to response to oxidative stress (*HYR1, CCP1, SRX1, GAD1, PRX1, GPX1, TRR2,* ORF YCL033C, *HSP12*), genes of oxidative phosphorylation (*NDE2, ATP4, GSM1, ATP3, SDH2*), genes of the TCA cycle (*KGD1, LPD1, ACO1, IDP2*) and the pentose phosphate pathway (*SOL4, GND2, TKL2*). Cluster 13 contained genes of fatty acid oxidation (*FOX2, CTA1, POX1, TES1, PSC60, DCI1, INP1*), oxidative phosphorylation (*ATP19, QCR9, ATP2, QCR8, QCR5, COX12, COX6, QCR2, ATP20, SDH1, SDH4, COX9, ATP14, QCR10, ATP16, COX8, COX4, COX5a, COR1, NDI1, ATP15, COX13, ATP18, ATP1, COX7, CYT1, QCR7, SDH3, ATP7, ATP5*), the TCA cycle (*MDH1, FUM1, KGD2, PYC2, IDH2, LSC2, CIT1*), and genes related to response to reactive oxygen species (*SOD2, SOD1, CTA1, POS5, GRX2, MCR1*). Genes in cluster 13 were enriched in two putative 3'UTR motifs [(A/U)AUAUUC and A/C)UUUAU(G/U)(A/U)], and in binding sites of Ume6p transcription factor, regulating genes encoding activities related to the cell cycle, and a putative transcription factor [A(A/T)C(C/T)CCG]. Cluster 14, genes of which showed transient upregulation during 0.2 to 8 h, contained genes of glutathione metabolism (*GPX2, GTO1, GTT1, GLO1, GTT2*) and carnitine metabolism (*YAT1, YAT2*).

Transient upregulation of reserve energy metabolism

In the initially fully aerobic cultures, genes of glycogen and trehalose metabolism were transiently upregulated during 0.2 to 8 h. Cluster 9 included genes related to synthesis of glycogen (*PCL6, GLG1, GSY2, GLC8*) and synthesis of trehalose (*TPS1*) while cluster 12 included genes related to synthesis of glycogen (*GLC3, PIG2*), mobilisation of glycogen (*GDB1, GPH1*) and mobilisation of

trehalose (*NTH1*). In the initially oxygen-limited cultures, genes of related to synthesis of glycogen (*GSY2*), synthesis of trehalose (*TPS1*), mobilisation of glycogen (*GDB1*) and mobilisation of trehalose (*NTH1*) were transiently upregulated between 0.2 and 3 h. In addition, the genes related to synthesis of glycogen (*PCL6, GAC1, PCL7, REG1, PCL8, RIM11*), synthesis of trehalose (*TPS2, TSL1*) and mobilisation of trehalose (*NTH2, TPS2*) in cluster 9 had a trend of transient upregulation between 0.2 and 1 h. Binding sites for Msn2/4p and Gisp1 transcription factors were enriched in both clusters of oxygen-limited cultures and in addition, binding sites for Ume6p and a putative transcription factor [A(A/T)C(C/T)CCG] were enriched in cluster 9. Cluster 18 which was transiently upregulated before final downregulation contained genes of glycogen metabolism (*BMH2, GLC3, GLG2, SGA1, GLG1, GLC8, GPH1*). In this cluster, binding sites for Msn2/4p, Gisp1 and Ume6p, and a 3'UTR motif [(A/U)AUAUUC] were enriched.

Analysis of transcriptional responses in context of regulatory networks using Reporter Features analysis

We further analysed the transcriptional response in context of the network of all known interactions between transcription factors/other regulatory proteins and genes and performed a multidimensional Reporter Features analysis (Oliveira *et al.* 2008; Patil and Nielsen 2005). From the network, the analysis identified regulators (i.e. transcription factors or regulatory proteins) whose surrounding genes had expression profiles with significantly high correlation during the time-course of adaptation to the anaerobic steady state. Thus, it identified transcription factors and other regulatory proteins that most probably determined the expression profiles of the sets of responding genes. Regulatory networks of the Reporter Regulators (with Reporter p-values less than or equal to 0.01) of the present data are shown in Figures 4 and 5. The open reading frames associated with the regulators are presented in Additional data files: TR21_multiD_reporters_p01.sif and TR21_multiD_reporters_p01.sif.

The regulatory network of Reporter Regulators identified for the initially fully aerobic cultures switching to anaerobicity contained 29 regulatory proteins and a total of 297 nodes of regulators and genes (Figure 4). The regulatory network of Reporter Regulators identified for the initially oxygen-limited cultures switching to anaerobicity contained 28 regulatory proteins and a total of 327 nodes of regulators and genes (Figure 5). The two regulatory networks shared the stress response regulators Msn2/4p, Hsf1p and Hog1p, growth-related regulators Basp1, Rap1p, Ifh1p, Gts1p, Rsc30p and Esa1p, and the protein degradation-related regulators Rpt6p and Snf7p.

Additionally, both networks contained regulators of genes of fatty acid β-oxidation Oaf1p and Pip2p, the Upc2p regulator of genes of sterol biosynthesis, the carbon-source responsive factor Adr1p and the Met1p regulator of methionine biosynthesis.

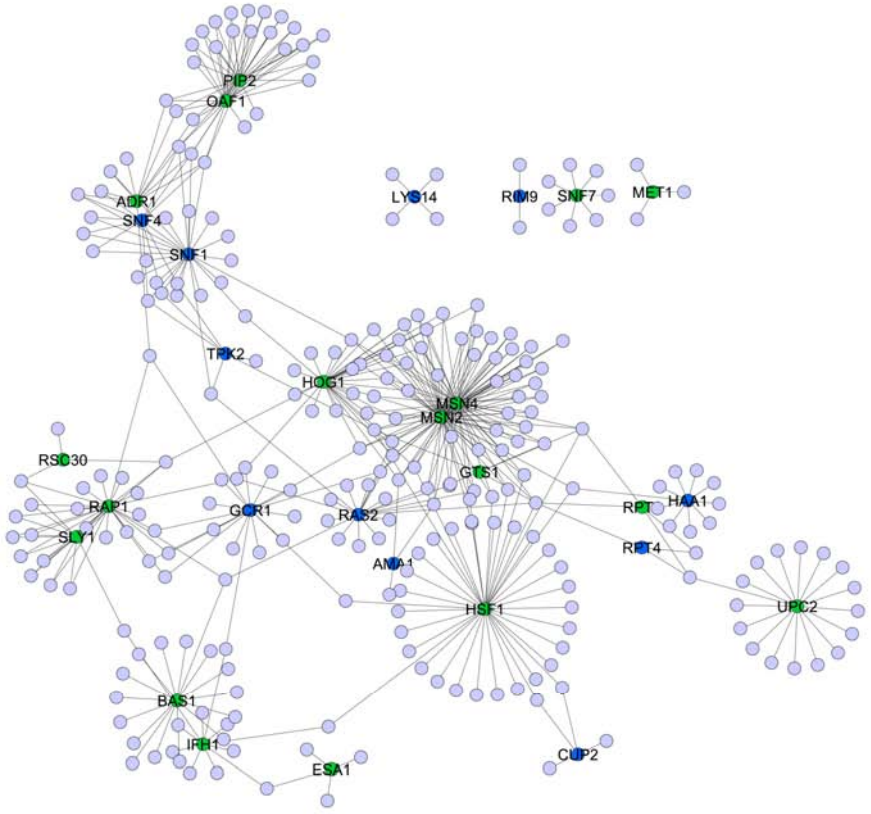


Figure 4. Active regulatory network in adaptation of initially fully aerobic cultures of S. cerevisiae to anaerobic conditions. *The transcription factors and regulatory proteins that most probably mediated regulation throughout the adaptation to anaerobic conditions were identified by multidimensional Reporter Features analysis (Oliveira et al. 2008; Patil and Nielsen 2005). The Reporter transcription factors and regulatory proteins are shown with their interactions to genes in the regulatory network. The reporters specific for the initially fully aerobic cultures and those shared with the initially oxygen-limited cultures are highlighted in blue and green, respectively.*

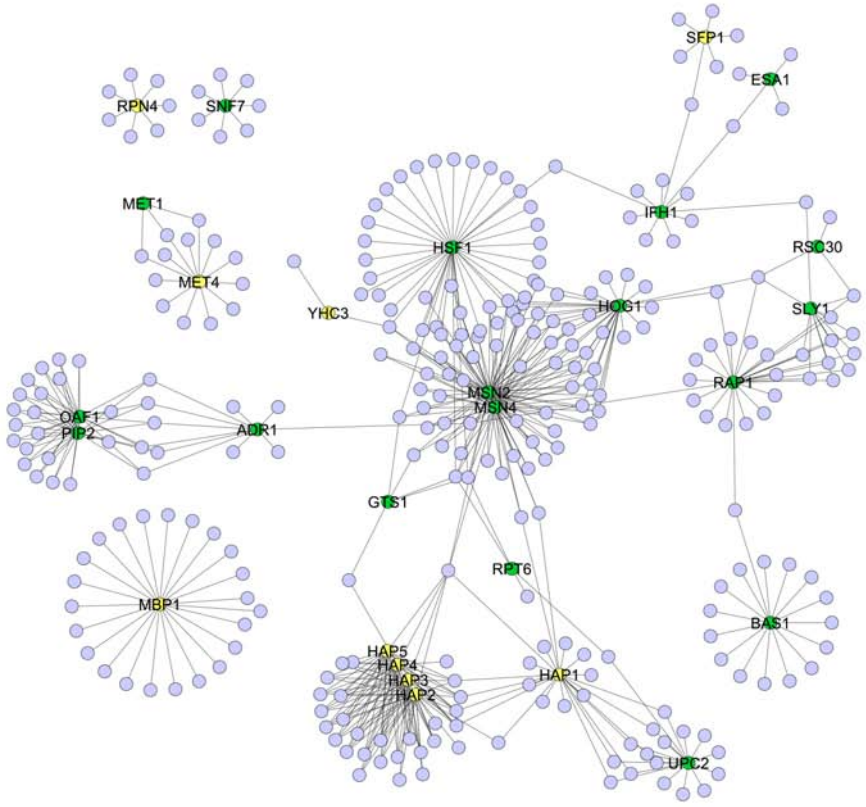


Figure 5. Active regulatory network in adaptation of initially oxygen-limited cultures of S. cerevisiae to anaerobic conditions. *The transcription factors and regulatory proteins that most probably mediated regulation throughout the adaptation to anaerobic conditions were identified by multidimensional Reporter Features analysis (Oliveira et al. 2008; Patil and Nielsen 2005). The Reporter transcription factors and regulatory proteins are shown with their interactions to genes in the regulatory network. The reporters specific for the initially oxygen-limited cultures and those shared with the initially fully aerobic cultures are highlighted in yellow and green, respectively.*

The Gcr1p activator of glycolytic genes, the cAMP dependent protein kinase Tpk2, and the Snf1p and Snf4p protein kinases were identified as Reporter Regulators only for the initially fully aerobic cultures. The Hap1p regulator was specific in the regulation of the initially oxygen-limited cultures, with a Reporter p-value threshold of 0.01, but with a threshold of 0.05 it was identified as an active regulator of both cultures.

Analysis of the transcriptional response in context of the metabolic network

Since the cultures studied were different in their initial metabolic state, being either fully respiratory or respiro-fermentative, the transcriptional response of the cultures to sudden oxygen depletion was also studied in the context of a genome-wide metabolic network with the Reporter Metabolites algorithm (Patil and Nielsen 2005). This algorithm identified the metabolites in the metabolic network whose surrounding enzymes, i.e. the genes encoding them, had significantly differential

expression at different times. The identified Reporter Metabolites (threshold reporter p-value less than or equal to 0.05) in the pathways of central carbon metabolism of *S. cerevisiae* after sudden depletion of oxygen from fully aerobic and oxygen-limited cultures are shown in Figure 6. The open reading frames associated with the metabolites of central carbon metabolism are presented in Additional data file: central_carbon_metabolism.sif.

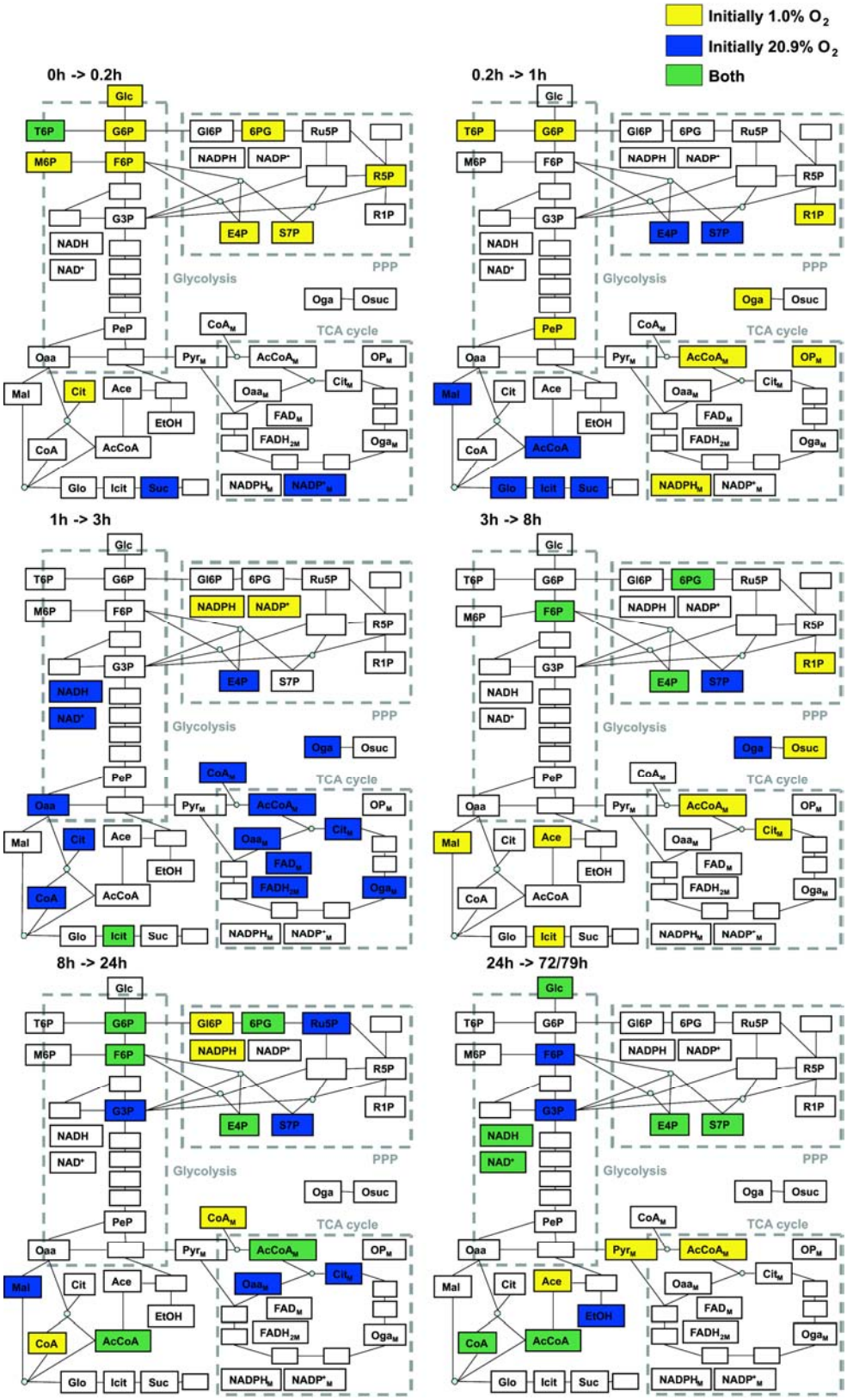


Figure 6. Reporter Metabolites observed in central carbon metabolism during the adaptation of S. cerevisiae to anaerobic conditions. *The series of figures shows the Reporter Metabolites identified (p-value < 0.05) at the indicated times after sudden oxygen depletion. Reporter Metabolites for initially fully aerobic cultures and initially oxygen-limited cultures are highlighted in green and red, respectively. Reporter Metabolites shared by both initial culture conditions are highlighted in yellow. Reporter Metabolites are metabolites in a metabolic network, around which significant transcriptional changes have occurred (Patil and Nielsen 2005).*

During the first interval, 0–0.2 h, the initially oxygen-limited cultures showed Reporter Metabolites in upper glycolysis and in the pentose phosphate pathway, whereas in the initially fully aerobic cultures Reporter Metabolites were not observed in the pentose phosphate pathway until after 0.2 h. In the latter cultures, likewise between 0.2 and 1 h, Reporter Metabolites were also observed in the glyoxylate cycle. Between 1 and 3 h after the oxygen depletion, the initially fully aerobic cultures showed Reporter Metabolites in the TCA cycle. 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.

Discussion

In response to oxygen depletion, both the fully aerobic and oxygen-limited cultures showed transient downregulation, but full recovery to the level of the initial steady state of genes encoding activities related to growth and cell proliferation. As a function of time, the expression profiles of these genes differed in the two culture sets, the initially oxygen-limited cultures responding more rapidly. However, the overall response was very similar. In addition, the same transcription factors and regulators, related to growth and cell proliferation were identified in the initially fully aerobic and the oxygen-limited cultures when the results of the FIRE and Reporter analyses were combined. The downregulation of genes encoding activities involved in these processes is likely due to the decrease observed in the specific growth rate to 0.06 h^{-1} almost immediately after the shift to anaerobiosis (Wiebe *et al.* 2008). This hypothesis is supported by the earlier observation that genes related to cell cycle, DNA replication and repair, rRNA processing and protein synthesis are transiently downregulated as a response to anoxia in batch cultivations on galactose, when the metabolism is strongly respiratory and specific growth rate is reduced as a response to anoxia, but not on glucose when the metabolism is partially fermentative, even in the presence of oxygen, and no change in specific growth rate is observed (Lai *et al.* 2006; Lai *et al.* 2005). However, it is interesting that although the specific growth rate was below 0.1 h^{-1} for approximately 15 h, the level of transcription of especially the ribosomal genes had returned to that observed in the initial steady state within 3 to 8 h. The transcription of genes encoding ribosomal proteins is positively correlated to the specific growth rate in steady state chemostat cultures (Regenberg *et al.* 2006; Fazio *et al.* 2008), but it has been suggested that these genes are regulated by the external environment rather

than the specific growth rate (Levy *et al.* 2007). Signalling in response to environmental changes may have developed to enable a faster response than feedback regulation by metabolic pathways could mediate (Levy *et al.* 2007). Supporting this hypothesis, Zaman *et al.* recently concluded that growth-rate specific transcription in yeast generally results from cells sensing their nutritional environment (Zaman *et al.* 2009).

mRNAs of genes encoding subunits of ribosomes have a half-life of 22 ± 6 min in *S. cerevisiae*, and the average half-life of mRNAs in yeast is 30 min (Wang *et al.* 2002). Thus, the 2 to 5-fold decrease in mRNA levels within 10 minutes after the oxygen depletion, observed in particular with mRNAs of genes related to ribosome biogenesis and RNA processing indicates active degradation of these mRNAs. The control of degradation of mRNAs involve 3'UTRs which also have important roles in the translation and localisation of mRNAs (Grzybowska *et al.* 2001; Ulbricht and Olivas 2008; Saint-Georges *et al.* 2008; Lawless *et al.* 2009). In the present study, binding sites of specific 3'UTRs motifs were found to be enriched in the genes related to growth and cell proliferation. The transient downregulation profiles of genes in which these motifs were enriched, suggest that the motifs may have a role in the degradation of mRNAs. In fact, the PUF4 motif, known to be involved in the decay of mRNAs of genes related to rRNA synthesis and processing and ribosomal biogenesis (Gerber *et al.* 2004; Grigull *et al.* 2004) and PUF5, associated with mRNAs encoding nuclear components (Gerber *et al.* 2004), were identified in clusters of the initially oxygen-limited cultures. In addition, co-localised with PUF4, a putative 3'UTR motif AUAGA was identified in the initially oxygen-limited cultures and also in the initially fully aerobic cultures as a part of a longer putative motif [A/U/G)(G/U)AUAGA]. In addition, another putative 3'UTR motif [UAUA(A/C)(G/U)A] was identifed in the initially fully aerobic cultures. The fact that different motifs were identified under the different culture conditions is intriguing, but it remains unclear if these differences reflect the small differences in the timing of the transcriptional responses under these two conditions.

,

In both the initially fully aerobic and oxygen-limited cultures, transient upregulation of genes encoding activities of processes of protein degradation was observed upon oxygen depletion. In the initially oxygen-limited cultures, some of these activities continued to be upregulated in the new anaerobic steady state compared to the initial steady state. Further, two regulators related to protein degradation (Rpt6p, Snf7p) (Glickman *et al.* 1999; Robinson *et al.* 1988) were identified as regulators under both conditions. Protein degradation may be related to the transiently occurring reduced specific growth rate of the cultures. However, it may also indicate a more global

remodelling of cellular functions and need for novel activities upon anaerobicity. Proteolysis plays an important role in the response to stress conditions (Hilt and Wolf 1992) and in fact binding sites of stress response-related transcription factors (MSN2/4, GIS1) (Martinez-Pastor *et al.* 1996; Zhang *et al.* 2009) were identified as enriched in these genes in the initially oxygen-limited cultures. Additionally, other indications of stress response were observed in the present study. Reporter Features analysis identified stress response-related regulators (Msn2/4p, Hsf1p, Hog1p) (Martinez-Pastor *et al.* 1996; Sorger and Pelham 1987; Westfall *et al.* 2004) in both cultures as a response to oxygen depletion and among the earlier mentioned genes related to growth and cell proliferation, the stress response-related transcription factor Xbp1p (Mai and Breeden 2000; Mai and Breeden 1997) was enriched.

Genes encoding mitochondrial membrane proteins and proteins with activities related to mitochondrial translation or protein import to mitochondria were transiently downregulated upon introduction of anoxia in all cultures. Again, the initial metabolic state made little difference to the response; the profiles of the temporal downregulation were close to identical. Among these genes, the PUF3 motif, which promotes the degradation of, and controls the localisation of mRNAs encoding mitochondrial proteins (Saint-Georges *et al.* 2008; Gerber *et al.* 2004; Olivas and Parker 2000), was enriched. Interestingly, in the initially fully aerobic cultures the transcription of these genes recovered to a higher level than in the initial steady state. The same was observed in the comparison of different steady states with varying levels of oxygen provision: the lowest expression level of these genes was observed in the fully aerobic cultures, compared to the oxygen-limited and anaerobic cultures (Rintala *et al.* 2009). This may be an indication of still unknown functions of mitochondria under oxygen-limited and anaerobic conditions.

When both the fully aerobic and the oxygen-limited cultures were switched to anaerobiosis, a rapid and transient downregulation of genes encoding proteins of sulphate assimilation and methionine biosynthesis occurred. The genes encoding activities for methionine biosynthesis are known to be downregulated as a response to increase in the intracellular concentration of S-adenosylmethione (AdoMet) or methionine (Thomas *et al.* 1989), and several transcriptional activators regulate the expression of these genes (Blaiseau *et al.* 1997; Kuras *et al.* 1996; Thomas *et al.* 1992). AdoMet provides activated methyl groups for phospholipid synthesis, and for protein and histone methylation, so downregulation of these genes could be related to a general transient downregulation of biosynthesis (Kaiser *et al.* 2006). Methionine biosynthesis is also closely linked to the synthesis of glutathione which is the main component in the maintenance of cellular redox

balance (Lopez-Mirabal and Winther 2008), and thus the rapid and transient changes observed in the genes encoding activities of methionine biosynthesis are likely to reflect altered redox balancing as a result of oxygen depletion (Lopez-Mirabal and Winther 2008).

Genes encoding plasma membrane phosphate transporters Pho84p and Pho89p were upregulated immediately after the shift to anaerobic conditions in both cultures. According to Gonzales *et al.* (Gonzalez *et al.* 2000) the intracellular phosphate and polyphosphate levels start to increase in less than 10 minutes after a shift to anaerobiosis and phosphate levels stay high in anaerobic, compared to aerobic conditions. This increase results from transient increase in transport of extracellular phosphate into the cell during the first 15 minutes after the shift to anaerobiosis. The function of this increase is not known, but regulation of glycolytic enzymes has been suggested (Gonzalez *et al.* 2000), and it appears to be important in metabolic regulation in response to sudden oxygen depletion. The increase in the transcription of phosphate transporter encoding genes observed in the current study was only transient and their expression was lower in the anaerobic than in the oxygen-receiving steady states.

In both the initially fully aerobic and the oxygen-limited cultures, genes related to fatty acid oxidation and peroxisomal biogenesis, response to oxidative stress, oxidative phosphorylation, TCA cycle and the pentose phosphate pathway were downregulated in anaerobic conditions. However, some of the genes of these pathways were transiently upregulated in the initially oxygen-limited cultures, before being downregulated to the new anaerobic steady state levels. Similar transient upregulation of genes of oxidative phosphorylation and the TCA cycle has been previously observed during adaptation to anaerobic conditions in batch cultures on galactose, but not on glucose, suggesting that the response is linked to cessation of respiration (Lai *et al.* 2006; Lai *et al.* 2005). Additionally, respiratory-deficient yeast cells in aerobic conditions respond to loss of oxidative phosphorylation by upregulating genes related to peroxisomal activities, including fatty acid oxidation and anaplerotic reactions, to increase supplies of acetyl-CoA and OAA (Epstein *et al.* 2001). As this response was not seen in the adaptation of the initially fully aerobic cultures to anaerobiosis, it seems to be specific for the transition from respiro-fermentative conditions. The expression of genes encoding some of these processes was also oxygen-dependent in steady state conditions (Rintala *et al.* 2009), and the expression levels may have already been maximal in 20.9% oxygen, allowing no further upregulation to occur.

As in the clustering analysis, Reporter Regulators related to stress, growth, protein degradation, fatty acid catabolism, sterol biosynthesis and carbon source regulation were identified in the adaptation to anaerobiosis, independent of the initial metabolic state of the culture. The regulatory network of the initially fully aerobic cultures specifically contained an additional regulator of glycolysis, indicative of changes upon switching from respiration to fermentation and initiating a high specific carbon flux through the glycolytic and fermentative pathways. The key metabolic regulator kinases Snf1p/Snf4p involved in several different processes (stress response, translation, lipid and glycogen biosynthesis, glucose derepression) and Tpk2p involved in the response to nutrients and stress, identified in the initially fully aerobic cultures as Reporter Regulators, suggest a coordinated transcriptional adjustment of metabolic genes.

Central carbon metabolism encompasses the major energy-generating pathways in the cell, the respiratory and the fermentative pathways, but it also generates precursors and reducing power for biosynthetic reactions. Reporter Metabolite analysis revealed that the temporally differential expression of genes encoding activities of the central carbon metabolism as a response to oxygen depletion was dependent on the initial metabolic state of the culture. In the initially oxygen-limited cultures, sudden oxygen depletion led to a rapid response around the metabolites of the upper part of glycolysis and the pentose phosphate pathway, whereas in the initially fully aerobic cultures, , the hierarchical regulation of the central carbon metabolism seemed not to be the first priority since slower responses around the metabolites of the pentose phosphate pathway, and the glyoxylate and TCA cycles were observed. Changes in the expression of genes encoding enzymes producing or consuming the metabolites are likely to lead to changes in the metabolite concentrations which are variables in the metabolic regulation of the system. However, in our previous study, the effect of oxygen depletion on the concentrations of the metabolites of central carbon metabolism was observed to be generally faster than the effect on gene expresssion (Wiebe *et al.* 2007). Then again, the differential regulation of especially the oxidative part of the pentose phosphate pathway was seen also in the study of Wiebe *et al.* (2007) as the concentration of 6-phosphogluconate was found to be dependent on the initial oxygen concentration of the cultures (Wiebe *et al.* 2007).

The changes observed in the expression of the genes encoding the upper glycolytic enzymes are probably not reflected in the metabolite concentrations as glycolytic flux has been shown to be mainly controlled at the post-transcriptional level (de Groot *et al.* 2007; Daran-Lapujade *et al.* 2004). Upper glycolysis is the entry point of storage carbohydrates into metabolism and thus its metabolites as reporters may also indicate the mobilisation of storage carbon due to the reduced production of energy equivalents as respiration suddenly ceases. In fact, the concentration of

trehalose 6-phosphate, an intermediate of trehalose biosynthesis, was observed to be dependent on the initial oxygen concentration by Wiebe *et al.* (2007). In the current study, genes related to both mobilisation and storage of glycogen and trehalose were found to be transiently upregulated in both conditions. This seemingly futile response could not be explained. However, the simultaneous upregulation of genes acting in mobilisation and storage of glycogen and threhalose has previously been seen as a response to stress and shown to be dependent on Msn2/4p transcription factors (Parrou *et al.* 1997; Hottiger *et al.* 1987).

Conclusions

The transcriptional responses of *S. cerevisiae* grown under glucose-limitation in either fully aerobic or oxygen-limited conditions (resulting in respiratory and respiro-fermentative metabolic states, respectively) to sudden depletion of oxygen were very similar to each other. During the adaptation to anaerobic conditions, and thus to fermentative growth and energy generation, the cells responded by transient downregulation of genes related to growth and cell proliferation. Additionally, the adaptation to anaerobiosis evoked stress response-related regulatory networks independent of the initial metabolic state of the culture. To enable global remodelling of the activities needed for the new mode of growth, a transient upregulation of genes related to protein degradation was observed.
In the initially oxygen-limited cultures the shift to anaerobiosis led to specific regulation of aerobic genes by the Hap2/3/4/5p-complex and to transient upregulation of genes involved in oxidative phosphorylation, TCA cycle, fatty acid oxidation, peroxisomal biogenesis, oxidative stress and pentose phosphate pathway. As the cell senses the decrease in the oxygen concentration, it may try to enhance the use of oxygen by upregulation of genes encoding the above mentioned activities. Although a similar transient response was not observed in the cells initially grown in the fully respiratory conditions, it may have been too short to have been observed with the sampling frequency used in this study and thus it remains unclear whether Hap2/3/4/5p-complex controls a similar response in the transition from fully aerobic to anaerobic conditions.

.

Acknowledgements

We thank Pirjo Tähtinen, Eila Leino and Tarja Laakso for excellent technical assistance. Prof. Jens Nielsen provided fruitful discussions and shared his expertise in yeast systems biology. The microarray analyses were carried out at the Finnish DNA Microarray Centre at Turku Centre for Biotechnology. The financial support of Tekes, The Finnish Funding Agency for Technology and

Innovation (Project numbers 40135/04 and 40537/05) and 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) is gratefully acknowledged.

References

ABRAMOVA, N. E., COHEN, B. D., SERTIL, O., KAPOOR, R., DAVIES, K. J. and LOWRY, C. V. (2001) Regulatory mechanisms controlling expression of the DAN/TIR mannoprotein genes during anaerobic remodeling of the cell wall in *Saccharomyces cerevisiae*. Genetics **157,** 1169–1177.

ALIMARDANI, P., REGNACQ, M., MOREAU-VAUZELLE, C., FERREIRA, T., ROSSIGNOL, T., BLONDIN, B. and BERGES, T. (2004) *SUT1*-promoted sterol uptake involves the ABC transporter Aus1 and the mannoprotein Dan1 whose synergistic action is sufficient for this process. Biochem. J. **381,** 195–202.

ANDREASEN, A. A. and STIER, T. J. (1953) Anaerobic nutrition of *Saccharomyces cerevisiae*. I. Ergosterol requirement for growth in a defined medium. J. Cell. Physiol. **41,** 23–36.

BECERRA, M., LOMBARDIA-FERREIRA, L. J., HAUSER, N. C., HOHEISEL, J. D., TIZON, B. and CERDAN, M. E. (2002) The yeast transcriptome in aerobic and hypoxic conditions: effects of hap1, rox1, rox3 and srb10 deletions. Mol. Microbiol. **43,** 545–555.

BENJAMINI, Y. and HOCHBERG, Y. (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society Series_ B **57,** 289–300.

BLAISEAU, P. L., ISNARD, A. D., SURDIN-KERJAN, Y. and THOMAS, D. (1997) Met31p and Met32p, two related zinc finger proteins, are involved in transcriptional regulation of yeast sulfur amino acid metabolism. Mol. Cell. Biol. **17,** 3640–3648.

BOUROT, S. and KARST, F. (1995) Isolation and characterization of the *Saccharomyces cerevisiae SUT1* gene involved in sterol uptake. Gene **165,** 97–102.

BRUCKMANN, A., HENSBERGEN, P. J., BALOG, C. I., DEELDER, A. M., BRANDT, R., SNOEK, I. S., STEENSMA, H. Y. and VAN HEUSDEN, G. P. (2009) Proteome analysis of aerobically and anaerobically grown *Saccharomyces cerevisiae* cells. J. Proteomics **71,** 662–669.

CHAN, K. and ROTH, M. B. (2008) Anoxia-induced suspended animation in budding yeast as an experimental paradigm for studying oxygen-regulated gene expression. Eukaryot. Cell, **10**, 1795–808

DARAN-LAPUJADE, P., JANSEN, M. L., DARAN, J. M., VAN GULIK, W., DE WINDE, J. H. and PRONK, J. T. (2004) Role of transcriptional regulation in controlling fluxes in central carbon metabolism of *Saccharomyces cerevisiae*. A chemostat culture study. J. Biol. Chem. **279,** 9125–9138.

DAVID, P. S. and POYTON, R. O. (2005) Effects of a transition from normoxia to anoxia on yeast cytochrome *c* oxidase and the mitochondrial respiratory chain: implications for hypoxic gene induction. Biochim. Biophys. Acta **1709,** 169–180.

DE GROOT, M. J., DARAN-LAPUJADE, P., VAN BREUKELEN, B., KNIJNENBURG, T. A., DE HULSTER, E. A., REINDERS, M. J., PRONK, J. T., HECK, A. J. and SLIJPER, M. (2007) Quantitative proteomics and transcriptomics of anaerobic and aerobic yeast cultures reveals post-transcriptional regulation of key cellular processes. Microbiology **153,** 3864–3878.

DIRMEIER, R., O'BRIEN, K. M., ENGLE, M., DODD, A., SPEARS, E. and POYTON, R. O. (2002) Exposure of yeast cells to anoxia induces transient oxidative stress. Implications for the induction of hypoxic genes. J. Biol. Chem. **277,** 34773–34784.

ELEMENTO, O., SLONIM, N. and TAVAZOIE, S. (2007) A universal framework for regulatory element discovery across all genomes and data types. Mol. Cell **28,** 337–350.

EPSTEIN, C. B., WADDLE, J. A., HALE, W.,4TH, DAVE, V., THORNTON, J., MACATEE, T. L., GARNER, H. R. and BUTOW, R. A. (2001) Genome-wide responses to mitochondrial dysfunction. Mol. Biol. Cell **12,** 297–308.

FALCON, S. and GENTLEMAN, R. (2007) Using GOstats to test gene lists for GO term association. Bioinformatics **23,** 257–258.

FAZIO, A., JEWETT, M. C., DARAN-LAPUJADE, P., MUSTACCHI, R., USAITE, R., PRONK, J. T., WORKMAN, C. T. and NIELSEN, J. (2008) Transcription factor control of growth rate dependent genes in *Saccharomyces cerevisiae*: a three factor design. BMC Genomics **9,** 341.

FUTSCHIK, M. (2007) Mfuzz: Soft clustering of time series gene expression data. R package version 1.6.0.

FUTSCHIK, M. E. and CARLISLE, B. (2005) Noise-robust soft clustering of gene expression time-course data. J. Bioinform Comput. Biol. **3,** 965–988.

GERBER, A. P., HERSCHLAG, D. and BROWN, P. O. (2004) Extensive association of functionally and cytotopically related mRNAs with Puf family RNA-binding proteins in yeast. PLoS Biol. **2,** E79.

GLICKMAN, M. H., RUBIN, D. M., FU, H., LARSEN, C. N., COUX, O., WEFES, I., PFEIFER, G., CJEKA, Z., VIERSTRA, R., BAUMEISTER, W., FRIED, V. and FINLEY, D. (1999) Functional analysis of the proteasome regulatory particle. Mol. Biol. Rep. **26,** 21–28.

GONZALEZ, B., DE GRAAF, A., RENAUD, M. and SAHM, H. (2000) Dynamic in vivo (31)P nuclear magnetic resonance study of *Saccharomyces cerevisiae* in glucose-limited chemostat culture during the aerobic-anaerobic shift. Yeast **16,** 483–497.

GRIGULL, J., MNAIMNEH, S., POOTOOLAL, J., ROBINSON, M. D. and HUGHES, T. R. (2004) Genome-wide analysis of mRNA stability using transcription inhibitors and microarrays reveals posttranscriptional control of ribosome biogenesis factors. Mol. Cell. Biol. **24,** 5534–5547.

GRZYBOWSKA, E. A., WILCZYNSKA, A. and SIEDLECKI, J. A. (2001) Regulatory functions of 3'UTRs. Biochem. Biophys. Res. Commun. **288,** 291–295.

GUZY, R. D., MACK, M. M. and SCHUMACKER, P. T. (2007) Mitochondrial complex III is required for hypoxia-induced ROS production and gene transcription in yeast. Antioxid. Redox Signal. **9,** 1317–1328.

HA, N., HELLAUER, K. and TURCOTTE, B. (1996) Mutations in target DNA elements of yeast *HAP1* modulate its transcriptional activity without affecting DNA binding. Nucleic Acids Res. **24,** 1453–1459.

HILT, W. and WOLF, D. H. (1992) Stress-induced proteolysis in yeast. Mol. Microbiol. **6,** 2437–2442.

HON, T., DODD, A., DIRMEIER, R., GORMAN, N., SINCLAIR, P. R., ZHANG, L. and POYTON, R. O. (2003) A mechanism of oxygen sensing in yeast. Multiple oxygen-responsive steps in the heme biosynthetic pathway affect Hap1 activity. J. Biol. Chem. **278,** 50771–50780.

HOTTIGER, T., SCHMUTZ, P. and WIEMKEN, A. (1987) Heat-induced accumulation and futile cycling of trehalose in *Saccharomyces cerevisiae*. J. Bacteriol. **169,** 5518–5522.

IRIZARRY, R. A., HOBBS, B., COLLIN, F., BEAZER-BARCLAY, Y. D., ANTONELLIS, K. J., SCHERF, U. and SPEED, T. P. (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics **4,** 249–264.

KAISER, P., SU, N. Y., YEN, J. L., OUNI, I. and FLICK, K. (2006) The yeast ubiquitin ligase SCFMet30: connecting environmental and intracellular conditions to cell division. Cell. Div. **1,** 16.

KURAS, L., CHEREST, H., SURDIN-KERJAN, Y. and THOMAS, D. (1996) A heteromeric complex containing the centromere binding factor 1 and two basic leucine zipper factors, Met4 and Met28, mediates the transcription activation of yeast sulfur metabolism. EMBO J. **15,** 2519–2529.

KWAST, K. E., BURKE, P. V. and POYTON, R. O. (1998) Oxygen sensing and the transcriptional regulation of oxygen-responsive genes in yeast. J. Exp. Biol. **201,** 1177–1195.

KWAST, K. E., BURKE, P. V., STAAHL, B. T. and POYTON, R. O. (1999) Oxygen sensing in yeast: evidence for the involvement of the respiratory chain in regulating the transcription of a subset of hypoxic genes. Proc. Natl. Acad. Sci. U. S. A. **96,** 5446–5451.

KWAST, K. E., LAI, L. C., MENDA, N., JAMES, D. T.,3RD, AREF, S. and BURKE, P. V. (2002) Genomic analyses of anaerobically induced genes in *Saccharomyces cerevisiae*: functional roles of Rox1 and other factors in mediating the anoxic response. J. Bacteriol. **184,** 250–265.

LAI, L. C., KISSINGER, M. T., BURKE, P. V. and KWAST, K. E. (2008) Comparison of the transcriptomic 'stress response' evoked by antimycin A and oxygen deprivation in *Saccharomyces cerevisiae*. BMC Genomics **9,** 627.

LAI, L. C., KOSORUKOFF, A. L., BURKE, P. V. and KWAST, K. E. (2006) Metabolic-state-dependent remodeling of the transcriptome in response to anoxia and subsequent reoxygenation in *Saccharomyces cerevisiae*. Eukaryot. Cell. **5,** 1468–1489.

LAI, L. C., KOSORUKOFF, A. L., BURKE, P. V. and KWAST, K. E. (2005) Dynamical remodeling of the transcriptome during short-term anaerobiosis in *Saccharomyces cerevisiae*: differential response and role of Msn2 and/or Msn4 and other factors in galactose and glucose media. Mol. Cell. Biol. **25,** 4075–4091.

LAWLESS, C., PEARSON, R. D., SELLEY, J. N., SMIRNOVA, J. B., GRANT, C. M., ASHE, M. P., PAVITT, G. D. and HUBBARD, S. J. (2009) Upstream sequence elements direct post-transcriptional regulation of gene expression under stress conditions in yeast. BMC Genomics **10,** 7.

LEWIS, T. L., KEESLER, G. A., FENNER, G. P. and PARKS, L. W. (1988) Pleiotropic mutations in *Saccharomyces cerevisiae* affecting sterol uptake and metabolism. Yeast **4,** 93–106.

LEVY, S., IHMELS, J., CARMI, M., WEINBERGER, A., FRIEDLANDER, G. and BARKAI, N. (2007) Strategy of transcription regulation in the budding yeast. PLoS ONE **2,** e250.

LOPEZ-MIRABAL, H. R. and WINTHER, J. R. (2008) Redox characteristics of the eukaryotic cytosol. Biochim. Biophys. Acta **1783,** 629–640.

LOWRY, C. V. and ZITOMER, R. S. (1984) Oxygen regulation of anaerobic and aerobic genes mediated by a common factor in yeast. Proc. Natl. Acad. Sci. U. S. A. **81,** 6129–6133.

MAI, B. and BREEDEN, L. (2000) CLN1 and its repression by Xbp1 are important for efficient sporulation in budding yeast. Mol. Cell. Biol. **20,** 478–487.

MAI, B. and BREEDEN, L. (1997) Xbp1, a stress-induced transcriptional repressor of the *Saccharomyces cerevisiae* Swi4/Mbp1 family. Mol. Cell. Biol. **17,** 6491–6501.

MARTINEZ-PASTOR, M. T., MARCHLER, G., SCHULLER, C., MARCHLER-BAUER, A., RUIS, H. and ESTRUCH, F. (1996) The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). EMBO J. **15,** 2227–2235.

OLIVAS, W. and PARKER, R. (2000) The Puf3 protein is a transcript-specific regulator of mRNA degradation in yeast. EMBO J. **19,** 6602–6611.

OLIVEIRA, A. P., PATIL, K. R. and NIELSEN, J. (2008) Architecture of transcriptional regulatory circuits is knitted over the topology of bio-molecular interaction networks. BMC Syst. Biol. **2,** 17.

PARROU, J. L., TESTE, M. A. and FRANCOIS, J. (1997) Effects of various types of stress on the metabolism of reserve carbohydrates in *Saccharomyces cerevisiae*: genetic evidence for a stress-induced recycling of glycogen and trehalose. Microbiology **143 (Pt 6),** 1891–1900.

PATIL, K. R. and NIELSEN, J. (2005) Uncovering transcriptional regulation of metabolism by using metabolic network topology. Proc. Natl. Acad. Sci. U. S. A. **102,** 2685–2689.

R DEVELOPMENT CORE TEAM. (2005) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. Vienna, Austria. Version **2.3.0.**

REGENBERG, B., GROTKJAER, T., WINTHER, O., FAUSBOLL, A., ÅKESSON, M., BRO, C., HANSEN, L. K., BRUNAK, S. and NIELSEN, J. (2006) Growth-rate regulated genes have profound impact on interpretation of transcriptome profiling in *Saccharomyces cerevisiae*. Genome Biol. **7,** R107.

RINTALA, E.., WIEBE, M.G, TAMMINEN A., RUOHONEN L. and PENTTILÄ, M. (2008) Transcription of hexose transporters of *Saccharomyces cerevisiae* is affected by change in oxygen provision. BMC Microbiology **8**:53.

RINTALA, E., TOIVARI, M., PITKÄNEN, J. P., WIEBE, M. G., RUOHONEN, L. and PENTTILÄ, M. (2009) Low oxygen levels as a trigger for enhancement of respiratory metabolism in *Saccharomyces cerevisiae*. BMC Genomics **10,** 461.

ROBINSON, J. S., KLIONSKY, D. J., BANTA, L. M. and EMR, S. D. (1988) Protein sorting in Saccharomyces cerevisiae: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. Mol. Cell. Biol. **8,** 4936–4948.

SAINT-GEORGES, Y., GARCIA, M., DELAVEAU, T., JOURDREN, L., LE CROM, S., LEMOINE, S., TANTY, V., DEVAUX, F. and JACQ, C. (2008) Yeast mitochondrial biogenesis: a role for the PUF RNA-binding protein Puf3p in mRNA localization. PLoS One **3,** e2293.

SHIANNA, K. V., DOTSON, W. D., TOVE, S. and PARKS, L. W. (2001) Identification of a UPC2 homolog in *Saccharomyces cerevisiae* and its involvement in aerobic sterol uptake. J. Bacteriol. **183,** 830–834.

SMITH, S. J., CROWLEY, J. H. and PARKS, L. W. (1996) Transcriptional regulation by ergosterol in the yeast *Saccharomyces cerevisiae*. Mol. Cell. Biol. **16,** 5427–5432.

SMYTH, G. K. (2005) Limma: linear models for microarray data. In:Bioinformatics and Computational Biology Solutions using R and Bioconductor. R package version 2.12.0.

SMYTH, G. K. (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. Biol. **3,** Article3.

SNOEK, I. and STEENSMA, H. (2007) Factors involved in anaerobic growth of *Saccharomyces cerevisiae*. Yeast **24,** 1–10.

SORGER, P. K. and PELHAM, H. R. (1987) Purification and characterization of a heat-shock element binding protein from yeast. EMBO J. **6,** 3035–3041.

TER LINDE, J. J., LIANG, H., DAVIS, R. W., STEENSMA, H. Y., VAN DIJKEN, J. P. and PRONK, J. T. (1999) Genome-wide transcriptional analysis of aerobic and anaerobic chemostat cultures of *Saccharomyces cerevisiae*. J. Bacteriol. **181,** 7409–7413.

TER LINDE, J. J. and STEENSMA, H. Y. (2002) A microarray-assisted screen for potential Hap1 and Rox1 target genes in *Saccharomyces cerevisiae*. Yeast **19,** 825–840.

THOMAS, D., CHEREST, H. and SURDIN-KERJAN, Y. (1989) Elements involved in S-adenosylmethionine-mediated regulation of the *Saccharomyces cerevisiae MET25* gene. Mol. Cell. Biol. **9,** 3292–3298.

THOMAS, D., JACQUEMIN, I. and SURDIN-KERJAN, Y. (1992) MET4, a leucine zipper protein, and centromere-binding factor 1 are both required for transcriptional activation of sulfur metabolism in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **12,** 1719–1727.

ULBRICHT, R. J. and OLIVAS, W. M. (2008) Puf1p acts in combination with other yeast Puf proteins to control mRNA stability. RNA **14,** 246–262.

WANG, Y., LIU, C. L., STOREY, J. D., TIBSHIRANI, R. J., HERSCHLAG, D. and BROWN, P. O. (2002) Precision and functional specificity in mRNA decay. Proc. Natl. Acad. Sci. U. S. A. **99,** 5860–5865.

WARNES, G. R. (2006) gplots: Various R programming tools for plotting data. Included R source code and/or documentation contributed by Ben Bolker and Thomas Lumley. R package version 2.3.0.

VERDUYN, C., POSTMA, E., SCHEFFERS, W. A. and VAN DIJKEN, J. P. (1992) Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation. Yeast **8,** 501–517.

WESTFALL, P. J., BALLON, D. R. and THORNER, J. (2004) When the stress of your environment makes you go HOG wild. Science **306,** 1511–1512.

WIEBE, M. G., RINTALA, E., TAMMINEN, A., SIMOLIN, H., SALUSJÄRVI, L., TOIVARI, M., KOKKONEN, J. T., KIURU, J., KETOLA, R. A., JOUHTEN, P., HUUSKONEN, A., MAAHEIMO, H., RUOHONEN, L. and PENTTILÄ, M. (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 Res. **8,** 140–154.

ZAMAMIRI, A. Q., BIROL, G. and HJORTSO, M. A. (2001) Multiple stable states and hysteresis in continuous, oscillating cultures of budding yeast. Biotechnol. Bioeng. **75,** 305–312.

ZAMAN, S., LIPPMAN, S. I., SCHNEPER, L., SLONIM, N. and BROACH, J. R. (2009) Glucose regulates transcription in yeast through a network of signaling pathways. Mol. Syst. Biol. **5,** 245.

ZHANG, N., WU, J. and OLIVER, S. G. (2009) Gis1 is required for transcriptional reprogramming of carbon metabolism and the stress response during transition into stationary phase in yeast. Microbiology **155,** 1690–1698.

ADDITIONAL DATA FILES

Additional data file 1 – Table_S1.doc

Table S1. Over-represented GO- and KEGG-classes in clusters of gene expression data of transition from respiratory to fermentative metabolism

Clusters were determined by fuzzy c-means clustering of gene expression in time-course experiment with initial steady state receiving 20.9% oxygen in feed gas and sampled in time points of 0.2, 1, 3, 8, 24 and 79 hours after switch to nitrogen feed. Clusters are illustrated in Figure 2.

Cluster (unknowns/genes)	**GO-class (p<0.01)**	**KEGG-class (p<0.01)**
1 (71/275)	pentose metabolic process generation of precursor metabolites and energy protein refolding peroxisomal transport methylglyoxal metabolic process vitamin metabolic process SRP-dependent cotranslational protein targeting to membrane, translocation	Citrate cycle (TCA cycle) Pentose phosphate pathway
2 (15/89)	oxidative phosphorylation generation of precursor metabolites and energy acetyl-CoA metabolic process response to pheromone ATP biosynthetic process conjugation tricarboxylic acid cycle translation mitochondrion organization and biogenesis peptide metabolic process	Oxidative phosphorylation Citrate cycle (TCA cycle) MAPK signaling pathway
3 (22/108)	regulation of transcription cellular protein catabolic process histone acetylation	Glycan structures - degradation
4 (37/259)	ribosome biogenesis and assembly rRNA metabolic process organelle organization and biogenesis DNA unwinding during replication	RNA polymerase Pyrimidine metabolism Purine metabolism Histidine metabolism Selenoamino acid metabolism
5 (33/121)	proteolysis anion transport autophagy	Proteasome
6 (31/168)	sulfur metabolic process aspartate family amino acid metabolic process RNA processing RNA splicing	Sulfur metabolism Selenoamino acid metabolism
7 (25/109)	glycolysis energy derivation by oxidation of organic compounds amino acid transport spore wall assembly	Glycolysis / Gluconeogenesis Fructose and mannose metabolism

8 (17/100)	cofactor metabolic process mRNA 3'-end processing glyoxylate metabolic process vitamin metabolic process ammonium transport	Biotin metabolism
9 (60/247)	proteolysis vacuolar transport non-recombinational repair regulation of gluconeogenesis glycogen biosynthetic process	SNARE interactions in vesicular transport Inositol phosphate metabolism Phosphatidylinositol signaling system
10 (22/114)	sulfur metabolic process glycolysis response to stimulus alcohol biosynthetic process gluconeogenesis protein folding mRNA processing age-dependent response to oxidative stress DNA repair	Glycolysis / Gluconeogenesis
11 (11/71)	transcription from RNA polymerase II promoter mRNA capping mitochondrial signaling pathway vesicle-mediated transport cell communication	
12 (41/182)	protein folding thiamin metabolic process nucleoside triphosphate biosynthetic process peroxisome degradation regulation of cell redox homeostasis energy reserve metabolic process cellular carbohydrate catabolic process	Glycolysis / Gluconeogenesis Nitrogen metabolism Starch and sucrose metabolism
13 (5/101)	mRNA export from nucleus ribosomal protein import into nucleus DNA replication, synthesis of RNA primer asparagine biosynthetic process from oxaloacetate axial bud site selection mitotic cell cycle glutathione metabolic process chromosome localization DNA repair isoprenoid metabolic process lipid metabolic process fatty acid metabolic process establishment of cell polarity	Phenylalanine metabolism Fatty acid biosynthesis
14 (18/103)	secretion vacuole organization and biogenesis signal peptide processing homoserine metabolic process pH reduction intracellular transport	

15 (19/148)	translation protein targeting to mitochondrion heterocycle metabolic process tRNA aminoacylation aerobic respiration heme a metabolic process DNA replication checkpoint	Aminoacyl-tRNA biosynthesis Porphyrin and chlorophyll metabolism
16 (18/128)	actin filament-based process transcription initiation growth	Basal transcription factors Glycerophospholipid metabolism
17 (53/89)	spore wall assembly cell differentiation cellular developmental process	
18 (23/166)	translation cellular localization secretory pathway nucleoside metabolic process nuclear pore organization and biogenesis cell organization and biogenesis protein import into nucleus translational elongation organelle inheritance protein import into mitochondrion fatty acid elongation pyrimidine salvage telomere organization and biogenesis	Ribosome
19 (32/172)	cation homeostasis vacuolar acidification sterol transport siderophore transport ethanolamine biosynthetic process vacuole organization and biogenesis secondary metabolic process biopolymer glycosylation establishment of localization very-long-chain fatty acid metabolic process nucleotide-sugar transport heme biosynthetic process endocytosis	Oxidative phosphorylation Glycerophospholipid metabolism Porphyrin metabolism
20 (25/235)	ribosome biogenesis and assembly rRNA metabolic process organelle organization and biogenesis RNA modification ergosterol metabolic process base-excision repair response to zinc ion leading strand elongation microtubule nucleation pseudouridine synthesis chromatin silencing at silent mating-type cassette translation transcription ornithine biosynthetic process	Pyrimidine metabolism RNA polymerase Ribosome Purine metabolism Valine, leucine and isoleucine biosynthesis
21 (30/70)	response to singlet oxygen telomere maintenance via telomerase	

22 (29/294)	ribosome biogenesis and assembly translation rRNA processing amino acid metabolic process organelle organization and biogenesis tRNA modification nucleobase, nucleoside, nucleotide and nucleic acid metabolic process RNA methylation translational initiation protein export from nucleus cell organization and biogenesis IMP biosynthetic process NADPH regeneration purine salvage one-carbon compound metabolic process pentose-phosphate shunt heterocycle metabolic process	Ribosome Aminoacyl-tRNA biosynthesis Lysine biosynthesis Purine metabolism
23 (7/82)	nuclear import regulation of translational termination intracellular transport tRNA splicing DNA repair response to stress secretory pathway	
24 (33/185)	fatty acid oxidation propionate metabolic process ammonium transport generation of precursor metabolites and energy carnitine metabolic process response to reactive oxygen species peroxisome organization and biogenesis	Propanoate metabolism Pyruvate metabolism Glyoxylate and dicarboxylate metabolism Fatty acid metabolism Citrate cycle (TCA cycle) Glutathione metabolism

Additional data file 2 – Table_S2.doc

Table S2. Over-represented GO- and KEGG-classes in clusters of gene expression data of transition from respiro-fermentative to fermentative metabolism

Clusters were determined by fuzzy c-means clustering of gene expression during the transition from a steady state receiving 1.0% oxygen in feed gas to an anaerobic steady state. Samples were taken at 0.2, 1, 3, 8, 24 and 72 h after the switch to nitrogen feed. Clusters are illustrated in Figure 3.

Cluster (unknowns/genes)	**GO-class (p<0.01)**	**KEGG-class (p<0.01)**
1 (51/211)	sterol metabolic process secretory pathway protein amino acid glycosylation vacuole organization and biogenesis maintenance of cell polarity di-, tri-valent inorganic cation homeostasis membrane organization and biogenesis	N-Glycan biosynthesis Biosynthesis of steroids
2 (20/123)	mitotic cell cycle DNA packaging chromosome segregation negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process microtubule nucleation organelle organization and biogenesis regulation of cyclin-dependent protein kinase activity negative regulation of transcription axial bud site selection regulation of transferase activity ribosomal protein import into nucleus rRNA transport cytoskeleton organization and biogenesis nuclear pore organization and biogenesis gene silencing	Cell cycle
3 (38/293)	ribosome biogenesis and assembly rRNA processing organelle organization and biogenesis nucleobase, nucleoside, nucleotide and nucleic acid metabolic process DNA unwinding during replication nucleocytoplasmic transport pre-replicative complex formation nuclear export methylation one-carbon compound metabolic process	RNA polymerase Histidine metabolism Purine metabolism Pyrimidine metabolism Selenoamino acid metabolism
4 (13/165)	translation protein import into mitochondrion inner mitochondrial membrane organization and biogenesis cellular respiration	Aminoacyl-tRNA biosynthesis Valine, leucine and isoleucine biosynthesis

5 (24/98)	response to pheromone conjugation cell division cell morphogenesis regulation of cell size cell growth cytogamy filamentous growth plasma membrane fusion cell wall organization and biogenesis cell adhesion cell wall chitin biosynthetic process	MAPK signaling pathway Aminosugars metabolism
6 (27/132)	serine family amino acid biosynthetic process snoRNA metabolic process membrane lipid biosynthetic process monocarboxylic acid metabolic process	Aminophosphonate metabolism
7 (6/122)	nucleobase, nucleoside, nucleotide and nucleic acid metabolic process aromatic compound metabolic process nuclear mRNA splicing, via spliceosome G1-specific transcription in mitotic cell cycle chromatin remodeling amino acid biosynthetic process transcription	Basal transcription factors Phenylalanine, tyrosine and tryptophan biosynthesis Histidine metabolism
8 (21/104)	protein complex assembly aerobic respiration translation mitochondrion organization and biogenesis protein targeting to mitochondrion	Aminoacyl-tRNA biosynthesis Ubiquinone biosynthesis
9 (69/256)	energy reserve metabolic process regulation of glycogen catabolic process protein targeting to vacuole peroxisome organization and biogenesis trehalose metabolic process autophagy fluid transport sporulation mRNA polyadenylation	Starch and sucrose metabolism Regulation of autophagy Pentose and glucuronate interconversions
10 (18/90)	microtubule cytoskeleton organization and biogenesis conjugation nucleocytoplasmic transport	Ubiquitin mediated proteolysis
11 (41/71)	spore wall assembly monosaccharide transport telomere maintenance	
12 (22/82)	allantoin metabolic process heterocycle catabolic process cofactor catabolic process	
13 (38/210)	oxidative phosphorylation acetyl-CoA catabolic process tricarboxylic acid cycle ion transport fatty acid oxidation glutamate biosynthetic process proline catabolic process to glutamate response to reactive oxygen species	Oxidative phosphorylation Citrate cycle (TCA cycle)

14 (38/213)	ubiquitin-dependent protein catabolic process proteolysis vitamin metabolic process regulation of small GTPase mediated signal transduction glutathione metabolic process carnitine metabolic process	Proteasome Alanine and aspartate metabolism
15 (17/94)	metal ion homeostasis protein targeting to vacuole response to oxidative stress protein amino acid palmitoylation	
16 (21/136)	ubiquitin-dependent protein catabolic process proteolysis arginine biosynthetic process positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process gluconeogenesis monosaccharide biosynthetic process heteroduplex formation cell wall polysaccharide biosynthetic process (sensu Fungi)	Proteasome
17 (24/214)	ER to Golgi vesicle-mediated transport DNA synthesis during DNA repair DNA replication signal peptide processing nuclear pore organization and biogenesis tRNA transport regulation of S phase of mitotic cell cycle sterol biosynthetic process acetate biosynthetic process fatty acid elongation high affinity iron ion transport chromosome localization	
18 (93/372)	generation of precursor metabolites and energy coenzyme metabolic process autophagy vacuolar transport protein retention in Golgi pentose metabolic process response to stress peroxisome organization and biogenesis glycogen biosynthetic process fatty acid metabolic process vitamin metabolic process	Pentose phosphate pathway Citrate cycle (TCA cycle)
19 (21/112)	actin cytoskeleton organization and biogenesis bipolar bud site selection phosphate transport mitochondrion distribution filamentous growth cell communication	
20 (38/347)	ribosome biogenesis and assembly organelle organization and biogenesis rRNA metabolic process nuclear transport one-carbon compound metabolic process barrier septum formation DNA-dependent DNA replication gene silencing	RNA polymerase Pyrimidine metabolism Purine metabolism

	regulation of translational fidelity response to zinc ion nuclear organization and biogenesis transcription chromatin assembly protein targeting to membrane	
21 (39/335)	translation ribosome biogenesis and assembly organelle organization and biogenesis telomere organization and biogenesis mismatch repair sulfur amino acid metabolic process leading strand elongation cell organization and biogenesis cotranslational protein folding pentose-phosphate shunt nitrogen compound metabolic process chromosome organization and biogenesis RNA processing	Ribosome
22 (54/200)	protein catabolic process glucoside transport pyruvate metabolic process cell cycle arrest in response to pheromone vacuolar protein catabolic process MAPKKK cascade during cell wall biogenesis negative regulation of progression through cell cycle energy reserve metabolic process	Starch and sucrose metabolism
22 (54/200)	protein catabolic process glucoside transport pyruvate metabolic process cell cycle arrest in response to pheromone vacuolar protein catabolic process MAPKKK cascade during cell wall biogenesis negative regulation of progression through cell cycle energy reserve metabolic process	Starch and sucrose metabolism

Additional data file 3 – Figure_S1.pdf

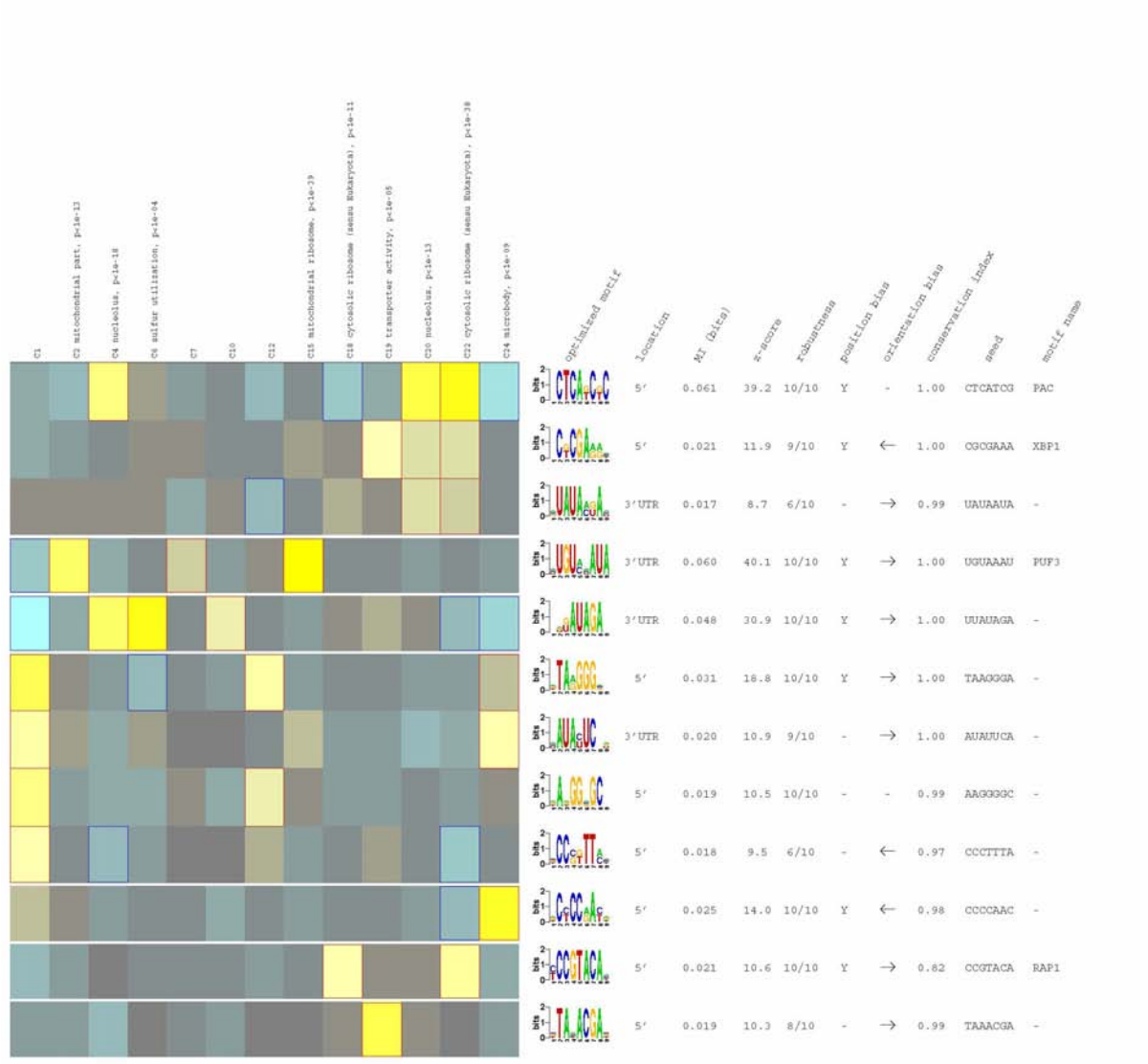


Figure S1. FIRE analysis for transcriptional regulatory motifs occurring in the clusters presented in Figure 2.

For each cluster, the most significant GO enrichments are shown at the top. Yellow indicates over-representation of a motif in a given cluster and significant ($p<0.05$) overrepresentation is highlighted with red frames. Similarly, blue blocks and blue frames indicate significant ($p<0.05$) under-representation. For each motif, the location (either 5' upstream or 3' UTR), mutual information (MI) value, Z score associated with the MI value, a robustness score ranging from 1/10 to 10/10, a position bias indicator ("Y" indicates position bias is observed), orientation bias indicator, conservation index, the seed that gave rise to the motif and name of the closest known motif are presented. For more details, see Elemento and co-workers 2007 (Elemento *et al.* 2007).

Additional data file 4 – Figure_S2.pdf

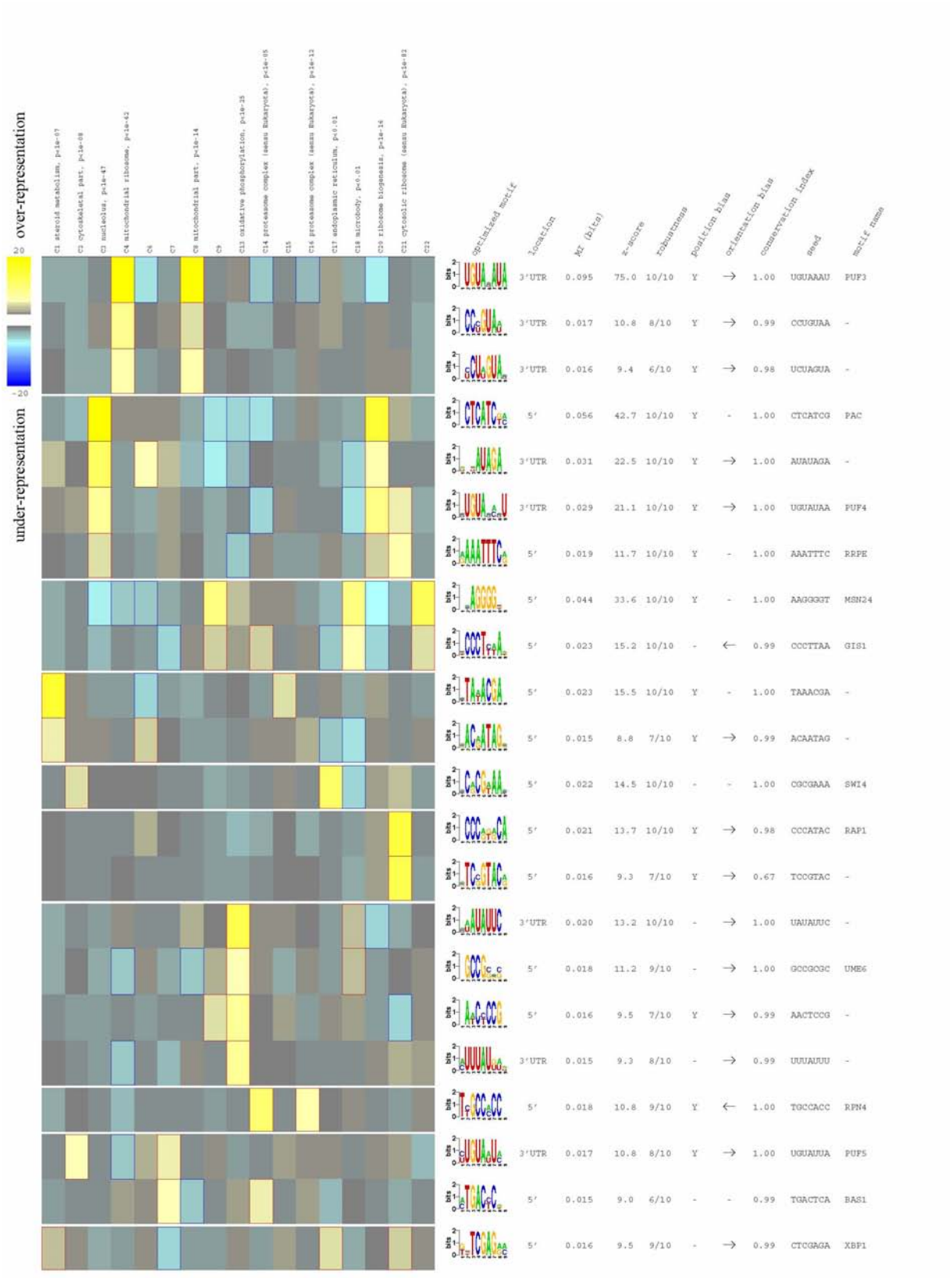


Figure S2. FIRE analysis for transcriptional regulatory motifs occurring in the clusters presented in Figure 3.

For each cluster, the most significant GO enrichments are shown at the top. Yellow indicates over-representation of a motif in a given cluster and significant ($p<0.05$) overrepresentation is highlighted with red frames. Similarly, blue blocks and blue frames indicate significant ($p<0.05$) under-representation. For each motif, the location (either 5' upstream or 3' UTR), mutual information (MI) value, Z score associated with the MI value, a robustness score ranging from 1/10 to 10/10, a position bias indicator ("Y" indicates position bias is observed), orientation bias indicator, conservation index, the seed that gave rise to the motif and name of the closest known motif are presented. For more details, see Elemento and co-workers 2007 (Elemento *et al.* 2007).

Additional data file 5 – Figure_S3.tif

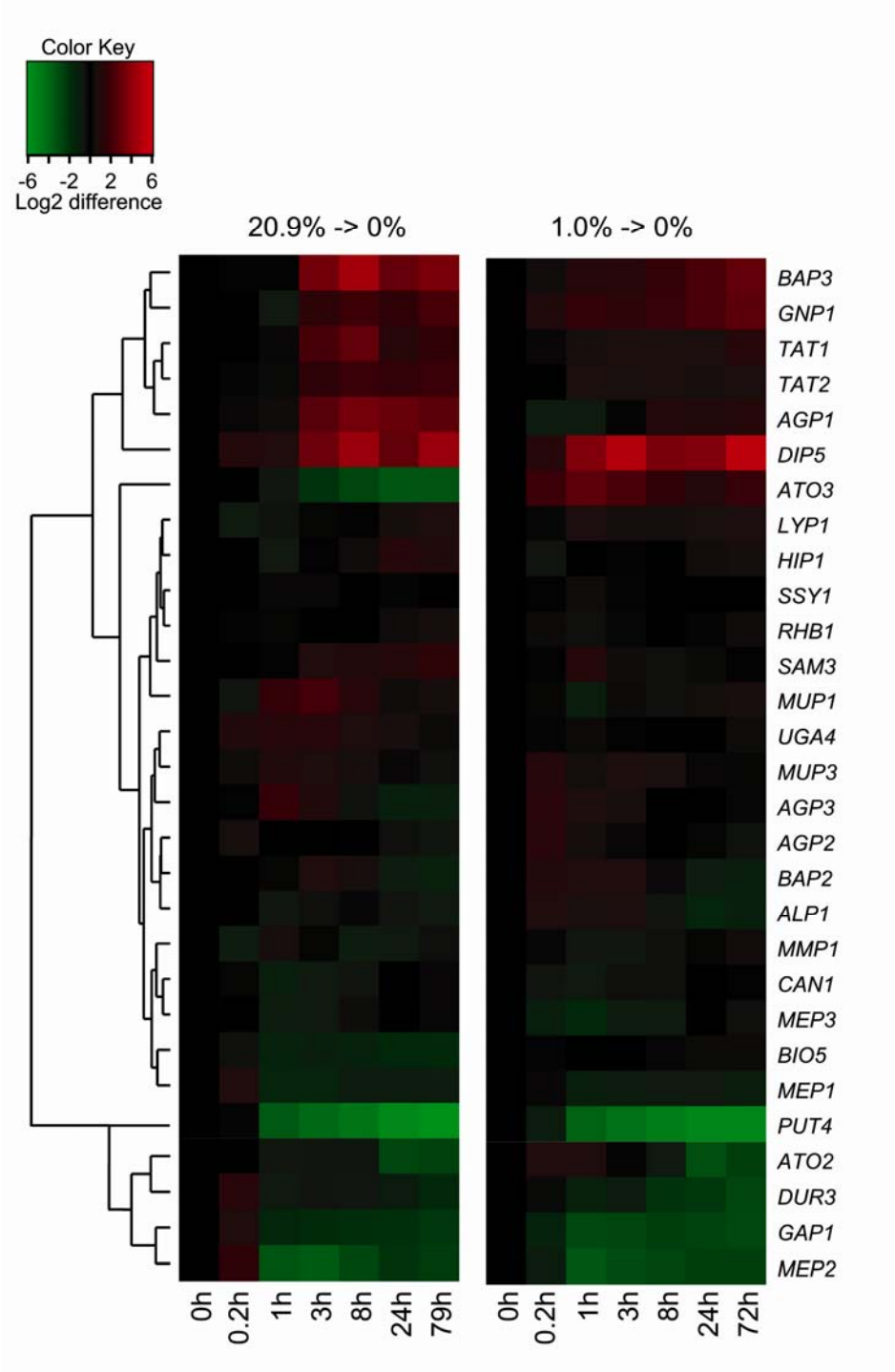


Figure S3. The heatmap of relative expression levels of genes encoding amino acid, ammonium and urea permeases. In the heatmap, green represents downregulation and red represents upregulation.

Series title, number and report code of publication

VTT Publications 747
VTT-PUBS-747

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.

ISBN
978-951-38-7413-1 (soft back ed.)
978-951-38-7414-8 (URL: http://www.vtt.fi/publications/index.jsp)

Series title and ISSN	Project number
VTT Publications 1235-0621 (soft back ed.) 1455-0849 (URL: http://www.vtt.fi/publications/index.jsp)	70174

Date	Language	Pages
November 2010	English, Finnish abstr.	82 p. + app. 93 p.

Name of project	Commissioned by

Keywords	Publisher
Saccharomyces cerevisiae, oxygen, transcriptome, proteome, hexose transporters, central carbon metabolism, trac, metabolites	VTT Technical Research Centre of Finland P. O. Box 1000, FI-02044 VTT, Finland Phone internat. +358 20 722 4520 Fax +358 20 722 4374

Julkaisun sarja, numero ja raporttikoodi

VTT Publications 747
VTT-PUBS-747

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.

ISBN
978-951-38-7413-1 (nid.)
978-951-38-7414-8 (URL: http://www.vtt.fi/publications/index.jsp)

Avainnimeke ja ISSN	Projektinumero
VTT Publications 1235-0621 (nid.) 1455-0849 (URL: http://www.vtt.fi/publications/index.jsp)	70174

Julkaisuaika	Kieli	Sivuja
Marraskuu 2010	Englanti, suom. tiiv.	82 s. + liitt. 93 s.

Projektin nimi	Toimeksiantaja(t)

Avainsanat	Julkaisija
Saccharomyces cerevisiae, oxygen, transcriptome, proteome, hexose transporters, central carbon metabolism, trac, metabolites	VTT PL 1000, 02044 VTT Puh. 020 722 4520 Faksi 020 722 4374

VTT PUBLICATIONS

729 Olavi Lehtoranta. Innovation, Collaboration in Innovation and the Growth Performance of Finnish Firms. 2010. 136 p. + app. 16 p.

730 Sami Koskinen. Sensor Data Fusion Based Estimation of Tyre-Road Friction to Enhance Collision Avoidance. 2010. 188 p. + app. 12 p.

731 Anja Oasmaa & Cordner Peacocke. Properties and fuel use of biomass-derived fast pyrolysis liquids. A guide. 2010. 76 p. + app. 46 p.

732 Venkata Gopalacharyulu Peddinti. Data integration, pathway analysis and mining for systems biology. Espoo 2010. 62 p. + app. 67 p.

733 Johanna Kirkinen. Greenhouse impact assessment of some combustible fuels with a dynamic life cycle approach. Espoo 2010. 63 p. + app. 58 p.

734 Antti Grönroos. Ultrasonically Enhanced Disintegration. Polymers, Sludge, and Contaminated Soil. 2010. 100 p. + app. 27 p.

735 Michael Lienemann. Characterisation and engineering of protein–carbohydrate interactions. Espoo 2010. 90 p. + app. 30 p.

736 Jukka-Pekka Pesola. Building Framework for Early Product Verification and Validation. Master Thesis. Espoo 2010. 75 p.

737 Virpi Oksman. The mobile phone: A medium in itself. Espoo 2010. 89 p. + app. 132 p.

738 Fusion Yearbook. Association EURATOM-TEKES. Annual Report 2009. Eds. by Seppo Karttunen & Markus Airila. 2010. 136 p. + app. 13 p.

739 Satu Hilditch. Identification of the fungal catabolic D-galacturonate pathway. Espoo 2010. 74 p. + app. 38 p.

740 Mikko Pihlatie. Stability of Ni-YSZ composites for solid oxide fuel cells during reduction and re-oxidation. Espoo 2010. 92 p. + app. 62 p.

741 Laxmana Rao Yetukuri. Bioinformatics approaches for the analysis of lipidomics data. Espoo 2010. 75 p. + app. 106 p.

742 Elina Mattila. Design and evaluation of a mobile phone diary for personal health management. Espoo 2010. 83 p. + app. 48 p.

743 Jaakko Paasi & Pasi Valkokari (eds.). Elucidating the fuzzy front end – Experiences from the INNORISK project. Espoo 2010. 161 p.

744 Marja Vilkman. Structural investigations and processing of electronically and protonically conducting polymers. 2010. 62 p. + app. 27 p.

746 Jarkko Kuusijärvi. Interactive visualization of quality Variability at run-time. Espoo 2010. 111 p.

747 Eija Rintala. Effects of oxygen provision on the physiology of baker's yeast *Saccharomyces cerevisiae*. Espoo 2010. VTT Publications 747. 82 p. + app. 93 p.

ISBN 978-951-38-7413-1 (soft back ed.)
ISSN 1235-0621 (soft back ed.)
ISBN 978-951-38-7414-8 (URL: http://www.vtt.fi/publications/index.jsp)
ISSN 1455-0849 (URL: http://www.vtt.fi/publications/index.jsp)