Physiological impact and context dependency of transcriptional responses: A chemostat study in *Saccharomyces cerevisiae*

PROEFSCHRIFT

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Chapter 1

General Introduction

Successful industrial fermentations are the resultant of many process parameters that have generally been optimized by trial and error. At the very centre of the fermentation process are the microorganisms, whose consistent performance is essential for the quality of the final product. In the past, efforts on 'what the cell does and how it does it' were mainly focused on biochemical and physiological aspects. In recent years, molecular genetics and genomics have become an integral and essential part of strain improvement programmes. One of the 'high-profile' techniques used in present day genomics is DNA microarray analysis, which allows for the high-information-density screening of the gene complement and transcriptional activity of microbial genomes.

'Googling' *Saccharomyces cerevisiae* (the Latin name for bakers' yeast) yields approximately 9 million hits. On top of the list is the *Saccharomyces* genome database (www.yeastgenome.org) (52). The complete yeast genome sequence has been available since 1996 (114) and consists of approximately 6300 open reading frames (ORFs) within 16 chromosomes. The number of ORFs is still a matter of debate and fluctuates from 5700 to 6300 genes (53, 161). With the availability of the genome sequence and with the help of advanced technologies for analysing gene function and gene expression (including transcriptome analysis via DNA microarrays), the proteins encoded by 4404 *S. cerevisiae* genes now have been assigned biochemical functions (June 2006), while 1376 ORFs still remain unclassified and as many as 824 ORFs are dubious.

1. *Saccharomyces cerevisiae*: past, present and future

In 1680, the Delft-based Dutch scientist Antonie van Leeuwenhoek used his homemade microscopes to become the first human to observe individual cells of *S. cerevisiae*. It was not until the second half of the 19th century that Pasteur, the famous French scientist, realized that these cells were actually living organisms (307).

S. cerevisiae plays an important role in the brewery and bakery industries, in which it is applied on a massive scale for food production. Throughout the ages, small households have been active as biotechnologists by brewing wine and beer, or making bread, in their own backyards. In this process, mankind's taste and expertise has selected for the best yeast for each application. In wine and beer fermentations, this is a somewhat fashion-dependent balance of taste, aroma and clarity of the final brew (183). In bread making, yeast with high fermentative capacities are favoured, but storage stability of the yeast and its contribution to dough (in)stability are also important parameters (22). However, modern day large-scale fermentations no longer can rely on rules of thumb, as these fermentations rely on industrial standards for product quality. New technologies and yeast strains are constantly being developed to predict and control for desirable fermentation outputs (352). With the recent boom in genetic engineering, yeast itself is being studied at the molecular level, in search of knowledge on what, to a significant extent, is still a black box. It should be stressed here that this application of molecular techniques is not always focused on the eventual genetic engineering of this food organism, which might meet with serious issues in terms of consumer acceptance. Instead, molecular techniques are used to rationally improve process conditions and to guide classical strain improvement programmes.

In its wine making, brewing and baking applications, *S. cerevisiae* is generally regarded as safe (GRAS). It has also a fairly simple nutritional requirement: it is able to grow on simple sugars like glucose or maltose and on ammonia or an amino acid (e.g. methionine or asparagine) as the sole nitrogen source (200). Its ease of use and its low propagation cost availability have made yeast one of the most prolific organisms to work with in the production of alcoholic beverages. In the down-stream processing, *S. cerevisiae* is easily separated from its broth via its inherent flocculation properties (294, 347), and its robustness allows for easy storage and recycling. However, nutritional and growth conditions in industrial fermentations are not always optimal. The nutritional value of the medium greatly depends on factors that are difficult to control. For instance, the nutritional value of wort (the substrate for beer fermentation) depends on the quality and pre-treatment of barley (244) while the ripeness of grapes is an important factor in determining growth and metabolic activity of wine yeasts (210, 220). Stuck fermentations or production of off flavours can be the consequence of suboptimal process conditions and may cause substantial economic losses. Application of novel analytical tools such as DNA microarrays may help us understand the physiological responses of yeasts to process conditions and thereby help to design robust processes and to select for strains with a stable performance. This subject is further discussed in chapter 1.3.

Rapid production of ethanol is not only central in the application of *S. cerevisiae* in classical food fermentations. Yeast-based ethanol production is also rapidly gaining attention as a means of producing fuel ethanol from renewable agricultural feedstocks. Consequently, *S. cerevisiae* has been intensively engineered to ferment alternative carbon sources such as the pentose sugars xylose and arabinose (179). The experimental accessibility and wealth of biochemical knowledge on this organism has also engaged many new lines of interest, mainly as a model eukaryotic organism for molecular and cell biology studies. Possessing a fully annotated genome, *S. cerevisiae* is an ideal model system for other eukaryotic systems. For example, yeast has been applied for identifying genes related to human diseases (44, 109, 367) and for unravelling gene functions in bacteria, flies, worms and humans (311). All in all, *S. cerevisiae* possesses prominent useful features: the ease and low cost of cultivation, short generation times, comprehensive genetic and biochemical data and its excellent accessibility to (molecular) genetic techniques for functional studies and metabolic engineering.

The future direction of yeast metabolic engineering is bright, with the current demand for better performing organisms to enable a switch from petrochemistry to industrial biotechnology. However, in the food industry the implementation of genetically modified organisms is still limited due to consumer-acceptance issues. There are presently some signs that public view may be slowly changing towards acceptation of the potential benefits of molecular science. A recent, interesting example is the recent market introduction of a genetically engineered malolactic wine yeast strain ML01 (143) that has obtained GRAS status from the FDA. Although engineered yeasts are only now (perhaps) gaining grounds in the food industry, there are already many commercialised genetically modified (GM) yeasts applied in the pharmaceutical and industrial bulk biochemical production ('white biotechnology'). *S. cerevisiae* has been used as a recombinant organism in heterologous protein production, e.g. Hepatitis B vaccine (27, 222), human insulin (61, 81), chymosin (223) and metabolically engineered for improved and novel product formation, e.g. glycerol (253), ethanol (178), lactate (341). In general, metabolic engineering methods have been applied for strain improvement strategies to include: (i) utilization of a broader range of substrates (ii) introduction of new product formation pathways (iii) reduction of by-product formation (iv) improvement of productivity or yield and/or (v) improvement of cellular properties such as resistance to stresses in the industrial environment (242).

1.1 *S. cerevisiae* meets transcriptomics

1.1.1 From genome to expression analysis

For *S. cerevisiae*, the 'post-genomic era' started in 1996. Since then, the study of the transcriptome (i.e., the full complement of messenger RNA's in the cell) has become the most accessible tool for genome-wide analysis in yeast (89). DNA microarrays have been developed to explore the genome in a high-information-density fashion. The original goal of DNA microarray technology (199, 287) was to shed light on

changes of expression on the global transcriptome level when a designated perturbation/stimulus or genetic intervention was implemented. Most microarray studies in yeast concentrate on analysing gene expression responses to for example a stress stimulus (49, 107, 165), in response to a change in culture condition over time (38, 129, 147, 251, 280) or in steady state conditions (137, 316) and in different nutritional sources (32, 70, 368).

Alternatively, microarrays can be used to identify sequence (dis)similarity between *S. cerevisiae* strains. In the study of Winzeler *et al.* (365), a comparison of 14 different yeast strains showed 11,115 single-feature polymorphisms (SFPs) compared to the sequenced strain S288C that was used as the template for the Affymetrix Genechip® platform. A study by Daran-Lapujade *et al.* (69) used the same platform to compare two laboratory strains of *S. cerevisiae*. The laboratory strain CEN.PK113-7D (the strain used in this PhD project) was shown to have as few as 288 (4.5 %) genes with lower hybridisation signal and as many as 25 genes called absent compared to the sequenced strain S288C. In more recent developments of microarrays, a system to detect all single-nucleotide differences between genomes has been developed from a single hybridisation to a whole-genome DNA (tiling) microarray (Chapter 1.1.2) (116).

In Delft, analytical tools have also moved beyond Van Leeuwenhoek's microscopes. In recent years, the Industrial Microbiology Section of the Delft University of Technology have developed a platform that combines the Affymetrix Genechip® technology for DNA-microarray analysis with chemostat fermentation of *S. cerevisiae* (Figure 1). The central approach consists of the design of sets of chemostat experiments that enable the researcher to 'isolate' the transcriptional responses to individual process parameters or genetic interventions. Changes of cultivation conditions can be explored either in steady-state cultures or during controlled perturbations, which is always relative to one or more reference conditions. The next step, which is at the heart of transcriptome analysis, is sample preparation and detection. Strict working conditions and good laboratory practice need to be maintained in order to achieve high reproducibility and data quality. For instance, the aid of the Agilent 2100 Bioanalyser ensures that each step taken in preparing the RNA for hybridisation is of the utmost quality. Before any biological meaning can be distilled from the microarray data, statistical analysis and data visualization/clustering is performed *in silico*. Although detailed laboratory protocols have been published, the computational tools necessary to analyse the data are constantly evolving and no definitive consensus exists as to the best method for revealing patterns of gene expression (268). Finally, by associating the co-expression of genes, microarrays allow for the identification of new targets for functional analysis, for unravelling transcriptome regulation (possible eventual use for diagnostic purposes) and for metabolic engineering strategies.

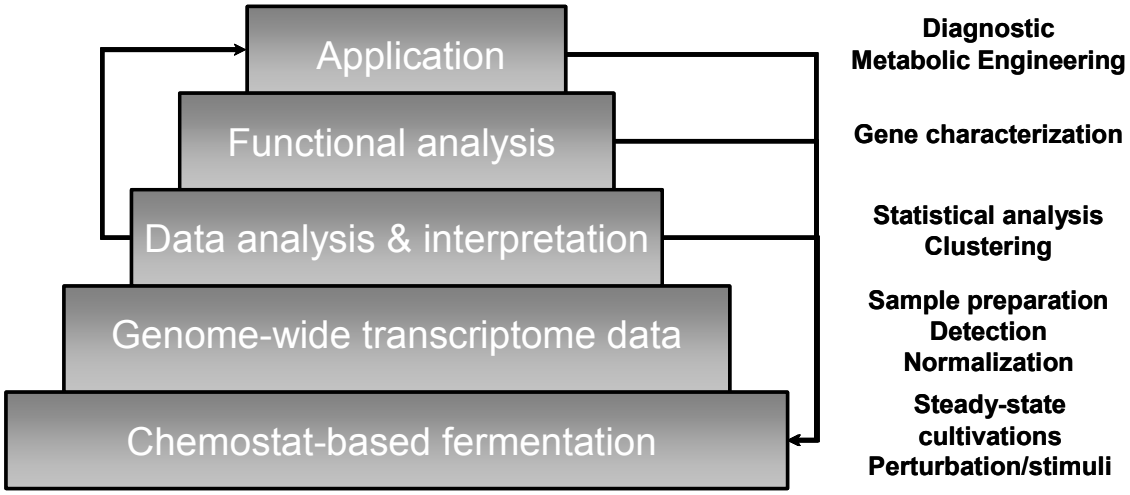

Figure 1: **Central approach for chemostat-based transcriptomics**

1.1.2 The Affymetrix Genechip® platform

There are three main types of microarray platforms that are most widely used and they can be distinguished by methods by which the probes have been placed on the array surface, the size of the array probes and how the samples are labelled for analysis. The first type uses short oligonucleotide probes that are synthesized directly on a glass surface (28, 98, 140, 159, 306). The Affymetrix Genechip®, which is used throughout this thesis work, is based on this technology. The technology behind the Genechip® relies on the hybridisation of non-colour-coded fluorescent-labelled fragmented RNA onto short unique 25-mer oligonucleotides probes attached directly to the gene chip. Each gene is generally represented by a probe set of 16 distinct 25-mer, perfectly matching oligonucleotide. This feature is amplified with another 16 imperfect matches, which has the 13th oligonucleotide mismatches to the perfect matched oligonucleotide. By these standards, the Genechip® is able to distinguish any cross-hybridisation due of sequence similarities of the probe design and selects strongly for the amplification of the selected gene alone.

The other types of microarray platforms are based on spotted long oligonucleotide and cDNA microarrays, in which the probe synthesis is separate from the array manufacture (93, 140, 162, 288). These microarrays rely on hybridising long cDNA probes (500-5000 bases long) or oligonucleotides (50-80 bases long) with two differentially labelled mRNA samples (labelling is generally based on the fluorescent dyes Cy3 and Cy5). In cDNA microarrays, these long probes have as a disadvantage that specific hybridisation becomes more difficult because of increased possibilities of cross-hybridisation. Because of these many disadvantages (Table 1), long oligonucleotide platforms will probably replace cDNA microarrays in the near future. Along side, long oligonucleotide platforms are becoming more popular for the

improved sensitivity and flexibility of the probe array design (15, 140, 159, 260, 375). Table 1 lists the pros and cons of these three different types of microarrays.

Other types of microarray platforms may have a combination of the technologies across these platforms. For instance, Applied Biosystems uses long oligonucleotide probes but with a non-colour-coded fluorescent-labelling method (chemiluminescence). This platform has the combinatorial benefits of the above microarrays but the application span is still limited to only few organisms (305, 353).

Table 1: Technical concerns over microarrays (adapted from (306))

Short oligonucleotide microarrays e.g. Affymetrix Genechip®	***Advantages*** • Synthesis of oligonucleotides directly onto glass surface • High-density spotting (1 million spots/cm^2) • Short probes enhances reproducibility, measurements of absolute expression levels and detection of SNPs • Distinguish splice variants and members of gene families • Multiple probes to each gene • Comparison to multiple sample conditions by fluorescent dye staining method ***Disadvantages*** • Requires accurate sequence databases • Costly arrays and materials
cDNA microarrays e.g. Incyte UniGEM V Long oligonucleotide microarrays e.g. Operon, Agilent	***Advantages*** • Cheap operating cost • Flexible design of array: custom made arrays • Ease of manufacture of array • Strong binding of probes (long oligonucleotide) ***Disadvantages*** • Probe generation separate from array synthesis: incorrect spotting • Only ~10,000 spots/cm^2 (cDNA arrays) • Long cDNA allows cross-hybridisation (cDNA arrays) • Unable to distinguish splice variants and member of gene families (cDNA arrays) • Choice of reference is crucial for data interpretation • Limited to pair-wise dye-swap analysis

Conventional dye-swap (Cy3/Cy5) microarrays also have limited use as it has the capability of comparing only two conditions in each experimental context. A comparison with three or more conditions becomes cumbersome and costly as each experiment will need to be compared to one another (36). This inherently reduces the richness of the data obtained and limits the potential of microarrays. The Genechip® on the other hand is a photolithographic microarray which is based on fluorescently labelled nucleic acids. Each experiment is treated individually with each expressed gene assigned with a signal intensity that can be converted to numerical values via the Affymetrix software GCOS (GeneChip® Operating Software). This method of scanning allows for greater mobility of data, i.e. any one array can be compared to any other. This diverges the traditional pair-wise comparisons to allow for more

dynamic analyses like time-course, parallel-comparison or multi-dimensional experiments.

A recent further development of microarrays involves better detection of transcriptional changes. A new generation of microarrays has been developed with the whole genome sequence on an array (72, 116). This oligonucleotide array for *S. cerevisiae* contains 6.5 million probes and interrogates both strands of the full genomic sequence with 25-mer probes tiled at an average of eight nucleotide intervals on each strand. These arrays have already been used to identify all single-nucleotide differences between genomes of two *S. cerevisiae* strains (116). More interestingly, because RNA expression on both strands of the complete genome is studied, this enabled the identification of the boundary, structure and level of coding and non-coding transcripts. Apart from the expected transcripts already known, new operon-like transcripts, transcripts from neighbouring genes not separated by intergenic regions, and genes with complex transcriptional architecture where different parts of the same gene are expressed at different levels were discovered (72, 116).

1.2 Transcriptomics meet chemostats

Many of the transcriptome data available for *S. cerevisiae* have been obtained in conventional shake-flask cultures. In such cultures, conditions are by necessity dynamic (e.g. shake flask) (262). In a dynamic fermentation, many culture and environmental parameters change over time. This means that the time a sample is taken for transcriptome analysis or for any other purposes is critical in comparisons between different environmental conditions or between different mutants. Figure 2 shows the many advantages of using chemostats for transcriptome studies. First of all, chemostat-based fermentations are tightly defined and controlled steady states. In this manner, changes in transcriptional responses caused from a single stimuli/perturbation (e.g. oxygen availability, nutrient-limitation or temperature effects) can be singled out and studied while keeping the other parameters constant (e.g. pH, stirrer speed, non-limiting nutrients, etc.).

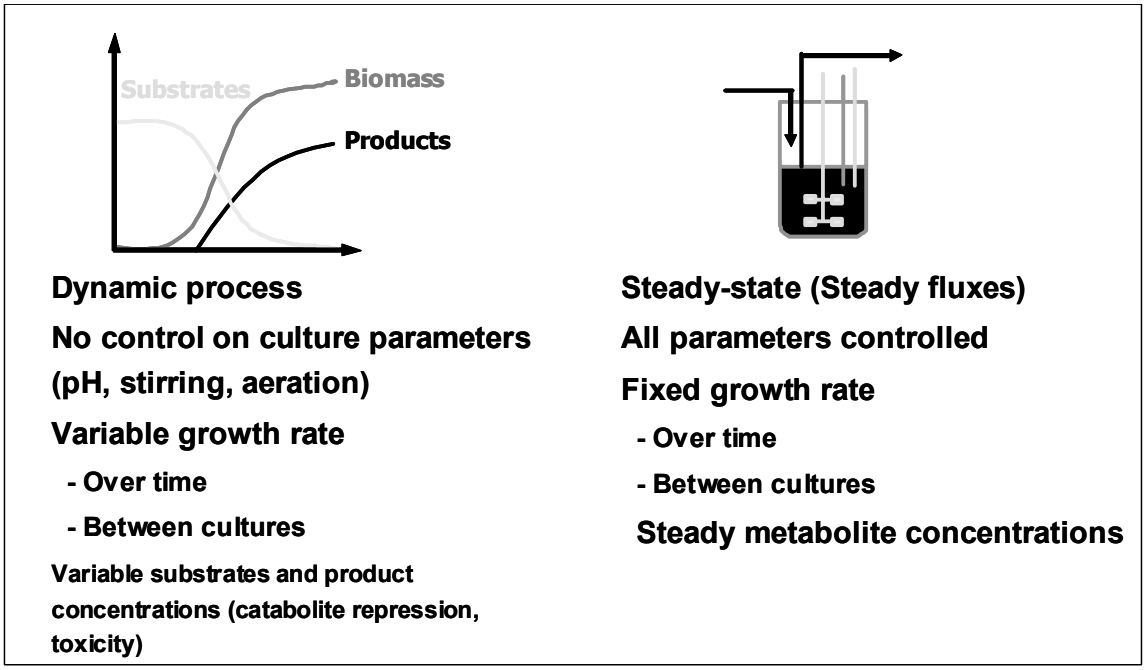

Figure 2: **Advantages of chemostats over batch cultures in transcriptome analysis**

In chemostat cultivations, growth conditions and metabolic activity of the microorganism can, in principle, be kept constant in time. An important limitation to this rule is that prolonged cultivation can lead to evolutionary changes (42, 148, 359). Such evolutionary changes might tremendously complicate the interpretation of microarray data. Hence, sampling for RNA should always be done on relatively 'young' chemostat cultures. In the Delft lab, sampling is done between 10-14 generations after starting the continuous medium feed. Another key issue with dynamic fermentations is the variable growth rates that occur during these processes. For example, a reduced temperature will lead to a reduced specific growth rate relative to the reference culture. Since specific growth rate does itself have an important impact on the transcriptome (187), this makes it difficult to discriminate between direct and indirect effects of process or genetic interventions. In chemostats, the specific growth rate (μ h^{-1}) is governed by the dilution rate (D, h^{-1}) of the chemostat, which can be easily manipulated. Hence, the specific growth rate of a chemostat culture can be controlled and does not vary over time and between different culture conditions.

The strict control of growth conditions in chemostats provides an ideal platform for reproducible transcriptome analysis (262). The combined use of the Genechip® and chemostats has contributed to an increase of data exchange between laboratories, because the low variations on data acquisition allowed inter laboratory comparisons without indications for significant laboratory bias (262). Current trends of study on the global level spanning from transcriptomics to proteomics to metabolomics has also called for a more reproducible, reliable and biologically homogeneous datasets (138, 139). Measurements in particular on the protein and metabolite levels require precision analysis and highly reproducible settings (219). Especially in the field of 'systems biology', which requires high-quality

and high-information-density analysis at different information levels ('omes'), chemostat cultivation is now strongly in the ascendant (71, 167, 172, 263).

1.3 Applications of microarrays

1.3.1 As a diagnostic tool in industrial fermentation

The performance of industrial fermentation processes can be influenced by a single parameter but, in practice, performance is likely to be the net result of a combination of many process parameters. Process parameters such as temperature, pH and dissolved oxygen concentration may be controlled easily with currently available tools for automated biochemical process control. However, this may still pose a problem when perturbations are localized in a large fermentor. Changes in the availability of nutrients may be even more difficult to control. Especially when complex fermentation media such as wort, must or molasses are used, it is not always evident from simple analyses which nutrient is limiting growth or which chemical compound in the complex medium has a (negative) effect on process performance and/or end-product quality. This is especially relevant in beer and wine fermentation, where the formation of minute amounts of metabolic by-products (e.g. sulphur-containing off flavours formed from the amino acids cysteine and methionine, (154) can have a tremendous impact of product quality). With the knowledge that yeast adjusts their transcriptional activity in response to their environment, measurements of global transcriptional activity may provide an information-rich and sensitive analytical tool to diagnose the quality of the yeast cell's industrial environment (Figure 3).

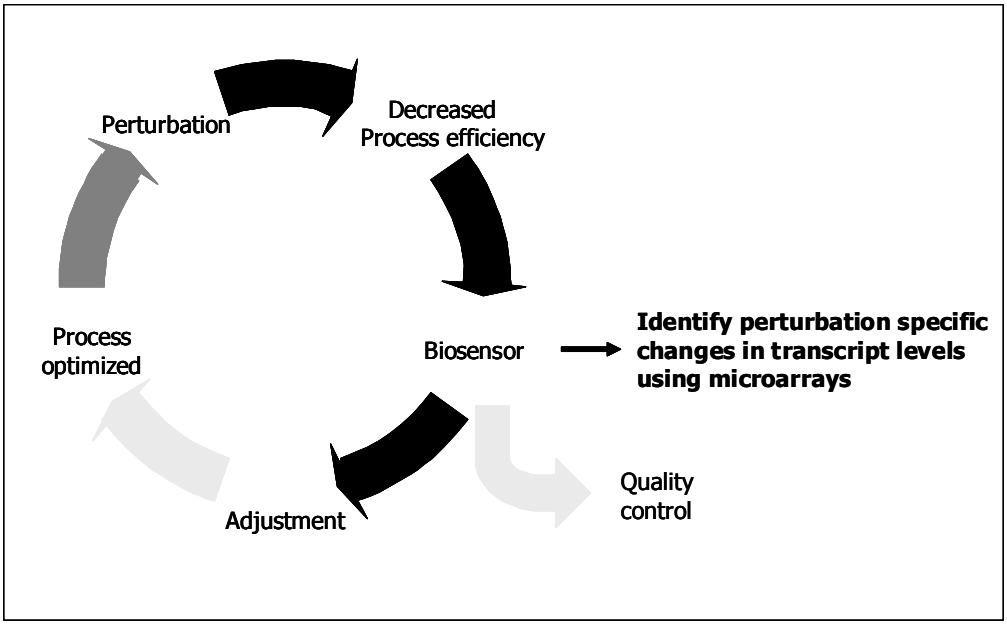

Figure 3: **Microarrays as a tool for process diagnostics in yeast-based fermentation processes.** A source of perturbation can be easily be identified and adjusted by analysing changes in transcript levels on a custom-made microarray with 'indicator genes'.

Routine use of microarrays in industrial fermentations for process optimisation has, to my knowledge, not yet been realized. Several reasons may contribute to this rather slow introduction of microarray technology in industrial environments. To start with, the processing of RNA and hybridisation procedures at present takes rather long (approximately 2-3 days for the Affymetrix platform). Furthermore, in order to utilize microarrays to identify various perturbations that can possibly decrease process efficiency, first and foremost, indicator or signature genes should be identified. Signature genes are genes that are consistently up- or down-regulated in response to a specific condition, regardless of the experimental background against which this response is recorded. Only when this information is available, cost effective custom arrays can be developed for routine process diagnostic purposes. This is in line with similar practices in the medical field where custom arrays are used for diagnostics of patients with diseases or for evaluation of pharmacological efficacy (198, 291, 298).

1.3.2 Functional genomics: giving the genome's parts a biological meaning

Since the biochemical function of a large fraction of the yeast genes (approximately 21 %) still remains unclassified, functional genomics remains one of the most exciting fields to explore. Functional genomics aspires to answer basic questions such as when is a gene expressed, where its product is localized and how gene products interact to produce a complex living system. There are several approaches in functional genomics, which the five core applications in yeast include (i) genome-wide knock-outs (ii) genetic mapping studies (iii) gene expression studies (iv) protein structure studies and (v) protein interaction studies. Of all these, the most readily accessible, high-throughput and advanced in technology is gene expression profiling with microarray assays.

Assigning gene function from the expression of mRNA levels is rather indirect. Most mRNA are not functional themselves; they are intermediates and transmitters of information from the genome to the proteome (306). The assumption that the expression of mRNA leads to a functional protein or is the reflection of the *in vivo* flux through a metabolic pathway (70) is not always true. Here, the limitation of microarrays needs to be realized to prevent meaningless speculation. If a function is to be annotated to a gene based on gene expression data, the following assumptions take place; that (i) a gene is expressed because it is required, (ii) no post-translational changes occur that may reflect the abundance of mRNA and (iii) each gene is transcribed independent of another and there is no competition for resources (306). More frequently, the combination of two or more of the above mentioned approaches in functional genomics are essential to fully functionalise a coding gene.

Microarrays have been used with other genome-wide techniques like random transposon mutagenesis to identify previously non-annotated genes in yeast (175, 252). In the cited study, the authors described the discovery of 137 genes that were excluded previously because they were either a protein of <100 amino acids or because the ORF by which they were encoded was wholly contained within another larger ORF. RNA expression data have also been used in combination with the yeast deletion library. Here, single deletion mutants were grown competitively and their

fitness was scored proportional with the expression of its gene under the experimental context (26, 112). These studies concluded that only a small percentage of genes that exhibit a significant increase in expression would also exhibit a significant fitness defect when knocked out.

1.3.3 Gene control and regulation: key for metabolic engineering

The control of gene expression for the production of biologically active proteins in eukaryotic cells is more complex than in prokaryotic cells. A major difference is the presence of a nuclear membrane in eukaryotes, which prevents the simultaneous transcription and translation that occurs in prokaryotes. This compartmentalization of proteins leads to control of gene expression at several information levels, which are listed in Table 2. Of all these controls, mRNA transcription is still generally considered to be the most important mode of regulation for gene control. In a growing yeast culture, transcription factors are normally deployed to either up-regulate or repress the transcription of genes that share common function and/or promoter sequence elements in the upstream regions of these genes (190, 196). In terms of metabolic engineering, it is not always clear which genes are involved in the regulation of fluxes in the pathway.

Table 2: Gene control in *S. cerevisiae* (adapted from http://web.indstate.edu/thcme/mwking/gene-regulation.html)

Level of interaction	***Description***
Chromatin structure	DNA compacted to chromatin affects the ability of transcriptional factors and RNA polymerase to bind and initiate transcription. Presence of histones effects accessibility.
mRNA transcription	Most important control. Influenced by the strength of promoter elements and the presence or absence of enhancer sequences (by binding specific transcription factors), and the interaction between multiple activator proteins and inhibitor proteins.
Transcript processing and modification	Capping and polyadenylating of mRNA and removal of introns.
RNA transport	mRNA must leave the nucleus in order to be translated into protein.
Transcript stability	mRNA can vary greatly in their stability. Unstable transcripts have sequences predominately, in the 3'-non-translated regions for rapid degradation.
Translation initiation	The ability of ribosomes to recognize and initiate synthesis from the correct AUG codon can affect the expression of a gene product.
Post-translational modification	Modifications include glycosylation, acetylation, fatty acylation, disulfide bond formations.
Protein transport	Proteins must be transported to their site of action to be biologically active.
Control of protein stability	Many proteins are rapidly degraded, whereas others are highly stable. Specific amino acid sequences in some proteins have been shown to bring about rapid degradation.

In modern molecular genetics, genetic intervention (metabolic engineering) is a strategy for changing fluxes through metabolic pathways. In practice, it is often exceedingly difficult to assess which (often multiple) enzymes control the flux. Consequently, it is often not possible to predict which (combination of) enzyme(s) needs to be overexpressed to achieve an increase of the flux. Indeed, in many cases, overproduction of individual enzymes is a fruitless strategy, as has been convincingly demonstrated in painstaking research (241, 285) on the glycolytic pathway. In these cases, because the link is not directly related to the pathway, engineering of the cell via an empirical approach is often too time consuming.

In some cases, reverse metabolic engineering may provide a solution. In inverse engineering, the genotype of strains with interesting properties is studied to identify the molecular basis for an industrially relevant trait. Subsequently, the insight derived from such studies is applied in knowledge-based metabolic engineering. For example, the genome-wide expression profile of an optimised production strain may be compared to a parent strain. These transcriptional changes are then implemented back on the parent strain to see if the gain observed in the optimised strain can be reconstructed (284). Genome-wide protein-DNA binding interaction studies (known as location analysis) (121, 190) have also been used to interpret the genome's regulatory code by binding tagged transcriptional regulators to promoter elements. By studying these interactions, complex network of global transcriptional response can be mapped and hence identify potential targets for metabolic engineering.

In industry, optimised production strains have traditionally been obtained via years of selection. Changes in the genome are often random and may involve point mutations or changes in expression profiles of a group of genes. Currently, commercially available microarrays nonetheless have limited use in the analysis of point mutations in the genome, as these changes need to be on the region of the designed probes on the chip. The new generation of 'tiled' arrays that incorporate the whole genome sequence are likely to be a much more useful tool for such research (116). For now, microarrays serve as better tools for identifying changes of gene expression levels when comparing different strains. Because of the complexity of transcriptional regulation, one may discover an unlikely link that will influence the production of a certain pathway. Very often though, the knowledge of transcriptome data alone does not suffice for a successful implementation of metabolic engineering on a pathway. As listed in Table 2, the many possible gene control mechanisms both on the DNA and protein levels may have considerable effects on the regulation of fluxes in a pathway. For example, fluxes may be controlled by (by)product feedback inhibition/activation. Therefore, the integration of different 'omics' studies will eventually be needed to provide a basis for an effective, knowledge-based metabolic reprogramming of a cell.

1.4 Beer fermentation

Barley, water, hops and yeast are the four ingredients needed to make beer. Before the fermentation step that converts sugars to alcohol, barley is first malted to break starch to more readily fermentable sugars (i.e. maltose, maltotriose etc). This malted barley is then milled (Figure 4) and steeped into hot water to produce a sweet and thick liquid called wort. The wort is boiled with hops in a brew kettle to give beer its characteristic bitterness and then yeast is introduced to convert the sugars to alcohol in a fermentation tank. The fermentation process is normally carried out at low temperature. In lager brewing the temperature is between 10 and 15 ^{o}C. After fermentation, the beer goes through various processes of carbonation, filtration and packaging. The quality of beer through this process is influenced by many factors. The flavour and aroma for instance are greatly dependent on the composition of wort, type of hops, characteristics of the brewing strain and the consistency of the fermentation process.

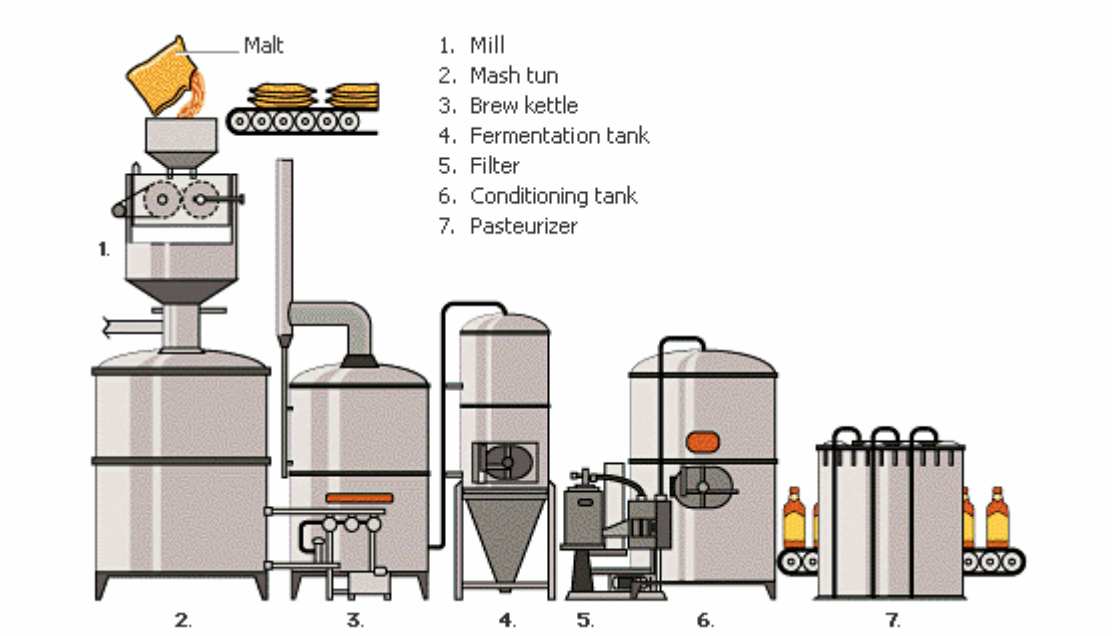

Figure 4: **Typical beer processing flowsheet.** Picture from http://encarta.msn.com/

The composition of wort is generally low in free amino nitrogen (FAN) levels. In cases when FAN levels are dramatically low, a variety of fermentation problems including slow or incomplete fermentations have been reported (39, 95). The amino acid pool in wort is normally the limiting factor for growth. Carbon sources on the other hand are abundantly available in wort. However, not all sugars may be used for fermentation, e.g. dextrins (39), which constitute about 20 % of all sugars in wort, cannot be fermented by yeast. Likewise, the amino acid proline is also not utilized because of the absence of oxygen during the major part of a beer fermentation (320). Although beer fermentations are generally aerated in the beginning of the fermentation to enable the synthesis of sterols and unsaturated fatty acids, the quality of the final product is many determined in anaerobic fermentation thereafter.

The alcohol yield during the fermentation process is also enhanced by the low temperature culture condition. At lower temperatures, yeast growth is decreased and the duration of alcoholic fermentation is maximized (328). At lower temperatures, fewer higher alcohols and greater proportions of acetate and ethyl esters are also produced resulting in the formation of less off-flavour compounds (327).

The two main types of beer, lager and ale, are fermented by strains of *Saccharomyces carlsbergensis* and *Saccharomyces cerevisiae*, respectively (307). The production of lager (bottom fermenting yeast) is the main beer produced by the major brewing industries. Brewing yeast are often tetraploid and consist of a hybrid of pure and mixed genetic lines of the *Saccharomyces* genus, mainly *S. bayanus*, *S. uvarum, S. pastorianus* and *S. cerevisiae* (270). However, because of the unavailability of genetic resources and the ambiguous taxonomic definition of lager yeast, *S. cerevisiae* has generally been utilized as a model for molecular studies on brewing-related subjects. It has recently been reported though that the sequencing of a lager strain has been completed and the DNA microarrays have been made – although, unfortunately, they are not available for researchers outside the company that commissioned the DNA sequencing (238). In the meantime, many studies on brewer's yeast using *S. cerevisiae* as a model have proven to be a valuable reference and resource in the investigation of the impact of fermentation conditions on yeast performance (38, 129, 147, 251). In the present study, chemostat-based transcriptomics was used to investigate genome-wide gene expression profiles to establish how *S. cerevisiae* responds to several process parameters relevant to beer fermentation as well as to other industrial fermentation processes.

The scope of this thesis

The PhD project described within this thesis is a part of a larger initiative to assess the applicability of chemostat-based microarray analysis for industrial and academic yeast research (Figure 5). Central to this research is a database of chemostat-derived transcriptome experiments that is meant to serve as a tool for understanding the behaviour of *S. cerevisiae* in particular for diagnostic, gene regulation, metabolic engineering and functional genomics purposes. In addition, this platform may provide a basis for the development of new custom arrays that consist of indicator genes that respond towards process parameters that are of immediate industrial relevance. In this thesis alone, four main issues relevant to large-scale yeast fermentation were investigated.

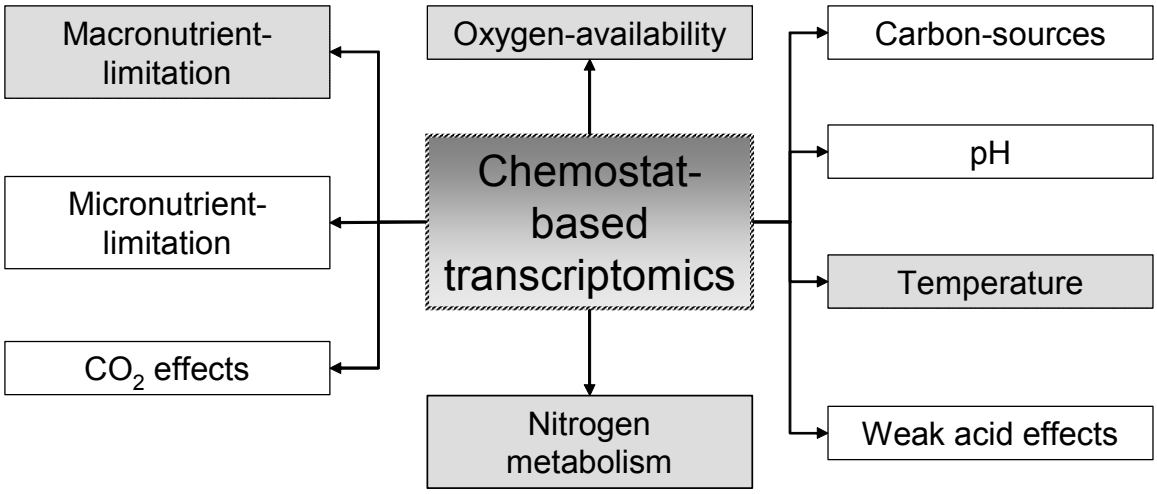

Figure 5: **Chemostat-based transcriptomics at TU Delft, Industrial Microbiology.** The categories shown are areas that have been studied so far. Grey boxes indicate are subjects addressed in this thesis.

In Chapter 2, we set out to investigate combinatorial effects of macronutrient limitation (carbon, nitrogen, phosphorus and sulfur) and oxygen availability (aerobic versus anaerobic) on transcription. Previously, the impact of macronutrient limitation on transcript profiles had already been studied under aerobic growth conditions (32). The main question in this Chapter was to what extent the transcriptional response to oxygen availability is influenced by the growth-limiting nutrient and vice versa. This question is relevant for industrial application as it determines to which extent web-based transcriptome databases can be applied to analyse transcriptional responses in industrial fermentations – which use completely different “background” conditions. From a fundamental science point of view, dissecting the combinatorial responses to different parameters can contribute to unravelling transcriptional regulation networks and guide functional analysis.

In Chapter 3, the signature transcripts that were consistently up-regulated under anaerobiosis (irrespective of the macronutrient limitation) were further investigated. Here the objective was to investigate the significance of transcript

profiles for directing functional analysis. In a more general sense, this chapter addresses the question whether, under a given experimental condition, increased transcript levels can be used to infer biochemical function of the corresponding gene products. To address this question, a set of the anaerobically up-regulated genes was deleted in tagged mutant strains. These mutants were then grown simultaneously in competitive anaerobic chemostat cultures and the fitness of each deletion strain was compared to a reference strain over a period of ten days. qPCR was used to track the concentration of DNA at each time point.

In laboratory experiments, the synthetic media are often based on a very limited set of preferred model substrates. For instance, ammonium salts are generally used as the sole nitrogen source and sulfate as the sole sulfur source. However, much industrial fermentation rely on organic substances such amino acids as the nitrogen and sulfur source. The question would then be how would the transcriptional response of these cultures be if an amino acid were supplied as the sole nitrogen source? Since amino acids are catabolized differently in pathways and in preference, we were interested in underlying a basis for understanding gene expression and control in these cultures. As part of the Delft transcriptome database project, Chapter 4 further explores the metabolism of a number of amino acids as the sole nitrogen source. Aerobic glucose-limited chemostat was used to examine transcriptional responses when ammonium, L-asparagine, L-phenylalanine, L-leucine, L-methionine or L-proline was supplied as the sole nitrogen source. Transcriptional regulation and identification of signature transcripts were key issues in this chapter.

Chapter 5 tackles transcriptional and physiological responses of *S. cerevisiae* to low temperature in chemostat cultivations limited for carbon and nitrogen. Low temperature is known to have drastic effects on the cell's homeostasis for instance in reduction of maximum specific growth rates, slower protein folding, RNA stabilization and increased viscosity of cellular fluidity. Here we discuss the differences of low temperature gene expression at 12 ^{o}C compared to 30 ^{o}C to the respective limitations and also regardless of the limitation in the hope to paint a clearer picture from the transcriptome responses on temperature effects. We also compared our results to other studies that have either been studied in a dynamic culture or as a cold-shock treatment.

As temperature change is a very profound change, it directly affects enzyme kinetics. In Chapter 6 we investigate how *S. cerevisiae* copes with low temperature effects on the *in vivo* fluxes through the glycolytic pathway. Chemostat cultures of glucose limitation grown at 12 ^{o}C were compared to cultures grown at the standard growth temperature of 30 ^{o}C. After exploring the transcriptional responses, focus was moved to the glycolytic *in vitro* enzyme activities, sugar transport and intracellular metabolite levels. Via this integrated 'systems' approach, we investigated at which levels the yeast controls glycolytic flux at different growth temperatures.

Chapter 2

Two-dimensional transcriptome analysis in chemostat cultures: combinatorial effects of oxygen availability and macronutrient limitation in *Saccharomyces cerevisiae*

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Abstract

Genome-wide analysis of transcriptional regulation is generally studied by determining sets of 'signature transcripts' that are up- or down-regulated relative to a reference situation when a single culture parameter or genetic modification is changed. This approach is especially relevant for defining small subsets of transcripts for use in high-throughput, cost-effective diagnostic analyses. However, this approach may overlook the simultaneous control of transcription by more than one environmental parameter. This study represents the first quantitative assessment of the impact of transcriptional cross regulation by different environmental parameters. As a model, we compared the response of aerobic as well as anaerobic chemostat cultures of the yeast *Saccharomyces cerevisiae* to growth limitation by four different macronutrients (carbon, nitrogen, phosphorus and sulfur). The identity of the growth-limiting nutrient was shown to have a strong impact on the sets of transcripts that responded to oxygen availability and vice versa. We concluded that identification of reliable signature transcripts for specific environmental parameters can only be obtained by combining transcriptome datasets obtained under several sets of reference conditions. Furthermore, the 2-dimensional approach to transcriptome analysis is a valuable new tool to study the interaction of different transcriptional regulation systems.

Introduction

Recent rapid developments in DNA-microarray technology have had a strong impact in research on the yeast *Saccharomyces cerevisiae*, an important industrial microorganism and model eukaryote. With the ability to study genome-wide transcriptome expression in a single microarray, large on-line transcriptome databases obtained from different mutants and under a wide range of cultivation conditions have become available as research tools (Gene Expression Omnibus http://www.ncbi.nlm.nih.gov/geo/ (86), Yeast Microarray Global Viewer (yMGV) http://www.transcriptome.ens.fr/ymgv/ (193)) .

Transcript profiles contain a wealth of information that may be applied in several ways for fundamental and applied research. When clear correlations are established between cultivation conditions and transcription of subsets of genes, such correlations can be used to guide functional-analysis studies on genes with as yet unknown biological functions. Furthermore, correlation of expression data with sequences of upstream regulatory elements can be applied to unravel the intricate networks of transcriptional regulation (371). In industrial biotechnology, one of the key applications of DNA-microarrays lies in diagnosing industrial fermentation processes. If transcriptional responses can be directly correlated to important parameters such as nutritional status of industrial microorganisms, or to the stresses to which they are exposed in industrial processes, transcriptome analysis can provide invaluable

information for process optimization (32, 130). For such diagnostic purposes, it would be preferable to construct small, cost-effective microarrays that contain a limited number of 'signature transcripts'. Such signature transcripts should respond uniquely to a single chemical or physical parameter that is relevant for the industrial process under study. This approach is analogous to the application of small diagnostic arrays used in clinical research for the rapid typing of tumors (291).

Hitherto, most transcriptome studies with *S. cerevisiae* have been done in shake-flask cultures (76, 107). In such cultures, it is not possible to control a number of important cultivation conditions (dissolved oxygen concentration, metabolite concentrations, pH, etc). Therefore, shake-flask cultivation by definition involves a continuously changing environment. Consequently, interpretation of transcriptome data from shake-flask cultivation is likely to be complicated by differences in specific growth rate, carbon catabolite repression, nitrogen catabolite repression, product accumulation, acidification, etc.

Chemostat cultivation offers a number of advantages for studies with DNA microarrays because it enables cultivation of microorganisms under tightly defined environmental conditions. In a chemostat, culture broth (including biomass) is continuously replaced by fresh medium, at a fixed and accurately determined dilution rate (D, h^{-1}). When the dilution rate is lower than the maximum specific growth rate of the microorganism (μ_{max}, h^{-1}), a steady-state situation will be established in which the specific growth rate equals the dilution rate ($\mu = D$). In such a steady-state chemostat culture, μ is controlled by the (low) residual concentration of a single growth-limiting nutrient. The option to accurately control and manipulate individual culture parameters (including medium composition, nature of the growth-limiting nutrient, pH, temperature and μ) under steady-state conditions makes chemostats excellent tools for studies on genome-wide transcriptional regulation. Indeed, a recent interlaboratory comparison of transcriptome data obtained in chemostat cultures demonstrated that the accuracy and reproducibility of this approach were superior to those obtained in previous studies with shake-flask cultures (262).

Chemostat cultures have recently been applied to study genome-wide transcriptional responses of *S. cerevisiae* to carbon-limited growth on different carbon sources (70), nutrient limitation for carbon, nitrogen, phosphorus and sulfur (32) and starvation (368), to the presence and absence of oxygen (262, 316) and oxidative stress responses (165). In each of these studies, it was attempted to vary a single cultivation parameter, while keeping all other parameters constant. This 'one-dimensional' approach resulted in sets of 'signature transcripts' that, within the experimental context, responded uniquely to a single cultivation parameter (e.g., uniquely up-regulated under nitrogen limitation, uniquely down-regulated during growth on ethanol). There is an important potential pitfall in this approach, as it does not include the possibility that expression of genes is simultaneously controlled by two or more environmental parameters. Such dual or multiple control would complicate the

identification of 'signature transcripts' and the interpretation of diagnostic transcriptome analysis.

So far, there have been no dedicated studies to investigate and quantify the way in which different transcriptional regulation responses overlap and interact. The goal of this study was to study this interaction by analyzing genome-wide transcriptional responses to four different nutrient-limitation regimes under aerobic and anaerobic conditions in chemostat cultures of *S. cerevisiae*. This 'two-dimensional' approach resulted in a new, robust set of 'anaerobic' and 'aerobic' signature transcripts for *S. cerevisiae*, as well as to a refinement of previous reports on nutrient-responsive genes. Moreover, the identification of genes regulated both by nutrient and oxygen availability provided new insight in cross-regulated network and hierarchy in the control of gene expression. These newly defined sets of signature genes were subjected to *in silico* promoter analysis to identify consensus regulatory elements.

Experimental Procedures

Strain and Growth Conditions

The prototrophic, haploid reference strain *S. cerevisiae* CEN.PK113-7D (*MAT***a**) (335) was grown at 30^{o}C in 2-liter chemostats (Applikon) with a working volume of 1.0 liter as described in (333). Cultures were fed with a defined synthetic medium that limited growth by either carbon, nitrogen, phosphorus, or sulfur with all other growth requirements in excess and at a constant residual concentration (32). The dilution rate was set at 0.10 h^{-1}. The pH was measured online and kept constant at 5.0 by the automatic addition of 2 M KOH with the use of an Applikon ADI 1030 biocontroller and the stirrer speed was set at 800 rpm. Anaerobic conditions were maintained by sparging the medium reservoir and the fermentor with pure nitrogen gas (0.5 liter.min^{-1}). Furthermore, Norprene tubing and butyl septa were used to minimize oxygen diffusion into the anaerobic cultures (348). The off-gas was cooled by a condenser connected to a cryostat set at 2^{o}C. Oxygen and carbon dioxide were measured offline with a NGA 2000 Rosemount gas analyzer. Steady-state samples were taken after approximately 10-14 volume changes to avoid strain adaptation due to long term cultivation (92, 148). Biomass dry weight, metabolite, dissolved oxygen and gas profiles were constant over at least 3 volume changes prior to sampling for RNA extraction.

Growth media

The synthetic medium composition was based on that described in (345). In all chemostats except for carbon, the residual glucose concentration was targeted to 17 g.$liter^{-1}$ to sustain glucose repression at the same level. For anaerobic cultivations, the reservoir medium was supplemented with the anaerobic growth factors Tween-80 and ergosterol as described previously (344). These media contained the following components (per liter). For carbon limited cultivation: 5.0 g of $(NH_4)_2SO_4$, 3.0 g of KH_2PO_4, 0.5 g of $MgSO_4.7H_2O$, and 25 g of glucose. For nitrogen-limited cultivation: 0.65 g of $(NH_4)_2SO_4$, 5.75 g of K_2SO_4, 3.0 g of KH_2PO_4, 0.5 g of $MgSO_4.7H_2O$ and 46 g of glucose. For phosphorus-limited cultivation: 5.0 g of $(NH_4)_2SO_4$, 1.9 g of K_2SO_4, 0.12 g KH_2PO_4, 0.5 g of $MgSO_4.7H_2O$, and 66 g of glucose. For sulfur-limited cultivation: 4.0 g of NH_4Cl, 0.05 g of $MgSO_4.7H_2O$, 3.0 g of KH_2PO_4, 0.4 g of $MgCl_2$ and 59 g of glucose. The medium composition for the aerobic chemostat cultures was as previously described in (32).

Analytical Methods

Culture supernatants were obtained after centrifugation of samples from the chemostats. For the purpose of glucose determination and carbon recovery, culture supernatants and media were analyzed by high

performance liquid chromatography fitted with an AMINEX HPX-87H ion exchange column using 5 mM H_2SO_4 as the mobile phase. Residual ammonium, phosphate and sulfate concentrations were determined with the use of cuvette tests from DRLANGE (Düsseldorf, Germany). Culture dry weights were determined via filtration as described by Postma *et al* (264).

Microarray analysis

Sampling of cells from chemostats, probe preparation and hybridization to Affymetrix Genechip® microarrays were performed as described in Chapter 1 and in Piper *et al* (262). The results for each growth condition were derived from three independently cultured replicates.

Data acquisition and analysis

Acquisition and quantification of array images and data filtering were performed using the Affymetrix software packages: Microarray Suite v5.0, MicroDB v3.0 and Data Mining Tool v3.0.

Before comparison, all arrays were globally scaled to a target value of 150 using the average signal from all gene features using Microarray Suite v5.0. To eliminate insignificant variations, genes with values below were set to 12 according to (262). From the 9,335 transcript features on the YG-S98 arrays a filter was applied to extract 6,383 yeast open reading frames of which there were 6,084 different genes. This discrepancy was due to several genes being represented more than once when sub-optimal probe sets were used in the array design.

To represent the variation in triplicate measurements, the coefficient of variation (C.V.; standard deviation divided by the mean) was calculated as previously described by Boer et al (32).

For further statistical analyses Microsoft Excel running the Significant Analysis of Microarrays (SAM v1.12) add in was used (330) for pair wise comparisons. Genes were considered as being changed in expression if they were called significantly changed using SAM (expected median false-discovery rate (FDR) of 1%) by at least 2-fold from each other condition. Hierarchical clustering of the obtained sets of significantly changed expression levels was subsequently performed by Genespring v6.1 (Silicon Genetics).

Promoter analysis was performed using web-based software Regulatory Sequence Analysis Tools (336). The promoters (from –800 to –1) of each set of co-regulated genes were analyzed for over-represented hexanucleotides. When hexanucleotide sequences shared largely common sequences, they were aligned to form longer conserved elements. All the individual promoter sequences contributing to these elements were then aligned, and the redundant elements were determined by counting the base representation at each position. The relative abundance of these redundant elements was then determined from a new enquiry of the co-regulated gene promoters and the entire set of yeast promoters in the genome.

The gene annotation was made according to the Comprehensive Yeast Genome Database (CYGD) (http://mips.gsf.de/genre/proj/yeast/index.jsp) at Munich Information center for Protein Sequence (MIPS) (229), Saccharomyces Genome Database (http://www.yeastgenome.org/) (SGD) (84) and the Yeast Proteome Database (YPD) at Incyte (https://www.incyte.com/).

Results

Experimental design and physiology of S. cerevisiae *in aerobic and anaerobic macronutrient-limited chemostat cultures*

In order to investigate the impact of transcriptional cross-regulation on the identification of 'signature transcripts', we designed a two-dimensional experimental approach (Figure 1). Four nutrient-limitation regimes (carbon, nitrogen, sulfur and phosphorus limitation) were studied. In one set of experiments, the four nutrient limitation regimes were studied in aerobic chemostat cultures. A second set of experiments was performed under the same nutrient limitation regimes, but in anaerobic chemostat cultures. The resulting set of eight fermentation conditions, each analyzed in three independent replicate cultures, enabled the identification of genes with a specific transcriptional response to one parameter only (e.g. induced under anaerobic conditions irrespective of the macronutrient-limitation regime). Furthermore, genes that transcriptionally responded to multiple parameters were identified (e.g. only induced under anaerobic conditions when growth was limited by the carbon source).

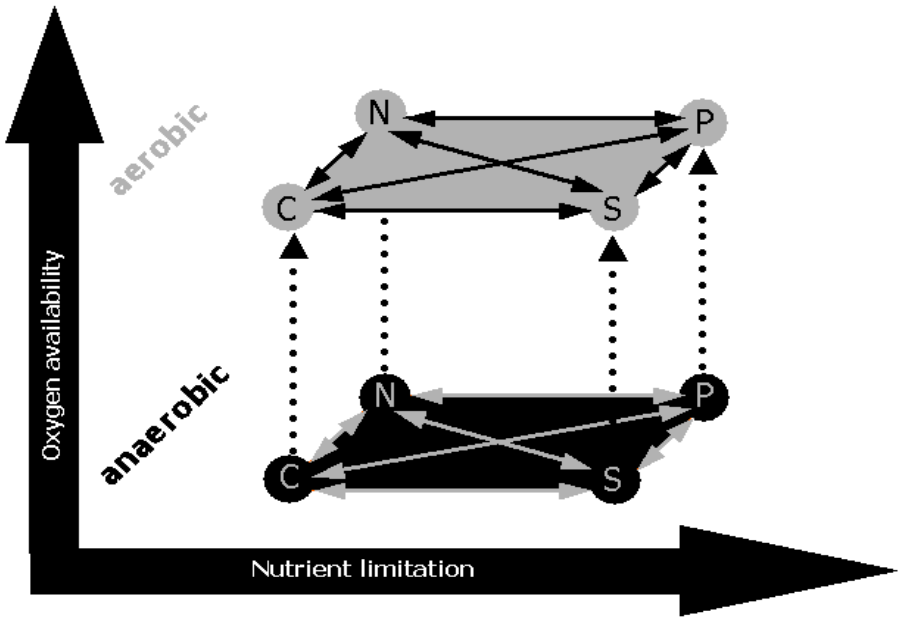


Figure 1: Two-dimensional experimental approach.

The experimental design for two-dimensional transcriptome analysis is shown. Each corner of the cube represents a chemostat condition. The upper horizontal surface represents the four aerobic macronutrient limitation regimes (carbon, nitrogen, phosphorus and sulfur). The lower horizontal surface represents the same macronutrient limitation regimes analysed under anaerobic conditions. The arrows indicate the pairwise comparisons included in the present two-dimensional transcriptome analysis.

To minimize experimental "noise", the composition of growth media was designed such that residual concentrations of non-growth-limiting nutrients were

essentially the same in all chemostat cultures (Table 1). Control experiments confirmed that the concentrations of the growth-limiting nutrients were below the detection limit of the respective assay procedures (Table 1). The option to control the steady-state concentrations of limiting and excess nutrients concertedly is a unique feature of chemostat cultivations.

The physiological parameters of the eight cultivation conditions are reported in Table 1. Under aerobic conditions, only the glucose-limited cultures exhibited a completely respiratory glucose metabolism, without production of ethanol. This resulted in a respiratory quotient close to unity (Table 1). Conversely, the aerobic cultures that were not limited by glucose exhibited a respiro-fermentative glucose metabolism, with simultaneous ethanol production and oxygen consumption (respiratory quotient > 1). In the anaerobic chemostat cultures, alcoholic fermentation was the sole mode of glucose dissimilation, as no oxygen was available for respiration. The ATP yield from alcoholic fermentation is much lower than that from respiratory glucose dissimilation (344), thus explaining the lower biomass yield on glucose of the anaerobic cultivations. The biomass yield on glucose in glucose-limited cultures was higher than in the non-glucose-limited cultures (Table 1). Under aerobic conditions, this can be partially explained by the involvement of alcoholic fermentation in the latter cultures. However, a reduction of the biomass yield in non-glucose-limited cultures was also observed under anaerobic conditions (Table 1). This may be related to the induction of energy-dependent transport systems during N-, P- and S- limited growth (187).

Microarray reproducibility, global transcriptome responses and data analysis

In order to obtain statistically robust, reproducible transcriptome datasets (262), independent triplicate chemostat cultivations and oligonucleotide DNA microarrays were carried out for each of the eight cultivation conditions. The average coefficient of variation for the triplicate transcriptome analyses (32, 262) for each of the eight conditions was below 0.21, except for the anaerobic glucose-limited chemostats (average coefficient of variation of 0.27). The level of the *ACT1* transcript, a common loading standard for conventional Northern analysis, did vary by less than 13% over the eight growth conditions (see Supplementary Table 1 online).

The eight different cultivation conditions would, in principle, allow for 56 different pair-wise comparisons. In the present study, we restricted analysis of the data to pairwise comparisons between cultivation conditions that differed in a single cultivation parameter only. Ultimately, this left 28 pair-wise comparisons. Four of these were pair-wise comparisons between aerobic and anaerobic cultures grown under the same macronutrient-limitation regime (Figure1, vertical arrows). A further 24 pair-wise comparisons involved all possible combinations of the four macronutrient-limitation regimes under either aerobic or anaerobic conditions (Figure 1, horizontal surfaces).

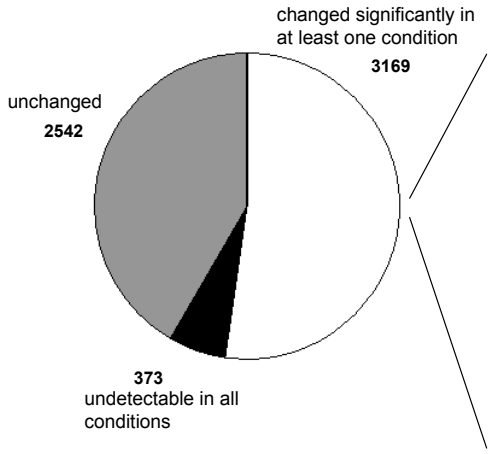
Each pair-wise comparison defined a set of genes that were significantly up- or down-regulated (fold change > 2 with a false discovery rate of 1%, see Experimental procedures section).

Table 1: Nutrient concentrations and physiological parameters of chemostat cultures used in this study.
(Unless indicated otherwise, data represent the mean ± S.D. of data from three independent steady-state chemostat cultivations.)

Growth-limiting nutrient	Residual nutrient measurements				Physiological parameters						
	glucose (g/l)	NH_4^+ (mM)	PO_4^{3-} (mM)	SO_4^{2-} (mM)	Y_{sx} (g/g)[a]	$q_{glucose}$[b]	$q_{ethanol}$[c]	q_{O2}[d]	q_{CO2}[e]	RQ[f]	Carbon recovery (%)
Aerobic											
Carbon	BD[h]	58.2 ± 1.3	19.8 ± 0.6	38.6[g]	0.49 ± 0.01	1.1 ± 0.0	0.0 ± 0.0	2.8 ± 0.3	2.8 ± 0.3	1.0 ± 0.0	98 ± 3
Nitrogen	16.7 ± 1.0	BD	18.6 ± 1.0[i]	40.7 ± 1.0	0.09 ± 0.00	5.8 ± 0.1	8.0 ± 0.1	2.7 ± 0.1	12.1 ± 0.2	5.8 ± 0.1	96 ± 1
Phosphorus	18.1 ± 1.0	54.3 ± 0.3	BD	47.5 ± 1.0	0.09 ± 0.00	6.1 ± 0.2	7.8 ± 0.1	4.0 ± 0.1	13.5 ± 0.2	6.1 ± 0.2	95 ± 2
Sulfur	17.4 ± 0.6	53.7 ± 2.4	18.4 ± 0.2	BD	0.14 ± 0.00	3.8 ± 0.1	4.4 ± 0.1	3.0 ± 0.0	8.0 ± 0.8	3.8 ± 0.1	96 ± 1
Anaerobic											
Carbon	BD	68.6 ± 2.8[i]	22.3 ± 0.6[i]	42.4 ± 1.6[i]	0.09 ± 0.0	6.0 ± 0.0	9.6 ± 0.1	NA[j]	10.3 ± 0.4	NA	101 ± 2
Nitrogen	16.2 ± 0.6	BD	21.9 ± 0.4	39.1 ± 0.8	0.07 ± 0.0	8.4 ± 0.0	13.5 ± 0.6	NA	14.8 ± 0.3	NA	101 ± 2
Phosphorus	19.1 ± 2.2	60.2 ± 2.6	BD	50.9 ± 0.9	0.06 ± 0.0	8.7 ± 0.2	13.9 ± 0.6	NA	15.8 ± 0.7	NA	101 ± 2
Sulfur	21.2 ± 0.2	61.1 ± 1.3	21.5 ± 0.2	BD	0.07 ± 0.0	7.9 ± 0.2	11.9 ± 0.4	NA	13.6 ± 0.8	NA	98 ± 1

[a] Yield of biomass (g/g of glucose consumed).
[b] mmol of glucose consumed/g of biomass/h.
[c] mmol of ethanol produced/g of biomass/h.
[d] mmol of oxygen consumed/g of biomass/h.
[e] mmol of carbon dioxide produced/g of biomass/h.
[f] RQ, respiratory quotient (q_{CO2}/q_{O2}).
[g] Single measurement.
[h] BD, below detection.
[i] Average of two measurements.
[j] NA, not applicable.

In total, 3169 genes (52 % of the genome) exhibited a significantly different transcript level in at least one of the 28 pair-wise comparisons. 2542 Genes (42 %) of the genome did not exhibit a significant difference in transcript level in any of the pair-wise comparisons. The remaining 373 transcripts (representing 6 % of the *S. cerevisiae* genome) remained below the detection limit under all eight conditions investigated (Figure 2) (see Supplementary Table 2 online).



regulatory profile	genes changed		
higher in anaerobic	65		
lower in anaerobic	90		
oxygen responsive genes	155		
regulatory profile	aerobic onlly	aerobic & anaerobic	anaerobic only
higher in C-lim	145	19	35
lower in C-lim	48	14	167
higher in N-lim	19	32	39
lower in N-lim	14	1	6
higher in P-lim	31	31	28
lower in P-lim	12	2	7
higher in S-lim	20	48	13
lower in S-lim	44	5	7
Nutrient specific genes	333	152	302

Figure 2: **Global transcriptional responses to growth in aerobic and anaerobic, macronutrient-limited chemostats**. The genome-wide transcript profiles of *S. cerevisiae* grown under different oxygen availability conditions and limitations (lim) for carbon, nitrogen, phosphorus, or sulfur are compared, and the classes of expression profiles were scored. About half of the predicted genome (48 %) was either unchanged or not measurable across all eight conditions. The remaining significantly changed genes (3169) were categorized into oxygen-responsive genes (155); genes that responded to macronutrient-limitation under solely aerobic conditions (333), solely anaerobic conditions (302) and irrespective of the presence of oxygen (152); and genes with a more complex transcription profile.

Transcripts that showed a consistent difference in the aerobic-anaerobic comparisons under all four macronutrient-limitation regimes were identified by combining the four relevant pair-wise comparisons (Figure 1, vertical arrows). This set of consistently oxygen-responsive genes contained 155 genes (2.6% of the genome; Figure 3A) (see Supplementary Table 3 online).

To investigate transcriptional responses to macronutrient limitation, we first identified transcripts that responded to a single nutrient-limitation regime under either aerobic or anaerobic conditions (Figure 3B, sets I and V). Combination of sets I and V for each of the four macronutrient-limitation regimes yielded a subset of transcripts that showed a consistent response to macronutrient limitation, irrespective of oxygen availability (Figure 3B, set III, 152 genes) (see Supplementary Table 4 online). In addition, this comparison yielded two sets of genes that only showed a transcriptional response to one of the four nutrient limitation conditions under either aerobic conditions (Figure 3B, set II, 333 genes) (see Supplementary Table 5 online) or anaerobic

conditions (Figure 3B, set IV, 302 genes) (see Supplementary Table 6 online). The data analysis approach described above enabled us to dissect the *S. cerevisiae* genome clusters of genes that either showed a consistent, robust response to oxygen availability or macronutrient limitation or, alternatively, showed a more complex dual-parameter transcriptional regulation.

Signature genes with a consistent transcriptional response to oxygen availability or macronutrient limitation

Ten clusters of genes that were identified showed a specific and consistent response to anaerobiosis, glucose limitation, nitrogen limitation, phosphorus limitation or sulfur limitation (Figure 4). In five of these clusters, the transcriptional response was defined as 'up-regulated' under the conditions indicated, in the other five clusters the transcriptional response was defined as 'down-regulated'. This terminology does not imply any mechanism of regulation. For example, down-regulation under nutrient limitation might, mechanistically, represent up-regulation under conditions of nutrient excess. In our discussion of these 'consistent-response' genes, we will restrict ourselves to a detailed analysis of the anaerobically up-regulated genes and some specific observations on the macronutrient-limitation-responsive genes.

Anaerobically up-regulated genes

Based on a statistically robust, two-laboratory transcriptome analysis of glucose-limited chemostat cultures of *S. cerevisiae*, Piper *et al* (262) identified 877 transcripts that were differentially expressed in anaerobic and aerobic cultures. These genes were distributed in 133 anaerobically up-regulated and 744 anaerobically down-regulated genes. In our 2-dimensional approach, the transcriptional response to oxygen availability of 722 of these genes (82 %) depended on the macronutrient limitation regime and only 155 genes showed a consistent response to anaerobiosis under all four macronutrient limitation regimes (65 up-regulated, 90 down-regulated) (Figure 3A).

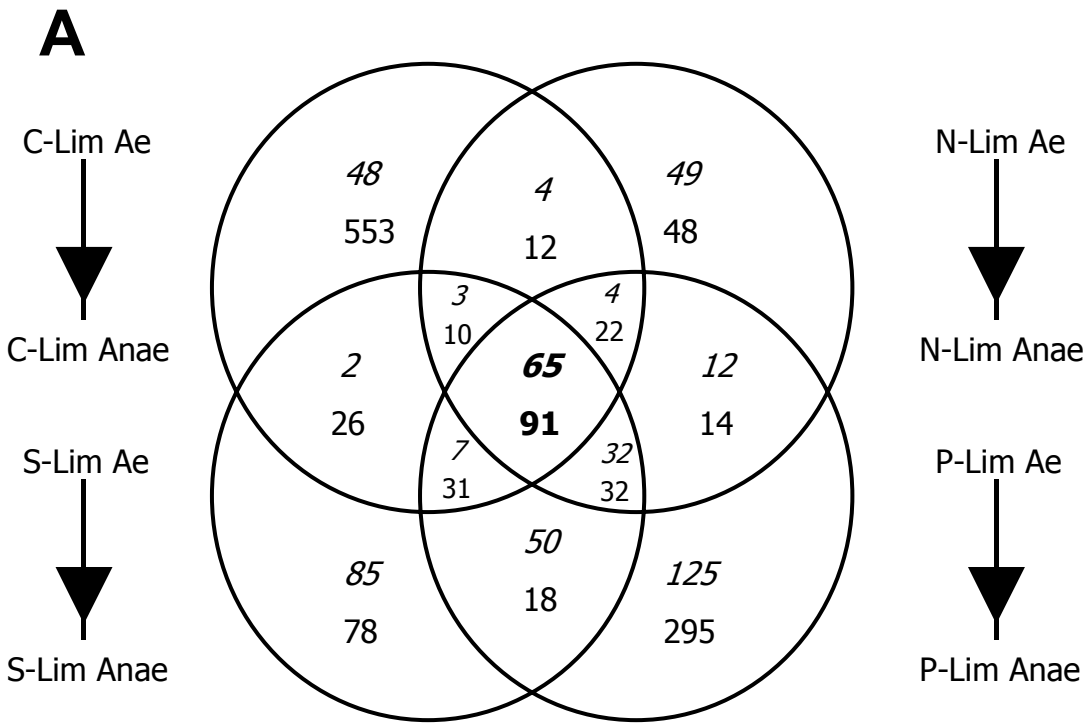
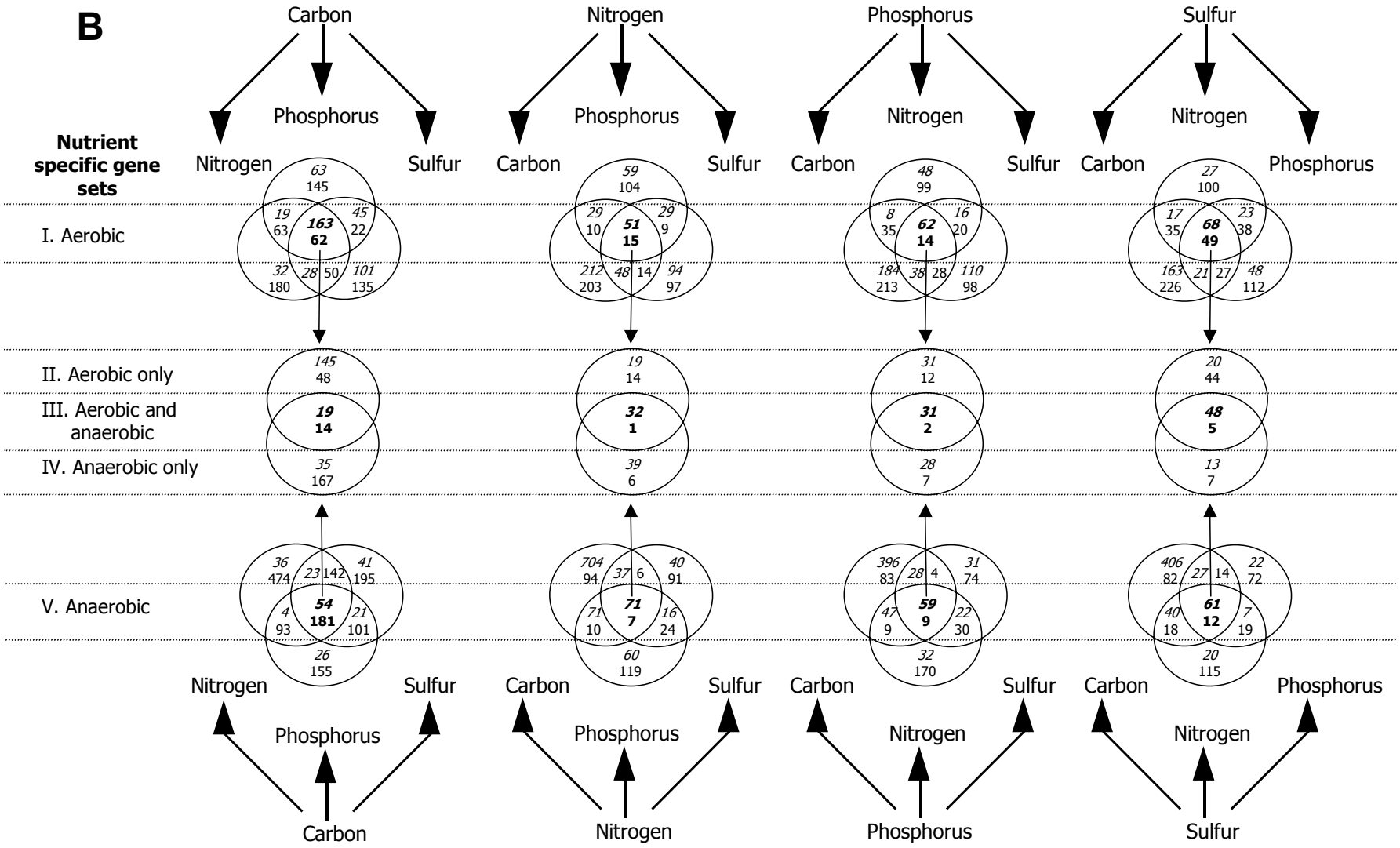


Figure 3: **Data mining strategy: dissection of the transcriptome response with respect to nutrient limitation and oxygen availability. A)** Venn diagram of signature anaerobic genes. Italic numeric and normal numeric represents up-regulation and down-regulation, respectively, under anaerobic conditions. Each of the four circles corresponds to a cluster of genes that showed a transcriptional response to oxygen availability under one of the four macronutrient limitation regimes. The overlap of the four clusters represents genes that show a consistent response to oxygen availability, irrespective of the nutrient limitation regime. **B)** (opposing page) Venn diagram of macronutrient-limitation-responsive genes. The diagram shows pair-wise transcriptome comparisons (see Figure 1) of each macronutrient limitation regime against the other three macronutrient limitation regimes, for aerobic and anaerobic cultures. Each circle represents the cluster of genes up-regulated (italic numeric) or down-regulated (normal numeric) for the reference macronutrient limitation. Sets I and V contain the genes that showed a consistent response to each of the four macronutrient limitation regimes in the three pair-wise comparisons under aerobic and anaerobic conditions, respectively. Combination of Sets I and V yielded three new subsets of macronutrient-limitation responsive genes. Set III represents signature genes that showed a consistent response to each of the macronutrient limitation regimes under aerobic and anaerobic conditions. Sets II and IV represent genes whose transcriptional response to a single macronutrient limitation regime was specific for aerobic or anaerobic conditions, respectively.


B
Nutrient specific gene sets
I. Aerobic
II. Aerobic only
III. Aerobic and anaerobic
IV. Anaerobic only
V. Anaerobic
Carbon
Nitrogen
Phosphorus
Sulfur
63 145 19 63 163 62 45 22 32 180 28 50 101 135
59 104 29 10 51 15 29 9 212 203 48 14 94 97
48 99 8 35 62 14 16 20 184 213 38 28 110 98
27 100 17 35 68 49 23 38 163 226 21 27 48 112
145 48 19 14 35 167
19 14 32 1 39 6
31 12 31 2 28 7
20 44 48 5 13 7
36 474 23 142 41 195 4 93 54 181 21 101 26 155
704 94 37 6 40 91 71 10 71 7 16 24 60 119
396 83 28 4 31 74 47 9 59 9 22 30 32 170
406 82 27 14 22 72 40 18 61 12 7 19 20 115

Of the 65 anaerobically up-regulated genes, 20 have an as yet poorly defined or unknown biological function. The 45 genes with known function were distributed over the functional categories, metabolism and energy (21 genes), transport (4 genes), cell rescue and defense (11 genes), protein synthesis (3 genes) and cell wall and organization (6 genes) according to the MIPS database (Comprehensive Yeast Genome Database http://mips.gsf.de/genre/proj/yeast/index.jsp) (229) (Figure 4). A closer inspection reflected the biosynthetic role of molecular oxygen in *S. cerevisiae* (276). Under anaerobic conditions, *S. cerevisiae* is not capable of *de novo* biosynthesis of sterols and unsaturated fatty acids and therefore these compounds are required as growth factors under anaerobic conditions (8, 9).

Although the anaerobic chemostat cultures were supplied with ergosterol and oleate, 22 of the consistently anaerobically up-regulated genes have been implicated in or associated with sterol or lipid metabolism. Of these genes, *UPC2* and *SUT1* are transcription factors for sterol uptake in yeast (272, 364) and *PDR11* and *AUS1* (members of the ABC membrane transporters) have been shown to be involved in sterol uptake for anaerobic growth. 13 members of the seripauperin family of possible cell wall mannoproteins (*DAN1*, *DAN2, DAN3, DAN4, TIR1, TIR2, TIR3, TIR4, PAU1, PAU3, PAU4, PAU5* and *PAU6*) that were consistently up-regulated in anaerobic cultures encode mannoproteins. These important determinants of cell wall permeability during anaerobiosis (3) may be involved in sterol uptake, as recently shown for *DAN1* (364). The *MGA2* gene product regulates the transcription of *OLE1*, which is involved in the biosynthesis of unsaturated fatty acids (153). *HES1*, *ARE1*, *YSR3* and *PLB2,* encode a putative oxysterol-binding protein, an acyl-CoA acetyl transferase, a putative regulator of sphingolipid metabolism and a phospholipase B2, respectively (104, 152, 213, 283). In addition to genes involved in sterol and fatty-acid metabolism, *COX5B* and *HEM13* displayed a consistent up-regulation in all anaerobic cultures. *COX5B* encodes the ‘anoxic subunit’ of cytochrome C oxidase, which is proposed to be involved in oxygen sensing (181). *HEM13* encodes a cytosolic coproporphrinogen III oxidase and has been described as the first, molecular oxygen-dependent and rate-controlling step of heme biosynthesis (276).

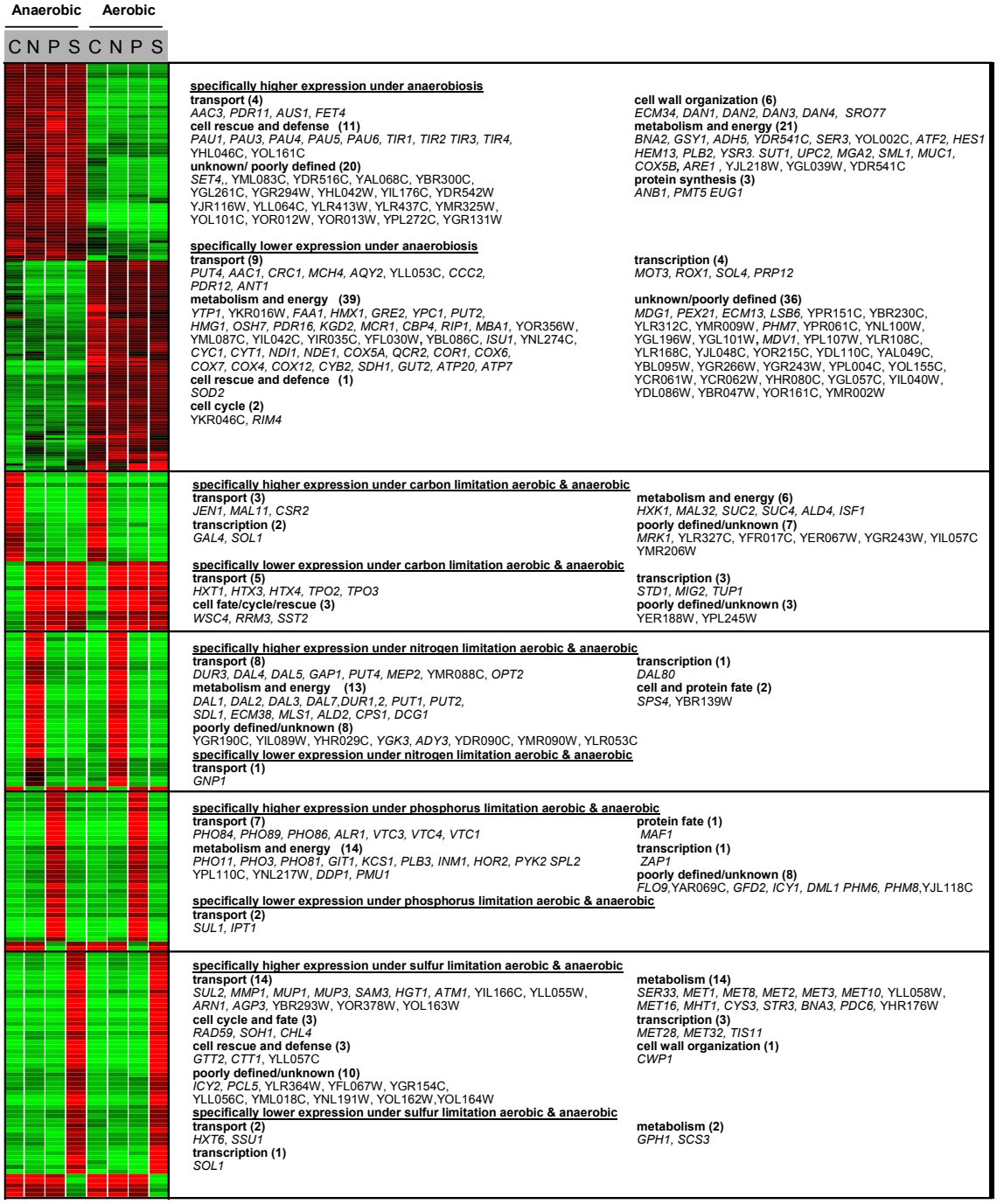


Figure 4: Signature genes that responded to a single environmental parameter. **The three independent transcriptome datasets for each condition were averaged and then compared. *Green* (relatively low expression) and *red* (relatively high expression) *squares* are used to represent the transcription profiles of genes deemed specifically changed. The signature genes were sorted by functional categories according to the Comprehensice Yeast Genome Database (229) and SGD (84) databases. The full dataset containing all transcript abundance measurements can be found at www.bt.tudelft.nl/2-Dtranscriptomics.**

As a further approach to assess the biological significance of the consistent transcriptional responses identified via the two-dimensional approach, we analyzed the enrichment of regulatory motifs in promoter sequences of the oxygen-responsive genes (Figure 3A, Figure 4, Table 2). Four over-represented sequences were recovered from the 65 anaerobic up regulated genes promoter regions (Table 2). At least one of the two overlapping sequences TCGTwyAG or CCTCGTwy was recovered from 34 genes (52%) in the cluster. These sequences are similar to the previously described binding site for Upc2p (CGTTT (55)), a transcription factor whose structural gene itself was consistently up-regulated in the anaerobic cultures. 17 genes (26 %) shared the element ATTGTTC, which is the known binding site for the anaerobic transcription factor Rox1p (204)). We also identified a new motif, AAGGCAC, within this cluster of genes, for which no DNA-binding protein has yet been identified. The Upc2p and AAGGCAC motifs showed a remarkable coincidence in the promoters of 12 genes of the cluster (Figure 5). In the upstream regions of these genes, the Upc2p binding site was present at –450 to –380 and the AAGGCAC element was present at –360 to –300 (Figure 5). The conservation of both the distance to the coding region and the distance between the elements strongly suggest biological relevance. 70% of the promoter sequences of the genes that were consistently up-regulated in the anaerobic cultures contain at least one of the three elements discussed above.

Transcriptional responses to macronutrient limitation: genes up-regulated under phosphate limitation

The four clusters of genes that were consistently (under aerobic as well as anaerobic conditions) up-regulated in response to growth limitation by a single macronutrient shared some conserved features. These involved induction of high-affinity uptake systems for the limiting macronutrient, excretion of nutrient-scavenging enzymes to the extracellular medium, induction of systems for mobilization and utilization of intracellular reserves and induction of systems for transport and assimilation of alternative sources of the limiting nutrient (Figure 4). This is exemplified by the transcriptional response to phosphorus limitation.

Previous comparison identified 62 up-regulated signature transcripts for aerobic phosphate limited growth (32). Introducing a second dimension (anaerobic phosphate limitation) resulted in a 50% decrease of the genes composing this cluster. Indeed, 31 genes showed a consistent up-regulation relative to the other macronutrient-limitation regimes in aerobic and anaerobic phosphate-limited chemostat cultures. Among these genes, seven are involved in transport, 14 in metabolism, one in protein fate, one in transcription and eight have an as yet unknown function according the MIPS database (229) and Saccharomyces Genome Database (84). 23 of these phosphate-limitation-induced genes (74%) could be directly related to phosphorus metabolism.

Table 2: Gene coverage of over-represented sequences retrieved from promoters of co-regulated genes
(Unless stated otherwise, elements were counted present in a gene promoter only if they occurred at least twice).

Regulatory cluster	Promoter element[a]		Putative binding protein	Gene coverage (%)			Genome coverage[b] (%)	Range[c]		
	forward	Reverse								
Specifically higher in anaerobiosis	AAGGCAC[d]	GTGCCTT	?	38			8	0-3		
	ATTGTTC[d]	GAACAAT	Rox1p	26			12	0-3		
	ddACGAGG[d]	CCTCGThh	Upc2p	40			18	0-2		
	TCGTwyAG[d]	CTrwACGA	Upc2p	38			7	0-3		
				Set II[e]	Set III[f]	Set IV[g]		Set II	Set III	Set IV
Specifically higher in C-lim	dCCCCdh	dhGGGGh	Mig1p	43	65	25	28	0-5	0-6	0-5
Specifically higher in N-lim	rGATAAs	sTTATCy	Gln3p/Gat1p/Dal80p/Gzf3p	11	61	14	6	0-3	0-5	0-3
	CAATGA	TCATTG	Dal82p	11	23	3	13	0-4	0-3	0-2
Specifically higher in P-lim	mACGTGs	sCACGTk	Pho4p	13	58	13	3	0-2	0-6	0-6
Specifically higher in S-lim	GCCACA	TGTGGC	Cbf1p/Met4p/Met28p	5	33	NS[h]	3	0-4	0-5	0-1
	CACGTGA	TCACGTG	Met31p/Met32p	NS	10	NS	2	0-1	0-3	0-1

[a] Redundant nucleotides are given by: r = A or G, y = C or T, s = G or C, w = A or T, k = G or T, m = A or C, b = C, G or T, d = A, G, or T, h = A, C or T, n = A, C, G or T.

[b] Relative to 6451 open reading frame upstream promoters in the yeast genome according to RSA Tools.

[c] Range of motifs present in each promoter of the specific gene cluster.

[d] Elements counted present in a gene promoter when occurring at least once.

[e] Set I, containing aerobic-only nutrient-specific genes, as in Fig 3B.

[f] Set II, containing aerobic and anaerobic nutrient-specific genes, as in Fig 3B.

[g] Set III, containing anaerobic-only nutrient-specific genes, as in Fig 3B.

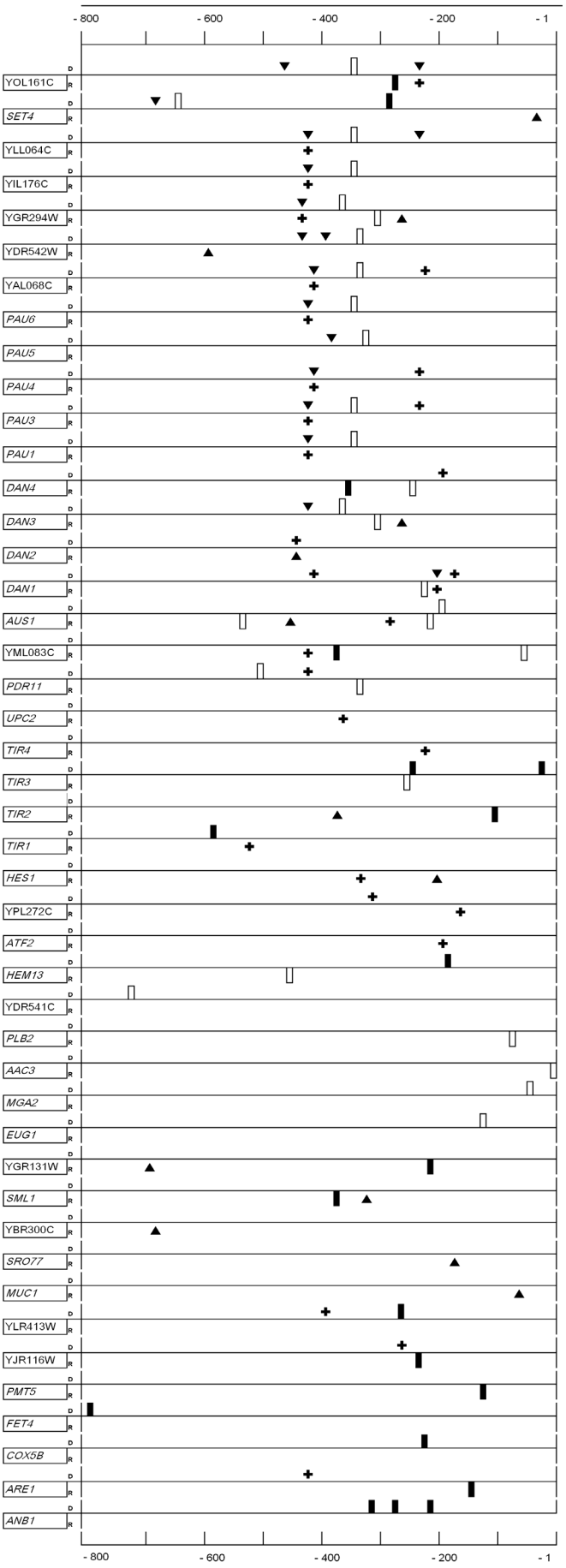
[h] **NS, no significant patterns retrieved by RSA Tools.**

All seven genes classified in the transport category were associated to phosphate transport (*PHO84*; high-affinity inorganic phosphate/proton symporter*, PHO89*; high-affinity sodium-dependent phosphate transporter (259), *PHO86*; protein associated with phosphate transport complex with phosphate transport complex (188)*, GIT1*; glycerophosphoinositol transporter (6) belonging to the major facilitator superfamily (MFS) , *VTC1, VTC3* and *VTC4*; subunits of the vacuolar membrane polyphosphate transporter complex (249)).

Of the remaining genes in this cluster, several are involved in phosphate mobililization: *PHO11* and *PHO3* encode phosphatases, *HOR2* a glycerol-3-phosphate phosphatase (230, 342), *INM1* an inositol monophosphatase (245), YNL217W a putative metallo-phosphatase (235), YPL110C a putative glycerophosphoryl diester phosphodiesterase, *DDP1* a diadenosine hexaphosphate hydrolase (46), *PLB3* a phospholipase B (224) and *PYK2* a glucose repressed pyruvate kinase (34). The proteins encoded by *PHM6* and *PHM8* are likely to encode proteins involved in phosphate metabolism (249) as well, and their promoter regions exhibit a Pho4p binding site. *PHO81* and *SPL2* are presumed inhibitors of the Pho80p-Pho85p cyclin-dependent protein-kinase complex and positive regulators of phosphate-related genes (96). Furthermore, *KCS1* an inositol (1, 2, 3, 4, 5, 6) hexaphosphate kinase involved in inositol metabolism was up-regulated (281). The remaining eight genes in the cluster (25 %) could not be directly associated to phosphate metabolism. Interestingly, two of these genes are involved in transcriptional regulation: *ZAP1* encodes a zinc-responsive transcriptional activator (374) and *MAF1* a putative repressor of RNA polymerase III transcription and a common component of multiple signaling pathways in *S. cerevisiae* that sense changes in the cellular environment (331).

In silico promoter analysis of the genes that were consistently up-regulated under phosphate limitation revealed an overrepresented mACGTGs motif (present in 58% of the genes in the cluster as opposed to 3% in the *S. cerevisiae* genome). This sequence shows strong similarity to the CACGTG consensus for the binding site of Pho4p (94), the main transcription factor required for expression of the phosphate-related genes (Table 2).

Figure 5 (opposing page): **Localization of consensus binding sites in promoter sequences of up-regulated genes in the absence of oxygen irrespective of nutrient limitation**. The promoter regions of genes from –800 to –1 were based on the sequences obtained from RSAT (336). ▲,Upc2p consensus sequence TCGTwyAG; ▼, Upc2 consensus sequence TCGTwyAG found on the Crick strand; **+**, Upc2 consensus sequence CCTCGThh; ▉ , Rox1p consensus sequence ATTGTTC; □ AAGGCAC consensus sequence. Consensus sequences on both strands are indicated. The open reading frames on the Watson strand and on the Crick strand are indicated by D and an R respectively. Redundant nucleotides are given by: y = C or T, w = A or T, h = A, C or T.


- 800
- 600
- 400
- 200
- 1
YOL161C
SET4
YLL064C
YIL176C
YGR294W
YDR542W
YAL068C
PAU6
PAU5
PAU4
PAU3
PAU1
DAN4
DAN3
DAN2
DAN1
AUS1
YML083C
PDR11
UPC2
TIR4
TIR3
TIR2
TIR1
HES1
YPL272C
ATF2
HEM13
YDR541C
PLB2
AAC3
MGA2
EUG1
YGR131W
SML1
YBR300C
SRO77
MUC1
YLR413W
YJR116W
PMT5
FET4
COX5B
ARE1
ANB1

Transcriptional cross regulation identified from two-dimensional transcriptome analysis

By combining the transcriptional responses to (an)aerobiosis in cultures subjected to four different macronutrient limitation regimes, it was possible to identify gene clusters that were subjected to transcriptional regulation by two environmental parameters. Identification of such clusters is not possible in conventional 'one-dimensional' pair-wise comparisons between cultivation conditions. Eight such clusters (Figure 3B set II and IV) could be assigned. To explore the biological significance of defining these clusters, we will discuss one of these clusters in more detail.

Of the 428 genes that showed a transcriptional response to carbon limitation in our analysis (Figure 3B set II, III, IV), only 33 genes showed a consistent response to carbon limitation irrespective of the availability of oxygen (Figure 3B set III). 193 genes (Figure 3B set II) only showed a significant transcriptional response under aerobic conditions. Of the remaining 202 genes (Figure 3B set IV), which only responded to carbon limitation in the anaerobic cultures, 167 genes were down-regulated in anaerobic, carbon-limited cultures and 35 genes up-regulated.

Of the 35 genes that were, at the level of transcription, uniquely up-regulated in anaerobic, carbon-limited chemostat cultures, 21 genes were related to mitochondrial function (Figure 6A) – even though glucose dissimilation in these cultures was completely fermentative. 15 of these mitochondrial-function-related genes were involved in oxidative phosphorylation and respiration, *QCR2, QCR6*, *QCR7* and *RIP1* as core subunits of ubiquinol cytochrome C reductase complex (complex III)*, COX4, COX5A, COX6, COX8, COX12* and *COX13* as core subunits of cytochrome C oxidase (complex IV), *ATP4, ATP15* and *ATP20* as core subunits of the F_O subunit of the mitochondrial ATP-synthase, *INH1* as the inhibitory subunit of the mitochondrial ATP-synthase and finally *CYC1* as the predominant aerobic isoform of cytochrome C. In addition, three of the four subunits of succinate dehydrogenase (*SDH1, SDH2* and *SDH4*) were significantly up-regulated in the carbon-limited anaerobic cultures. *DLD1* encodes mitochondrial D-lactate ferricytochrome-c-oxidoreductase (203), *MAM33* encoding a mitochondrial protein required for normal respiratory growth (295) and *NDE1* a mitochondrial cytosolically directed NADH dehydrogenase (205). The remaining 14 genes out the 35 genes of the discussed cluster were composed of two hexose-transporter genes (*HXT16* and *HXT17*), five genes encoding ribosomal proteins (RSP*10A, RPS25A, RPP1B, RPL4A* and *RPL9A*), and seven genes belonging to different metabolic routes (*SUT1, OSH7, AGP1, IMD2,* YLR089C, YAR075W and *GPA1*) (Figure 6B).

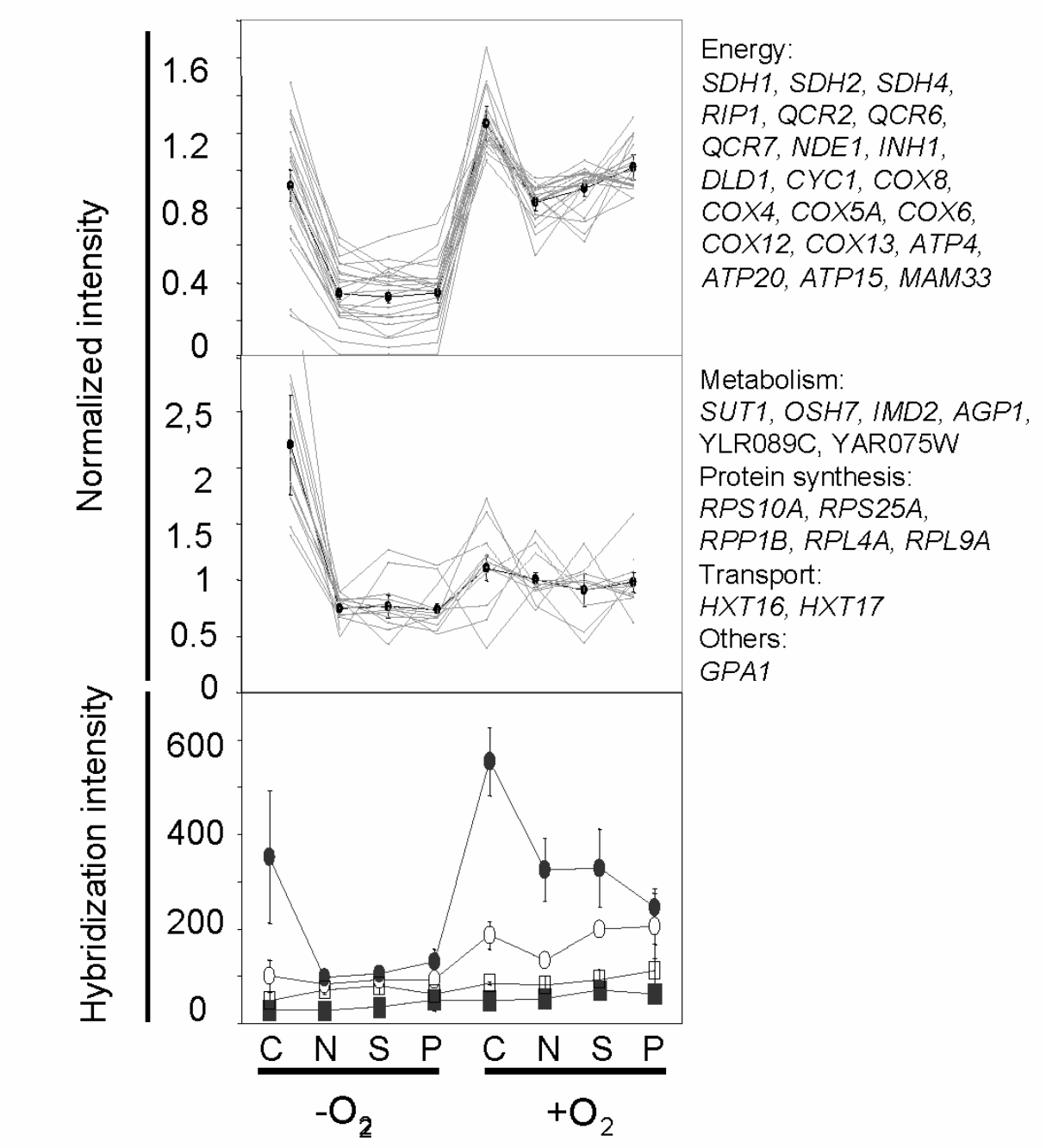


Figure 6: **Hierarchical regulatory control of gene expression.** *Upper panel*, normalized transcript profiles of mitochondrial function-related genes that clustered with a set of genes that showed a specific transcriptional up-regulation upon carbon limitation in anaerobic cultures. The transcript level across the four conditions for each gene was mean and variance normalized. The data represent the normalized abundance of all transcripts for each cultivation conditions, as well as the normalized average abundance of all transcript of the cluster, (thick black line). Error bars represent S.D. *Middle panel*, normalized transcript profiles of genes belonging to the cluster described under A that do not have a (known) relation to mitochondrial function. The data are presented as described for the *upper panel*. *Lower panel*, transcript profiles of the structural genes for the components of the Hap2/3/4/5p complex. Error bars represent S.D. ■, *HAP2*; ○, *HAP3*; ●, *HAP4*; □, *HAP5*.

The low mRNA levels of these genes in anaerobic chemostat cultures that were limited by nitrogen, phosphorus or sulfur, and thus had a high residual glucose concentration, strongly suggests that glucose catabolite plays an important role in their transcriptional regulation. Conversely, under aerobic conditions, the identity of the growth-limiting nutrient did not significantly affect transcription. In fact, closer inspection indicated that, in the aerobic cultures, high transcript levels were observed under all four macronutrient limitation regimes (Figure 6A). Furthermore, also in the aerobic cultures, the combined expression patterns of these genes suggest a moderate induction (Figure 6A) under glucose-limited conditions. However, the statistical criteria used for the definition of the cluster did not identify this induction as significant.

A simple model to explain these observations is that, for this particular subset of *S. cerevisiae* genes, induction by oxygen supersedes glucose catabolite repression. It is beyond the scope of this paper to analyze the molecular basis for this apparent hierarchy in transcriptional regulation. However several genes of this cluster such as *DLD1*, *QCR2, QCR7* and *CYC1* are known targets of the Hap2/3/4/5p complex (31, 100, 203, 250) (Figure 6A). *In silico* promoter analysis of the 35 genes of this subset revealed a significant over-representation (3-fold) of the ACCAATnA sequence, which overlaps the CCAAT core of the Hap2/3/4/5p binding site. Furthermore, the transcript level of *HAP4,* known as the regulatory subunit of the Hap2/3/4/5p complex, correlated with the expression pattern within this subset of genes (Figure 6C). Interestingly, *HAP4* expression is reported to be glucose repressible, being up-regulated after the diauxic shift and during growth on respiratory carbon sources (106). Further research is required to investigate which factors, in addition to regulation by the Hap2/3/4/5p complex, are involved in oxygen regulation of these genes and which factors determine the relative impact of glucose repression and oxygen induction.

Discussion

DNA microarrays as diagnostic tool for biotechnology

A detailed understanding of the environmental stimuli to which microorganisms are exposed in industrial fermentation processes is invaluable for rational design and optimization of such processes. DNA microarrays provide an interface that, in principle, allows the use of the microorganisms themselves as the ultimate ‘biosensor’. An unequivocal coupling between relevant environmental parameters and transcriptional responses is essential for this application of DNA microarrays. An important concept in this context is that of the ‘signature transcript’, which indicates a transcript of which the levels specifically increases (or decreases) in response to a single environmental stimulus.

This study indicates that, in general, robust signature transcripts cannot be identified by varying the process parameter of interest against a single, constant experimental background ('one dimensional transcriptome analysis'). Instead, identification of robust signature transcripts requires that transcriptional responses to an environmental parameter be analyzed against multiple experimental backgrounds. For example, the sets of 'signature transcripts' for (an)aerobiosis and growth limitation by 4 macro-nutrients that were previously established in 'one-dimensional' transcriptome comparisons (32, 262, 316, 368) were considerably reduced in size by the two-dimensional approach followed in the present study. Chemostat cultivation is an indispensable tool for this combinatorial approach as, in contrast to batch cultivation, it allows the manipulation of individual culture parameters while keeping other relevant parameters, including the specific growth rate, constant (32, 165, 262, 368).

Although this study covers only a minute fraction of the staggering diversity of environmental conditions to which *S. cerevisiae* may be exposed in nature and in industry, it clearly demonstrates the complexity of transcriptional regulation. In real life, transcriptional responses of cells are influenced by hundreds of extracellullar signals. The interplay of these signals results in a multidimensional space in which each possible combination of signals results in a unique transcriptome. It is therefore to be anticipated that the number of robust signature transcripts will decrease further when, in addition to nutrient limitation and oxygen availability, other chemical or physical process parameters are included.

The significance of the combinatorial nature of the regulation of gene expression extends beyond *S. cerevisiae* and industrial biotechnology. For example, in the medical field, it is to be expected that the transcriptional profiles coupled to a disease or pharmacological efficacy will be equally sensitive to other stimuli and variance received by the cells. While, in a statistical sense, such effects may be averaged out when the identification of disease-correlated signature transcripts is based on large numbers of healthy and ill individuals (1), this does not exclude a strong impact of transcriptional 'cross talk' in individual patients that have been exposed to special circumstances.

Unraveling transcriptional regulation

Despite the combinatorial nature of transcriptional regulation, identification of unequivocal 'signature transcripts' should be possible when mechanisms of transcriptional regulation are fully understood. Ideally, signature transcripts should be encoded by genes that respond to a single transcriptional regulator protein whose expression and activity are uniquely dependent on a single environmental stimulus. Identification of such genes and regulators requires detailed knowledge of the regulons and recognition sequences of all relevant transcriptional regulators. Such knowledge is also essential for rational and predictable reprogramming of transcriptional regulation to improve the performance of industrial microorganisms.

Even for well studied organisms like *S. cerevisiae*, the physiological role of many transcriptional regulators, as well as the sequence motifs they recognize, remain to be identified. The two dimensional, chemostat-based approach proposed in this paper provides a powerful new tool for unraveling transcriptional regulation networks. This is exemplified by the enrichment of regulatory motifs in the consistently anaerobically induced transcripts (Table 2). Clearly, regulation by known transcriptional regulators (relief of *ROX1* repression and transcriptional activation by *UPC2* (20, 182, 364)) was not sufficient to account for the transcriptional response of all 65 genes that were consistently up-regulated under anaerobic conditions. Indeed, our study strongly suggests that at least a third factor, recognizing an AAGGCAC motif, is involved in transcriptional regulation by oxygen availability. This motif had gone unnoticed in a previous 'one dimensional' aerobic/anaerobic comparison (316). In general, a combinatorial analysis of the transcriptional responses to environmental stimuli is likely to increase enrichment of relevant regulatory elements and facilitate their identification.

The approach used in this study also allows statements on the hierarchy of transcriptional regulation. This is exemplified by a subset of genes related to mitochondrial function. Under anaerobic conditions, these genes are regulated primarily by glucose repression-derepression. However, under aerobic conditions, a high transcript level was observed even under glucose-excess conditions. Together, these data indicate that, in the aerobic cultures, oxygen regulation supersedes glucose repression (Figure 6). By expanding datasets and combining them with an *in silico* analysis of promoter structure, combinatorial analysis of transcriptomes can accelerate the unraveling of transcriptional regulation networks.

Functional analysis

Assigning physiological functions to 'unknown-function' genes still poses a major challenge in the post-genomic era. By identifying groups of genes that appear to be co-expressed (269), DNA microarrays can guide functional analysis. Indeed, many studies have correlated mRNA levels to cultivation conditions. However, even when chemostat cultivation was used to change only a single environmental parameter, pairwise comparisons characteristically lead to large numbers of target genes, which complicates functional analysis (32, 70, 262, 316). Moreover, in a recent study on the genome-wide transcriptional responses to low temperature (137), a very poor correlation was observed between transcriptional responses of genes and the phenotype of the corresponding null mutants at low temperature.

Compared to previous one-dimensional studies, the combinatorial approach followed in the present study led to a clear enrichment in our 'robust-response sets' of (i) genes with known function related to the environmental status under study (Figure4) and/or (ii) genes with relevant regulatory elements (Table 2). By implication, also the unknown-function genes found in the corresponding datasets are more likely to have a direct functional relationship to the corresponding

nutritional/environmental status. We are currently testing this hypothesis for the subset of genes that showed a consistent upregulation under anaerobic conditions.

Among the 'robust response' signature genes identified in this study, 38 % do not have a clearly established biological function (Figure 4). It is noteworthy that some of these (YJL118C, YAR069c and YGR190C) belong to a group of ORFs for which it has been recently been proposed that they should be discarded from the yeast genome directory, based on genomic comparison of *S. cerevisiae*, *S. bayanus*, *S. mikatae* and *S. paradoxus* (161). The observation that three of these genes showed a consistent response to phosphate limitation (YJL118C and YAR069C) or nitrogen limitation (YGR190C) strongly suggests they are bona fide, biologically relevant genes.

Provided that yeast strain and cultivation procedures are standardized, DNA microarray analysis on chemostat cultures is well reproducible in different laboratories (262). We propose that a multi-laboratory effort to build an extensive, chemostat-based 'multidimensional' gene-expression database is an invaluable research tool for functional analysis of the *S. cerevisiae* genome and for yeast systems biology. Obviously, such a database should not necessarily be confined to transcriptome data, but could also cover other levels of information.

Footnote:

The complete dataset is available for download at Genome Expression Omnibus website (http://www.ncbi.nlm.nih.gov/geo/) as series with GEO accession number: GSE1723 and at our website www.bt.tudelft.nl/2-Dtranscriptomics where supplementary data can also be found.

Chapter 3

Competitive chemostat cultivation of *Saccharomyces cerevisiae* mutants indicates a weak correlation between oxygen-dependent transcriptional regulation and fitness of deletion strains under anaerobic conditions

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Abstract

The applicability of transcriptomics for functional genome analysis rests on the assumption that global information on gene function can be inferred from transcriptional regulation patterns. This study investigates whether *S. cerevisiae* genes that are consistently transcriptionally upregulated under anaerobic conditions do indeed contribute to fitness in the absence of oxygen. Tagged deletion mutants were constructed in 27 *Saccharomyces cerevisiae* genes that show a strong and consistent transcriptional upregulation under anaerobic conditions, irrespective of nature of the growth-limiting nutrient (glucose, ammonia, sulfate or phosphate). Competitive anaerobic chemostat cultivation showed that only 5 out of the 27 mutants (*eug1Δ*, izh2Δ, *plb2*Δ, ylr413wΔ and yor012wΔ) conferred a significant disadvantage relative to a tagged reference strain. Implications of this study are that: (i) transcriptome analysis has a very limited predictive value for the contribution of individual genes to fitness under specific environmental conditions, and (ii) competitive chemostat cultivation of tagged deletion strains offers an efficient approach for selecting relevant leads for functional analysis studies.

Introduction

While the number of completely sequenced microbial genomes continues to grow explosively, assignment of biochemical and physiological functions to the corresponding genes progresses at a much lower rate. A case in point is the extensively studied yeast *Saccharomyces cerevisiae*. Ten years after the completion of its genome sequence (114), 21 % of its genes neither have an experimentally confirmed function nor a function that can be predicted with a high degree of confidence based on similarity with genes from other organisms (Saccharomyces Genome Database, August 28, 2006 (http://www.yeastgenome.org/cache/genomeSnapshot.html) (132).

Accurate determination of gene function often requires sophisticated and costly experimental techniques. It is therefore worthwhile to select priority targets for functional analysis via high-throughput methods such as for synthetic-lethality screening (325, 326), mapping of physical interaction (110, 173) or expression analysis. With respect to the latter, DNA microarrays have been extensively used to map genome-wide transcriptional responses to a multitude of environmental parameters (32, 49, 70, 107). This approach yields sets of genes that show common and specific transcriptional responses to individual environmental parameters. The resulting sets of transcriptionally responsive genes often show enrichment for genes with known functions that can be directly correlated with the environmental conditions under study. Additionally, they

invariably yield sets of transcripts that encode proteins with unknown function or with a known biochemical function that cannot be readily linked to the conditions studied.

It is generally assumed that, in the case of upregulated transcripts, the biochemical functions of the encoded proteins contribute to the organism's physiological adaptation to the environmental parameter under study. However, there are few published studies that systematically investigate the extent to which this concept of 'transcriptomics-inferred function' is correct and applicable for guiding functional analysis research. Two large-scale comparisons suggest that the correlation between transcript profile and fitness of deletion strains may be far from perfect (26, 112, 113, 366).

Saccharomyces cerevisiae is the only yeast that can rapidly grow under aerobic as well as anaerobic conditions (348). This unique ability plays a major role in various industrial applications of *S. cerevisiae*, including beer fermentation, wine fermentation and large-scale production of fuel ethanol. Still, the genetic basis for rapid anaerobic yeast growth remains unknown. In a recent chemostat-based study (313), we used transcriptome analysis to investigate the response of the yeast *Saccharomyces cerevisiae* to anaerobic conditions. 65 Genes (ca. 1 % of the genome) were found to be significantly upregulated under anaerobic conditions, irrespective of the nature of the growth-limiting nutrient (glucose, ammonium, phosphate or sulfate). In separate experiments with the yeast deletion library (299), 24 genes were shown to be essential for anaerobic (but not for aerobic). Surprisingly, when these two sets of genes, obtained from different experimental approaches, were compared, no overlap was found.

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

Experimental procedures

Strains and growth conditions

S. cerevisiae CEN.PK113-7D (*MAT*a *MAL2-8c SUC2*) (335) was used as the prototrophic reference strain. All knockout strains (Supplemental Table 1 online) were constructed in this genetic background. Strains were constructed by using standard yeast media and genetic techniques (43). The kanamycin resistance cassette was amplified by PCR by using specific primers (Supplemental Table 1 online) and the pUG6 vector as template (119). As part of the deletion process, each gene disruption was replaced with a KanMX module and uniquely tagged with two 20mer sequences (http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.htm) (Supplemental Table 1 online). The gene YGR059W was either tagged with a unique downtag sequence or an uptag sequence. The deletion of YOR012W carried along inactivation of neighbouring and overlapping ORF YOR013W. The double mutant strain yor012WΔ/yor013WΔ will be referred as yor012WΔ in the rest of the manuscript. Strains were

routinely grown at 30°C on complete media (YPD). Supplemental data can be obtained online on www.bt.tudelft.nl.

Shake-flask cultivation was performed in 500 ml flasks containing 100 ml of medium, which were incubated at 30°C on an orbital shaker set at 200 rpm. The composition of the synthetic medium (SM) was as follows: 20 g liter^{-1} glucose, 5 g liter^{-1} $(NH_4)_2SO_4$, 6 g liter^{-1} KH_2PO_4, 0.5 g liter^{-1} $MgSO_4$, trace elements and vitamin solutions (343). The pH of the medium was adjusted to 5.0 and sterilized by autoclaving. Glucose was autoclaved separately. Vitamins were filter-sterilized and added to the medium. Growth of the various strains was monitored by OD measurements at 660 nm. After growing all strains to mid-exponential phase, an equivalent amount of each mutant strain, corresponding to 0.02 OD_{660nm} unit, was aseptically pooled to prepare a mixed inoculum (30 ml total volume) for the competition experiments.

Chemostat cultivation was performed at 30 °C in 1-liter working volume laboratory fermenters (Applikon, Schiedam, The Netherlands) at stirrer speed of 800 rpm, pH 5.0, with a dilution rate (D) of 0.10 h^{-1} as described previously (333). The pH was kept constant, using an ADI 1030 biocontroller (Applikon, Schiedam, The Netherlands), via the automatic addition of 2 M KOH. The fermentors were flushed with pure nitrogen gas for anaerobic growth and air for aerobic growth at a flow rate of 0.5 liter min^{-1} using a Brooks 5876 mass-flow controller (Brooks Instruments, Veenendaal, The Netherlands). The dissolved-oxygen concentration was continuously monitored with an Ingold model 34 100 3002 probe (Mettler-Toledo, Greifensee, Switzerland) and was 0 % for anaerobic growth and above 70 % for aerobic growth. To sustain anaerobiosis, the medium vessels were sparged with pure nitrogen gas and Norprene tubing was used to minimize oxygen diffusion into the fermentors. Anaerobic carbon-limited steady-state chemostat cultures of the reference strain *S. cerevisiae* ygr059wΔ::uptag (see Results section) were grown on a synthetic medium as described previously (345). Aerobic carbon-limited chemostat cultures contained the same medium but with 7.5 g liter^{-1} glucose and without the anaerobic growth factors Tween-80 and ergosterol. When steady state was achieved, the 30 ml competition mix was aseptically injected into the culture using a syringe. Samples were taken via the effluent line every 24 hours for a period of 216 hours. The samples were chilled on ice, spun down and frozen at -20°C for high-molecular-weight DNA extraction.

High-molecular-weight DNA extraction

DNA samples were purified using an adapted method described by (43). 40 ml of cell culture broth was spun down and resuspended in 1 ml of DNA extraction buffer (2 % Triton X-100, 1 % SDS, 100 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA pH 8.0). 400 µl of the resuspended cells was added to an equal volume of phenol/chloroform/isoamyl alcohol (25/24/1) pH 8.0 and 0.3 g sterile glass beads. The Bio101 Fastprep (Qbiogene, CA) was used to break the cell walls with a speed setting of 4.5 for 15 s. After centrifugation, the supernatant was transferred to 500 µl phenol/chloroform/isoamyl alcohol (25/24/1) pH 8.0 and vortexed. Supernatant was transferred to 1 ml of absolute ethanol (-20°C) for precipitation of DNA and centrifuged for 15 min (13,000 rpm) at room temperature. The DNA pellet was resuspended in 400 µl TE buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA pH 8.0), 15 µl RNAse cocktail (Ambion 2286) and placed at 37°C until fully dissolved. After centrifugation, the chromosomal DNA was re-precipitated with 5 µl 7.5 M ammonium acetate and 1 ml absolute ethanol (-20°C) and immediately centrifuged at 13,000 rpm for 15 min at room temperature. The air-dried DNA pellet was resuspended in 50 µl TE buffer. Quality of DNA was checked with 1% TAE agarose gel. DNA quantity was analysed at OD_{260}.

Quantitative real-time PCR

qrtPCR was run on an DNA engine Opticon I system (BioRad, Hercules CA) with the following settings: 94°C for 2 min. 94°C for 10 s, 55°C for 10 s, 72°C for 10 s and plate reading. The denaturation, annealing, elongation and reading steps were repeated for 49 cycles. A melting curve from 55 to 94°C was performed

at the end of the reaction. The reaction mixture of 20 µl consisted of 10 µl SybrGreen TAG readymix (Sigma S1816), 0.2 mM forward primer, 0.2 mM reverse primer and 50 ng DNA. The C(t) value was calculated with the Opticon Monitor^{TM} software version 1.08 (BioRad, Hercules CA) by setting the threshold for significant detection levels to 10-times the standard deviation over the cycle ranged from 1 to 15. Each time point was carried out in triplicate readings.

Data and statistical analysis

The C(t) values were converted to amounts of DNA concentration (X_{DNA}) via the exponential relationship of X_{DNA} and C(t): $X_{DNA} = a.exp^{-C(t)}$, where a is a variable constant for each strain due to qrtPCR efficiency. For each strain, all X_{DNA} values measured during the 216 hours competition experiment were normalized to the X_{DNA} value at t = 0 to eliminate bias from PCR efficiency. Fitness was calculated by taking the slope of the best-fit linear trend line. The relative reduction of the fitness of mutant strains was calculated from the biomass balance [1]:

[1] $X_t = X_o.exp^{(\mu - D)t}$

where t = time (hrs), X_t = biomass concentration at time t, X_o = initial biomass concentration, μ = growth rate and D = dilution rate. Statistical analysis was done using the modified Z-score (144) to identify mutants that showed significant reduction in fitness (outliers). The modified Z-score was then subjected to a two tailed T-distribution test with 2 degrees of freedom in accordance to the Grubbs' test (18) to calculate the significance *p*-values for each mutant strain. Only mutants with q-value < 0.01 were deemed significantly reduced in fitness.

Results

Selection of target genes and construction of deletion strains

A previous transcriptome analysis of *S. cerevisiae* chemostat cultures yielded 65 genes that showed a higher transcript level in anaerobic chemostat cultures than in aerobic cultures (we will refer to these genes as 'anaerobically upregulated'), irrespective of the growth-limiting macronutrient (313). From these 65 genes, a set of 24 genes was selected for further analysis (Figure 1), based on the following criteria:

1. High change in transcript level (> 3 fold). This led to the elimination of 3 genes whose transcript level varied between 2 and 3-fold.

2. Unclear or unknown function. For example, 8 of the 65 genes are related to sterols and unsaturated fatty acids metabolisms. As these processes require molecular oxygen, their anaerobic upregulation is understood and we therefore eliminated these genes from the present study.

3. Not part of a family of genes with high sequence similarity. For example, 21 of the 65 anaerobically upregulated genes, belong to the seripauperin family (*DAN*, *PAU* and *TIR* genes). Since multiple members of this family were present in the set, redundancy might well obscure the interpretation of the competitive cultivation experiments carried out with

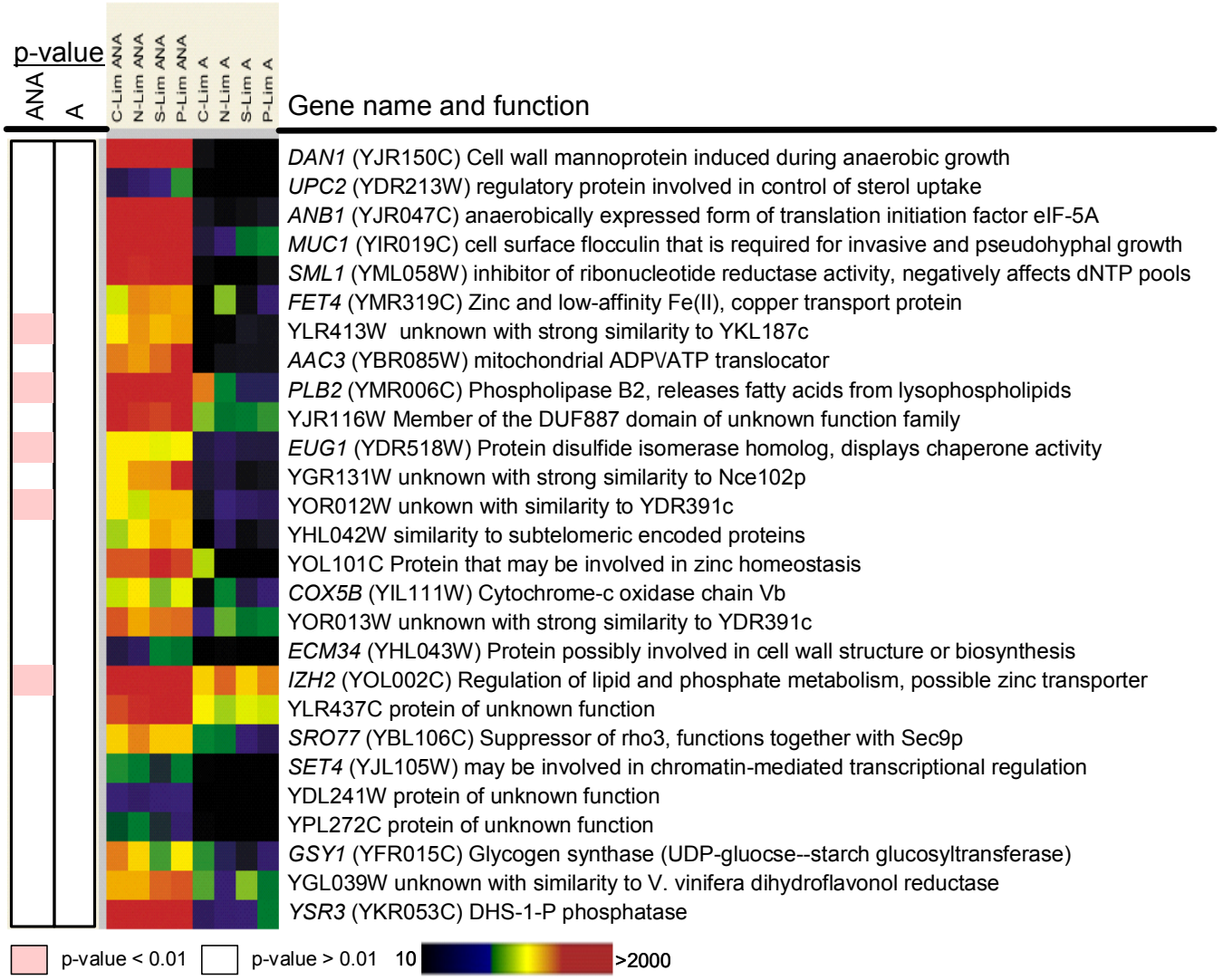

p-value
ANA
A
C-Lim ANA
N-Lim ANA
S-Lim ANA
P-Lim ANA
C-Lim A
N-Lim A
S-Lim A
P-Lim A
Gene name and function
DAN1 (YJR150C) Cell wall mannoprotein induced during anaerobic growth
UPC2 (YDR213W) regulatory protein involved in control of sterol uptake
ANB1 (YJR047C) anaerobically expressed form of translation initiation factor eIF-5A
MUC1 (YIR019C) cell surface flocculin that is required for invasive and pseudohyphal growth
SML1 (YML058W) inhibitor of ribonucleotide reductase activity, negatively affects dNTP pools
FET4 (YMR319C) Zinc and low-affinity Fe(II), copper transport protein
YLR413W unknown with strong similarity to YKL187c
AAC3 (YBR085W) mitochondrial ADP\/ATP translocator
PLB2 (YMR006C) Phospholipase B2, releases fatty acids from lysophospholipids
YJR116W Member of the DUF887 domain of unknown function family
EUG1 (YDR518W) Protein disulfide isomerase homolog, displays chaperone activity
YGR131W unknown with strong similarity to Nce102p
YOR012W unkown with similarity to YDR391c
YHL042W similarity to subtelomeric encoded proteins
YOL101C Protein that may be involved in zinc homeostasis
COX5B (YIL111W) Cytochrome-c oxidase chain Vb
YOR013W unknown with strong similarity to YDR391c
ECM34 (YHL043W) Protein possibly involved in cell wall structure or biosynthesis
IZH2 (YOL002C) Regulation of lipid and phosphate metabolism, possible zinc transporter
YLR437C protein of unknown function
SRO77 (YBL106C) Suppressor of rho3, functions together with Sec9p
SET4 (YJL105W) may be involved in chromatin-mediated transcriptional regulation
YDL241W protein of unknown function
YPL272C protein of unknown function
GSY1 (YFR015C) Glycogen synthase (UDP-gluocse--starch glucosyltransferase)
YGL039W unknown with similarity to V. vinifera dihydroflavonol reductase
YSR3 (YKR053C) DHS-1-P phosphatase
p-value < 0.01
p-value > 0.01
10
>2000

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

single deletion strains. We therefore decided to eliminate members of large gene families from this study.

4. No previously established clear relation with anaerobic growth.

Five additional genes were selected for inclusion in the further experiments. YGR059w was selected as a physiologically neutral marker gene based on transcript data. YGR059w encodes a sporulation-specific septin that functions in cytokinesis, meiosis I, and sporulation, and was not expressed in the haploid CEN.PK113-7D strain in 20 different chemostat conditions (see supplemental data table 2 online). *URA3*, which is essential for uracil biosynthesis, was included as a negative control: in the absence of uracil, *ura3Δ* strains should not grow. Additionally *DAN1*, *UPC2* and *ANB1* were included as extensively studied, anaerobically upregulated genes. *DAN1* encodes for a cell wall mannoprotein induced during anaerobic growth, initially excluded as a member of the seripauperin (PAU) family (349). *UPC2* (Uptake control 2) encodes a sterol regulatory element binding protein involved in the regulation of sterol biosynthetic gene expression and the uptake and intracellular esterification of sterols (364). Finally *ANB1* encodes the translation initiation factor eIF5A, that displays a specific and strong anaerobic transcriptional upregulation (358). In total, 29 genes were further studied by mean of competitive cultivations.

Competitive chemostat experimental design

An outline of the experimental design is presented in Figure 2. All 29 genes were deleted from the start to stop codon in *S. cerevisiae* CEN.PK113-7D and replaced with the *kanMX* deletion cassette flanked by two gene-specific 20-nucleotide tag sequences ((366) , see experimental procedure section). The kanMX cassette has previously been shown not to confer a selective (dis)advantage during prolonged chemostat cultivation of *S. cerevisiae* (14).

In contrast to previous large-scale functional profiling studies (112, 113, 366) where auxotrophic mutant collections were screened, all mutants used in this study were generated in the prototrophic CEN.PK113-7D strain (335). The use of prototrophic strains (with the exception of the *ura3* negative control strain) eliminates the risk that results are influenced by the nutritional requirements of auxotrophic strains (267).

Subsequently, steady-state chemostat cultures were grown with the neutral control mutant ygr059wΔ containing only the uptag (Figure 2). A second ygr059wΔ strain carrying a specific downtag sequence was also made and added to the mutant pool. This latter strain was used to normalize the population dynamics of the other mutants. The mix of deletion strains (see Methods section) was then injected into the steady-state chemostat culture. We prefer this approach over inclusion of the mutant pool during the start-up of the chemostat as previously reported by (14), where cultivation conditions are dynamic and the selective pressure may differ from that under steady-state conditions.

The culture was then sampled daily over a period of nine days (216 h). This time frame was chosen to reduce the impact of evolutionary adaptation, which would render a comparison of the fitness of individual tagged mutants impossible (149, 247) (Figure 2). After DNA isolation, samples were then analysed by quantitative real-time PCR, using the molecular tags to monitor the abundance of each mutant. After normalization to the initial sample, the abundance of the deletion strains was normalised to that of the ygr059wΔ::downtag reference strain included in the mutant pool.

Competitive anaerobic chemostat cultivation

During the competitive anaerobic chemostat experiments, strains that did not grow (μ = 0 hr^{-1}) were expected to disappear from the culture via washout kinetics at the dilution rate of 0.10h^{-1}. This is depicted by the washout line in Figure 3A. Indeed, the auxotrophic *ura3*Δ strain (negative control) closely followed this line (Figure 3A). After 96 h, the abundance of the *ura3*Δ strain did not decrease any further (Figure 3A). This abundance was taken to reflect the threshold for detection in the experimental set-up. The C(t) values measured for the reference strain ygr059wΔ::downtag did not vary by more than 3.6 % in the duplicate experiments over the period of 216 h.

The anaerobic competitive cultivation experiment was performed in two independent chemostat runs. The fitness of the mutants in the anaerobically upregulated genes observed in these two runs were generally in good agreement (Figures 1and 3). The fitness data from each strain were statistically evaluated by means of a statistical test, revealing 5 outliers (p-value < 0.01) from the set of 27 mutants (Figure 1). Consequently we noticed that it was not possible to make reliable statements about decreases in fitness below 20 %. While prolonging the chemostat experiment might lead to increased sensitivity, we decided against this because of the high risk of interference by evolutionary processes (149, 247).

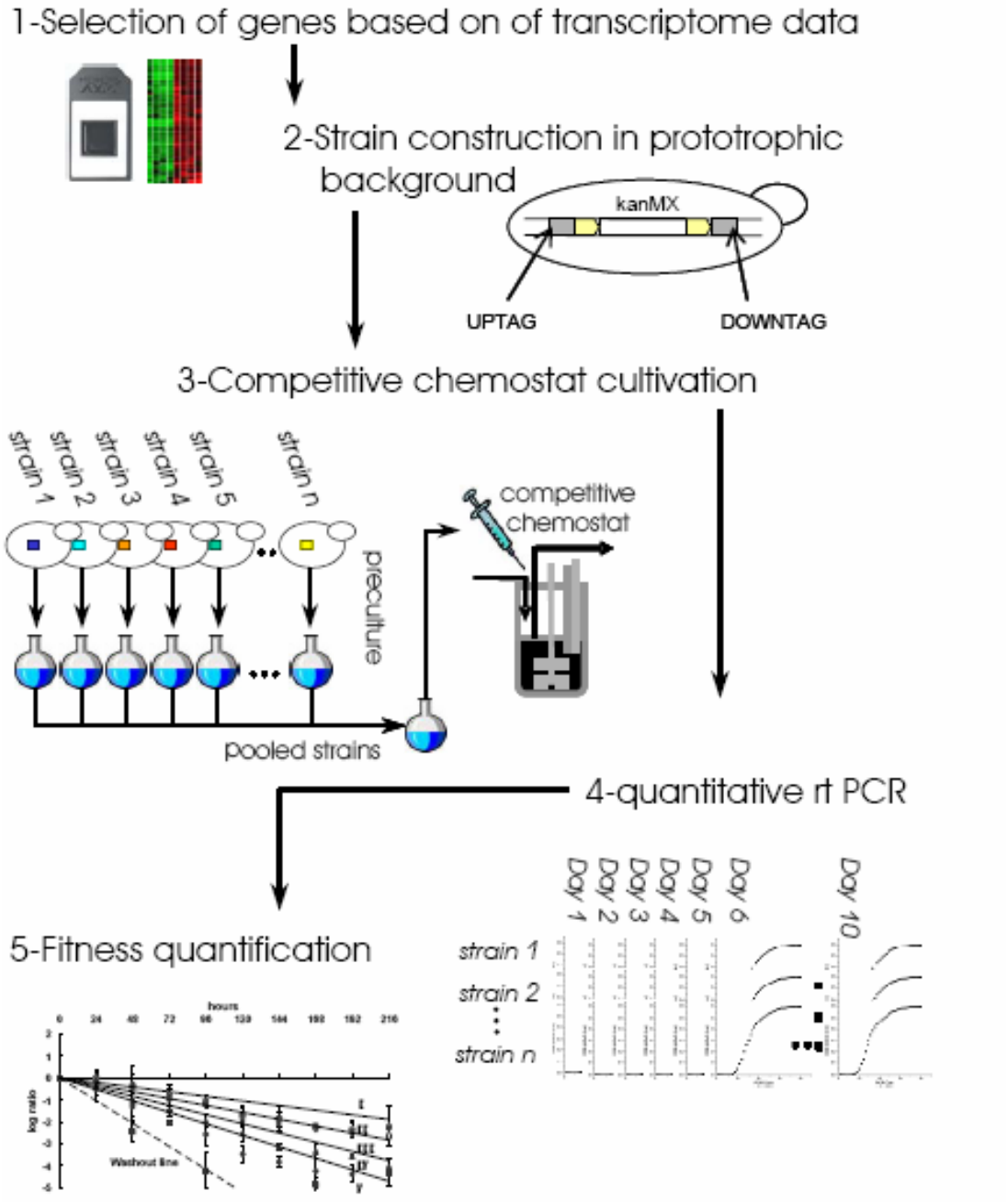
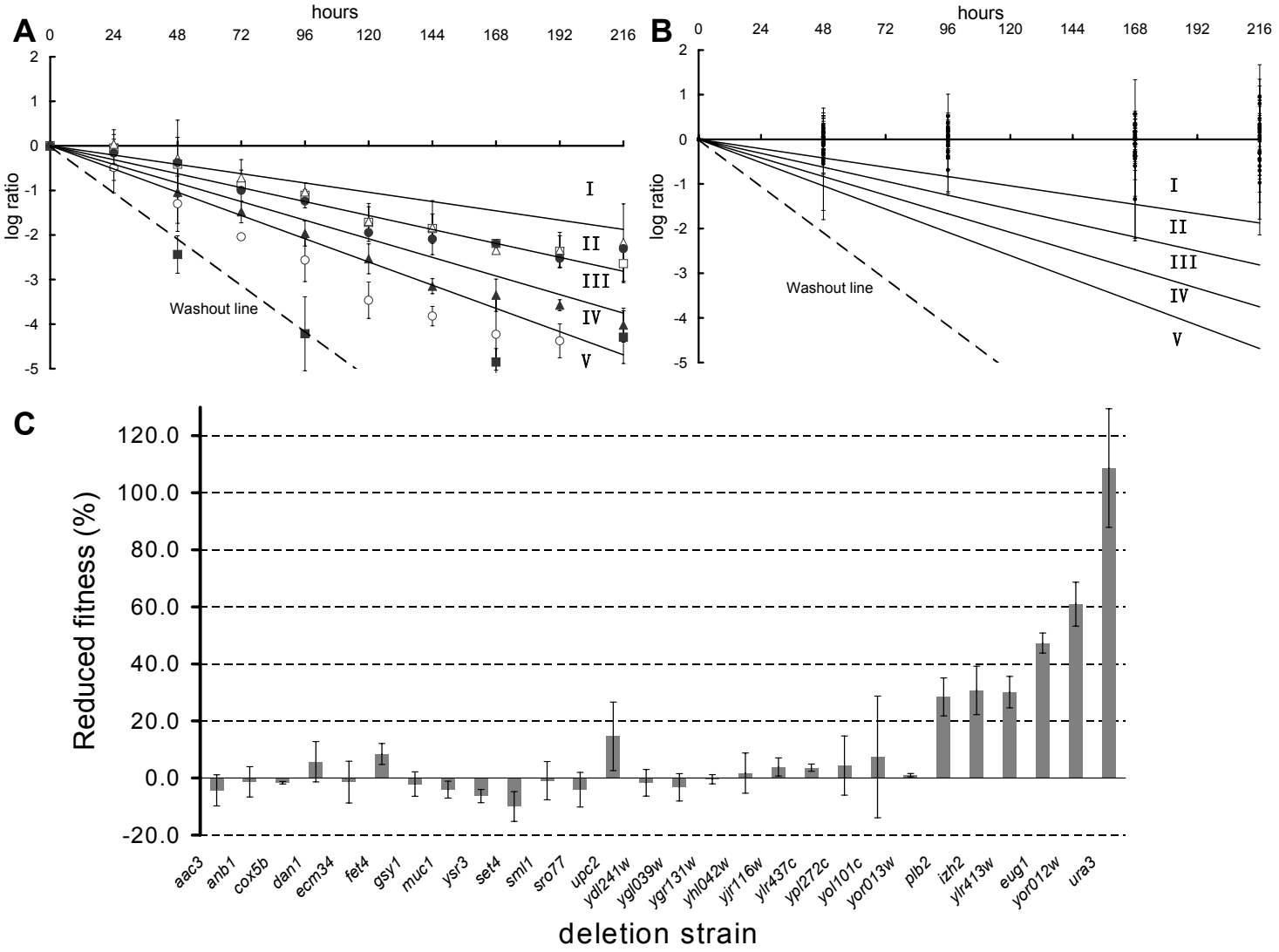


Figure 2: Experimental design


A
hours
0
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log ratio
2
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Washout line
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hours
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log ratio
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-3
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I
II
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IV
V
Washout line
C
Reduced fitness (%)
120.0
100.0
80.0
60.0
40.0
20.0
0.0
-20.0
aac3
anb1
cox5b
dan1
ecm34
fet4
gsy1
muc1
ysr3
set4
sml1
sro77
upc2
ydl241w
ygl039w
ygr131w
yhl042w
yjr116w
ylr437c
ypl272c
yol101c
yor013w
plb2
izh2
ylr413w
eug1
yor012w
ura3
deletion strain

Figure 3: **Results of anaerobic competitive chemostat cultures.** (A) Strains with fitness reduction: Log ratio ($\Delta C\{(t)_{mutant}/\Delta C(t)_{ref}$) as function of time. Graph areas (Roman numbers) indicate the following reductions of fitness (I): < 20 %; (II): 20-30 %; (III): 30-40 %; (IV): 40-50 %; (V): > 50 %. The dashed line denotes washout (zero specific growth rate). The graph only shows mutants that showed a > 20 % reduction of fitness. Symbols: ■ *ura3*Δ, □ ylr413wΔ, ● *izh2*Δ, ○ yor012wΔ, ▲*eug1*Δ, Δ *plb2*Δ. Error bars indicate mean deviation of two independent chemostat cultures with triplicate measurements for each time point. (B) Strains without fitness reduction: Log ratio ($\Delta C\{(t)_{mutant}/\Delta C(t)_{ref}$) as function of time. Error bars indicate mean deviation of two independent chemostat cultures with triplicate measurements for each time point. (C) Bar graph indicating fitness. Reduced fitness of each deletion strain was calculated from the slope of the best-fit linear line. Error bars indicate mean deviation of two independent chemostats.

None of the three anaerobic markers knockout strains *anb1*Δ, *dan1*Δ and *upc2*Δ displayed a significant fitness loss compared to the control strain (ygr059wΔ::downtag). While such a result could be anticipated in the case of *DAN1*, which is part of a large gene family, this result was more unexpected in the case of *ANB1* and *UPC2* that participate in central pathways as transcription and translation. It may be relevant to note that a larger variation in fitness between the two experimental runs was observed for the *upc2*Δ strain than for the *anb1*Δ and *dan1*Δ strains.

Regarding the remaining 24 mutants in anaerobically upregulated genes, only five (*eug1Δ*, *izh2Δ*, *plb2Δ,* ylr413wΔ and yor012wΔ; Figure 3A) showed a significant (20 – 60 %) reduction of fitness in independent replicate experiments (Figures 3A & 3C). Of the 5 genes whose deletion resulted in a reduction of fitness under anaerobic condition, *EUG1* is the most extensively documented. *EUG1* encodes a non-essential protein disulfide isomerase (312). The *S. cerevisiae* genome contains four additional protein disulfide isomerases (*PDI1, MPD1, MPD2* and *EPS1*) of which only *PDI1* is essential (246). In addition to their catalytic role in protein folding, protein disulfide isomerases act as chaperones (163). *IZH2/PHO36* has been proposed to be involved in metabolic pathways that regulate lipid and phosphate metabolism (160). Additionally, I*ZH2* is part of the *ZAP1* regulon and proposed to play a role in zinc homeostasis along with *IZH1*, *IZH3* and *IZH4* (206). *PLB2* encodes a lysophospholipase B involved in phospholipid metabolism (104, 224). Two additional lysophospholipase B are also found in *S.cereviisae* genome, Plb1 (62 % similarity) (189) and Plb3 (57 % similarity) (224) The two remaining genes are very poorly characterized. Several experiments indicate that Ylr413wp is localized at the cell surface ((80, 141) but, just like YOR012w, its function is totally unknown.

Aerobic reference experiments

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

1). As an additional control, the specific growth rates of the five mutant strains that showed a reduced fitness in the anaerobic cultures was measured in (semi-)aerobic shake-flask cultures and were found not to differ significantly from those of the isogenic reference strains CEN.PK113-7D and ygr059wΔ::downtag (Table 1). This implies that the reduction in fitness encountered in five of the mutant strains during anaerobic competitive growth was specific for anaerobiosis.

Table 1: Aerobic growth in shake flask and fitness reduction in aerobic competitive chemostats of the five mutants that showed significant disadvantage in anaerobic competitive chemostats. Data are presented as average ± mean deviation of results from two independent cultures for each strain. n.a. not applicable

Deletion mutant	Shake flask hr^{-1}	Fitness reduction (%)
*plb2*Δ	0.39 ± 0.00	7.0 ± 3.7
ylr413wΔ	0.38 ± 0.02	11.6 ± 3.3
*izh2*Δ	0.38 ± 0.01	15.8 ± 7.4
*eug1*Δ	0.37 ± 0.02	8.6 ± 0.1
yor012wΔ	0.34 ± 0.00	14.8 ± 5.6
ygr059wΔ	0.40 ± 0.02	-
CEN.PK 113-7D	0.39 ± 0.00	n.a.

Discussion

Previous systematic comparisons of transcript levels and fitness of yeast mutants in batch cultures (26, 112, 113, 366)) used the entire *S. cerevisiae* deletion library. The present study is the first to use transcriptome data for selecting target genes in chemostat-based competitive cultivation. We have reported a fitness profiling of knockout strains in genes that showed a significant upregulation under anaerobic conditions. Our experimental approach differs in several aspects from earlier *S. cerevisiae* (13, 14, 56) and *Escherichia coli* (50, 74, 75, 329) chemostat-based competition experiments by the injection of a mutant pool into a steady-state culture, use of qrtPCR for quantification and the selection of strains based on transcriptome studies. This novel setup was (i) sensitive (qrtPCR versus qPCR, colony plate count or Afymetrix™ tag3 array) (ii) cost-effective (goal orientated gene deletion selection) and (iii) yielded reproducible results (immediate fitness test from steady-state conditions and prototrophic strains).

Our study has yielded five priority targets for further functional analysis of the molecular basis for anaerobic growth in *S. cerevisiae*. Further analysis will involve the use of multiple mutations to narrow down gene function in the study. The available

literature provides some interesting leads. Lyons et al. (206) reported that *IZH2* is involved in coordinating both sterol and zinc metabolism under anoxia. The possibility that i*zh2* mutants may be impaired in uptake of sterols, which are essential for anaerobic growth of *S. cerevisiae* (8), merits further research. YLR413w encodes a protein with unknown function that has a 49 % sequence similarity to YKL187c, which is transcriptionally upregulated during growth on oleate (157). It is conceivable that these genes are implicated in the uptake of essential unsaturated fatty acids, which are essential for anaerobic growth. It is relevant to note that, in the present study, oleate was provided as Tween-80 (polyoxyethylene sorbitan monooleate). Tween-80 was introduced as a complex form to compensate for the inability of *S. cerevisiae* to synthesize *de novo* unsaturated fatty acids under anaerobiosis. However for utilization, the acyl-ester bound that links the oleate chain to the polyoxyethylene sorbitan complex must be cleaved. This might be relevant for the loss of fitness recorded for the *plb2*Δ strain. This gene might play an important role in cleaving Tween-80 as it would for lysophosphatidylcholine (104) at the single fatty acid ester bond to yield oleate. Besides, the incomplete functional complementation of *PLB1* and *PLB3* that were also expressed under anaerobic conditions might reflect difference in substrate affinity and specificity of all three yeast phospholipases B as already reported (225).

EUG1 encodes a protein disulfide isomerase of the endoplasmic reticulum rumen. It has been previously suggested (317) that *EUG1* might be involved in glycosylation and the isomerization of disulfide bonds during the folding of anaerobically synthesized Dan/Tir cell wall proteins, but this suggestion has not yet been experimentally followed up. The reason of the fitness loss of the yor012WΔ strain that actually corresponds to the double mutant yor012WΔ/yor013WΔ remained unknown. As a consequence of the overlap between the ORFs, a more elaborated knock-out strategy should be elaborated to study each deletion individually and sort out which of the two genes contributes to the reduction of fitness observed.

Of 24 *S. cerevisiae* genes that showed a strong and consistent transcriptional upregulation under anaerobic conditions but were not previously implicated in anaerobic metabolism based on other experimental approaches, only five were shown to contribute to fitness under anaerobic conditions via competitive cultivation of null mutants. At first glance, it might be argued that this low 'hit rate' is due to the low dilution rate in the chemostat cultures (0.1 h^{-1}, which is 3-fold lower than the maximum specific growth rate μ_{max} of *S. cerevisiae* CEN.PK113-7D under anaerobic conditions (180)). This interpretation is, however, not correct, as mutations that have a negative effect on the maximum specific growth rate will directly affect fitness because they lead to a lower affinity (μ_{max}/K_s) for the growth-limiting nutrient (where K_s is the substrate saturation constant) (45, 232).

Even though we sought to enrich the set of target genes by only including genes that showed a strong and consistent transcriptional upregulation under anaerobic conditions, the low 'hit rate' observed in our study was consistent with two earlier genome scale comparisons between transcript profiles and fitness where *S. cerevisiae* was exposed to DNA damaging agents (26) and grown in various stressful and growth conditions (1 M NaCl, 1.5 M Sorbitol, pH 8 and galactose) (112). Our observations show that high transcript levels cannot be interpreted as evidence for a unique physiological relevance of the encoded protein under the experimental conditions. This conclusion does not, however, imply that the observed transcriptional upregulation under anaerobic conditions is without biological significance.

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

1. Functional redundancy is an inherent problem in the analysis of (single) deletion mutants. While we have sought to reduce the impact of redundancy by eliminating members of highly related gene families from our study, several of the genes display sequence similarity with a single second yeast gene (Figure 1). For example, the role of the 'anaerobic' ATP/ADP translocase encoded by *AAC3* may well be taken over by its 'aerobic' counterparts Aac1p and/or Aac2p (83). *AAC1* is the only aerobic counter part since it is only expressed under aerobic conditions, however *AAC2/PET9* despite a higher expression in the presence of oxygen, is still expressed under anaerobic conditions (Table 2) (313). Similar functional complementation could occur for *UPC2* and *ANB1*, since *ECM22* and *HYP2* their respective homologue, were expressed irrespective of the oxygen regime (Table 2) (313).

   FET4 is another anaerobic marker gene. It encodes a (FeII) low-affinity iron/zinc/copper transport system, and its expression is coregulated by iron and oxygen (150). Under aerobic conditions iron uptake is mainly achieved through the product of *FET3* that encodes a (FeII) high affinity transport system (11). It is well conceivable that deletion of the gene *FET4* is compensated for by overexpression of one or more high-affinity transport systems (Figure 4). A comparable mechanism of gene expression autoregulation has been already reported. Upon deletion of *PDC1* that encodes the major pyruvate decarboxylase, growth on glucose is rescued by overexpression of *PDC5* (135). Overall, in *S.cerevisiae* a quarter of those gene deletions that have no phenotype are compensated by duplicate genes (118).

Table 2: Transcription intensities of genes with corresponding homologues in anaerobic (ANA) and aerobic (A) chemostat cultures with limitations in carbon (C), nitrogen (N), phosphorus (P) and sulfur (S). Mean ± deviations derived from three independent chemostat experiments.

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

2. The impact of the upregulation of a gene on fitness may be context dependent. For example, ammonia-limited growth of *S. cerevisiae* leads to a coordinated upregulation of transporters and enzymes involved in the assimilation of alternative nitrogen sources, even if these are not available in the growth medium (32, 211, 319). Similar mechanisms may underly the transcriptional upregulation under anaerobic conditions of some of the genes included in this study. For example, the oxidoreductase encoded by YGL039w may provide an excellent, energy-efficient redox sink for anaerobic growth – but only in the presence of its unknown substrate. This would also mean that assessing the contribution of transcriptionally upregulated genes would imply testing strains carrying multiple combinatorial deletion of differentially expressed transcripts.
3. The implied teleological relationship between transcript profiles and fitness does not necessarily have to exist for all genes that show a consistent transcriptional response to a given stimulus. For example, transcriptional regulation networks may have evolved to couple transcriptional responses to environmental stimuli that tend to coincide in the natural environment. When these stimuli are separated in the laboratory or in industry, not all transcriptional responses have a direct bearing on each individual stimulus.

The present study underlines that, in *S. cerevisiae*, increased transcript levels cannot be interpreted as evidence for a contribution of the encoded protein to the cell's fitness in the immediate experimental context. A similar conclusion has been drawn based on a comparison of metabolic fluxes and transcript levels of the corresponding genes, which showed that transcript levels cannot be used as 'flux indicators' (70). Rather than diminishing the value of transcriptome analysis, these observations underlines the need for integrated 'systems' approaches to understand functions of genes and genomes.

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Chapter 4

Transcriptional responses of *Saccharomyces cerevisiae* to preferred and non-preferred nitrogen sources in glucose-limited chemostat cultures

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Abstract

Aerobic, glucose-limited chemostat cultures of *Saccharomyces cerevisiae* grown with six different nitrogen sources were subjected to transcriptome analysis. The use of chemostats enabled an analysis of nitrogen-source-dependent transcriptional regulation at a fixed specific growth rate. A selection of preferred (ammonium and asparagine) and non-preferred (leucine, phenylalanine, methionine and proline) nitrogen sources was investigated. For each nitrogen source, distinct sets of genes were induced or repressed relative to the other five nitrogen sources. A total number of 131 of such 'signature transcripts' were identified in this study. In addition to signature transcripts, genes were identified that showed a transcriptional co-response to two or more of the six nitrogen sources. For example, 33 genes were transcriptionally up-regulated in leucine-, phenylalanine- and methionine-grown cultures, which was partly attributed to the involvement of common enzymes in the dissimilation of these amino acids. In addition to specific transcriptional responses elicited by individual nitrogen sources, their impact on global regulatory mechanisms such as nitrogen catabolite repression (NCR) could be monitored. NCR-sensitive gene expression in the chemostat cultures showed that, ammonia and asparagine were 'rich' nitrogen sources. By this criterion, leucine, proline and methionine were 'poor' nitrogen sources and phenylalanine showed an 'intermediate' NCR response.

Introduction

Its ability to use a broad range of nitrogen sources (16, 185, 319) has made *Saccharomyces cerevisiae* a popular model for studying nitrogen-source-dependent regulation. In *S. cerevisiae*, growth and gene expression strongly depend on the identity and concentration of the nitrogen source. Furthermore, carbon skeletons derived from several amino acids are converted to fusel alcohols (87), which are key contributors to flavor in beer and wine fermentation (19, 126, 127, 221, 221).

Based on the specific growth rate in glucose-containing media, nitrogen sources are classified as 'good' ('rich', 'preferred') or 'poor' ('non-preferred') (126, 211, 357). Good nitrogen sources are generally easily converted into glutamate and glutamine (126, 134, 357), with glutamine, asparagine and ammonia as prominent examples (57, 357). Leucine and phenylalanine are considered 'average' nitrogen sources while proline, methionine, γ-aminobutyrate and allantoin exemplify typical poor nitrogen sources (57, 126, 134, 211, 357).

In mixed-substrate cultures, *S. cerevisiae* exhibits a sequential use of good, average and poor nitrogen sources (126). This sequential use is controlled by a transcriptional regulation mechanism known as nitrogen catabolite repression (NCR) (58, 59, 211, 226). When a good nitrogen source is present, NCR shuts down pathways for the use of non-preferred nitrogen sources (57-59, 211). NCR is mediated by upstream activating sequences that contain the GATAA motif and a four-member family of GATA-binding transcription factors (Figure 1): Gln3p, Gat1p, Dal80p, and Gzf3p (30, 65, 66, 211, 304). In the presence of a rich nitrogen source,

Gln3p and Gat1p form cytosolic complexes with Ure2p, which prevents activation of NCR-sensitive transcription (Figure 1). In the absence or at limiting concentrations of a rich nitrogen source, Gln3p and Gat1p are dephosphorylated and targeted to the nucleus, where they activate transcription of NCR-sensitive genes. TOR kinases play a key role in the signal transduction pathway that couples nitrogen status to Gln3p and Gat1p phosphorylation (21, 25, 62-64, 174, 296).

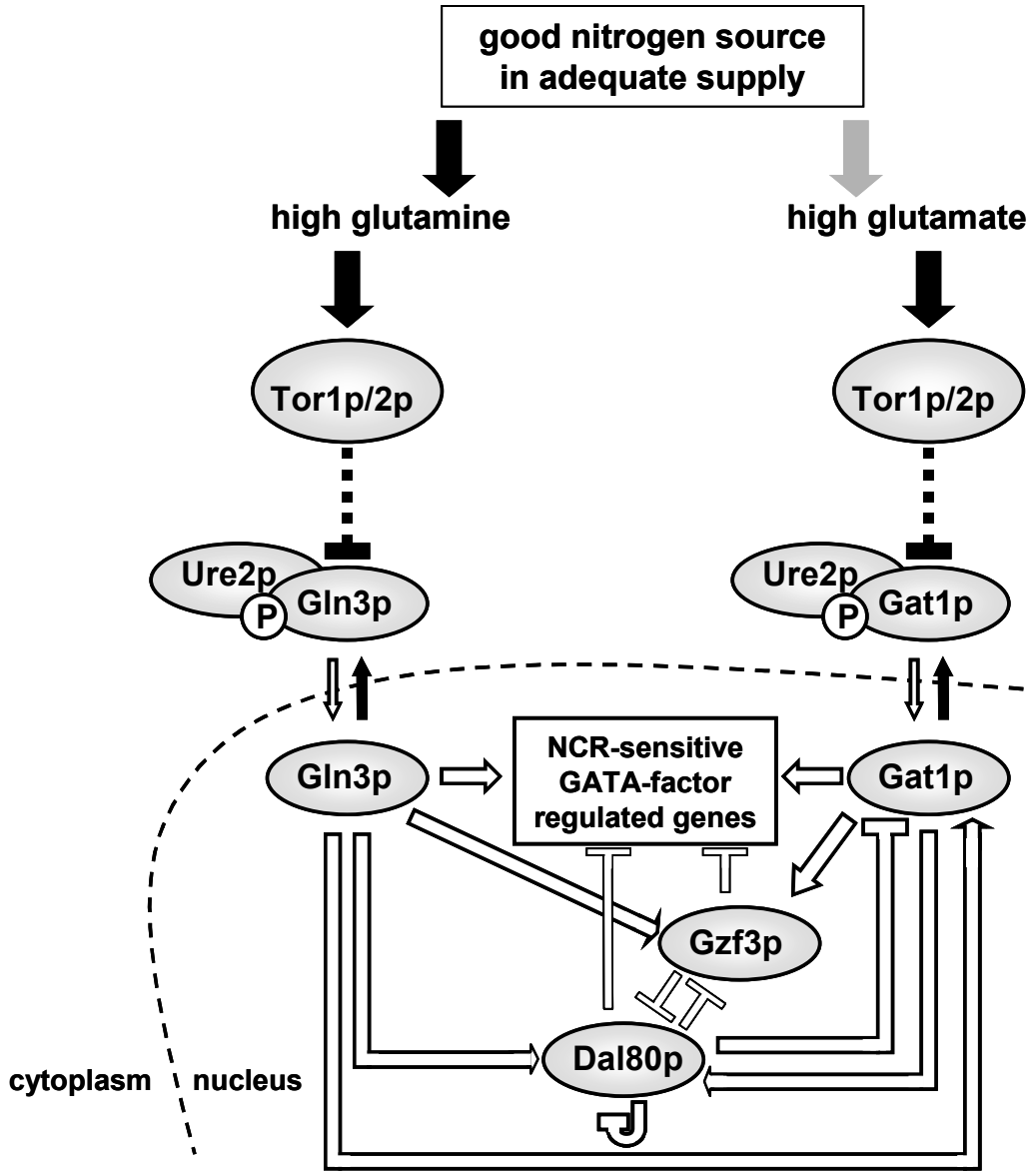


Figure 1: Mechanism of nitrogen catabolite repression. Tor kinases directly and/or indirectly regulate the localization of the transcription factors Gln3p and Gat1p in response to glutamine and glutamate concentrations respectively (21, 25, 63). Black lines indicate processes active during preferred nitrogenous conditions, white lines during non-preferred conditions. Interactions in the nucleus indicate regulation of transcription, outside the nucleus allosteric regulation (picture adapted from (54)).

With amino acids as the nitrogen source, NCR is complemented by specific regulation mechanisms. Most extracellular amino acids are sensed via the trans-

plasma-membrane *SSY1-PTR3-SSY5* (SPS) complex (99). Metabolism of some amino acids is regulated by specific transcriptional regulators, such as Leu3p for leucine (102), Aro80p for aromatic amino acids (146) and the Arg80p/Arg81p/Arg82p/Mcm1p complex for arginine (227, 228).

Research on nitrogen-source-dependent transcriptional regulation has mainly been performed in batch cultures (57, 126, 303, 357). In batch cultures, all nutrients are at least initially present in excess and parameters such as dissolved-oxygen concentration, metabolite concentrations and pH change over time. Furthermore, different nitrogen sources support different specific growth rates. Various physiological parameters, including *e.g.* amino acid pools (357) and protein and RNA levels (90), are strongly influenced by specific growth rate. It has been demonstrated that specific growth rate has a strong impact on transcriptional regulation (124). Consequently, shake flask cultures do not allow for a discrimination between direct effects of nitrogen sources on transcriptional regulation and indirect, growth-rate-dependent phenomena.

Chemostat cultivation is a useful tool for genome-wide transcription studies (32, 40, 262, 282, 302, 313), primarily because it allows the specific growth rate and other important culture parameters to be kept constant. This elimination of specific growth rate as an experimental variable should facilitate analysis of transcriptional responses to different nitrogen sources. For example, transcriptional analysis in chemostats should enable a separation of the role of TOR kinases in NCR (296) from their involvement in cell-growth control (156, 266, 290, 324).

The present study aims to investigate unique and shared transcriptional responses of *S. cerevisiae* to different nitrogen sources and to establish whether the nitrogen-source dependence of NCR is preserved in cultures grown at a fixed specific growth rate. To this end, glucose-limited chemostat cultures of *S. cerevisiae* grown on six different nitrogen sources (ammonium, asparagine, phenylalanine, leucine, methionine or proline) were subjected to transcriptome analysis.

Experimental Procedures

Strain and growth conditions

The prototrophic *S. cerevisiae* strain CEN.PK113-7D (*MAT***a**) (69, 335) was grown at 30°C in 1.0-liter working volume chemostats as described previously (333). Cultures were fed with a synthetic medium (345) that supported glucose-limited growth. The glucose concentration in the reservoir medium was 7.5 g·l-1. Different nitrogen sources were used, at the following concentrations: $(NH_4)_2SO_4$, 5.0 $g \cdot l^{-1}$; L-asparagine, 5.0 $g \cdot l^{-1}$; L-phenylalanine, 5.0 $g \cdot l^{-1}$; L-leucine, 10.0 $g \cdot l^{-1}$; L-proline, 8.8 $g \cdot l^{-1}$; L-methionine, 11.3 $g \cdot l^{-1}$. When an amino acid served as nitrogen source, the synthetic medium was supplemented with 6.6 $g \cdot l^{-1}$ K_2SO_4. The dilution rate was set at 0.10 h^{-1}. The pH was measured online and kept constant at 5.0 by the automatic addition of 2 M KOH with the use of an Applikon ADI 1030 biocontroller. Stirrer speed was 800 rpm and the airflow was 0.5 $l.min^{-1}$. Dissolved oxygen tension was measured online with an Ingold model 34 100 3002 probe, and was above 50% air saturation. A condenser connected to a cryostat set at 2 °C cooled the off-gas, and oxygen and carbon dioxide were measured off-line. Steady-state samples for arrays were taken after 10 to 14 volume changes to minimize the impact of

evolutionary adaptation that occurs after long term cultivation (70, 92). Dry weight, metabolite-, and gas profiles were constant over at least 3 volume changes prior to sampling for RNA extraction.

Analytical methods

Culture supernatants were obtained after centrifugation of chemostat samples and were analyzed by a high-performance liquid-chromatography set-up fitted with an AMINEX HPX-87H ion exclusion column using 5 mM H_2SO_4 as the eluent. Culture dry weights were determined via filtration as described by Postma *et al* (265). Total nitrogen and ammonium were determined with the use of DRLANGE cuvette tests (Düsseldorf, Germany).

Microarray analysis

Sampling of cells from chemostats, probe preparation and hybridization to Affymetrix GeneChip® Microarrays was performed as described by Piper *et al* (262). The results for each growth condition were derived from three independent chemostat cultures.

Data acquisition and statistical analysis

Acquisition and quantification of array images and data filtering were performed using the Affymetrix software packages: Microarray Suite v5.0, MicroDB v3.0 and Data Mining Tool v3.0. For comparison, all arrays were globally scaled to a target value of 150 using the average signal from all gene features using Microarray Suite v5.0. To eliminate insignificant fold changes, absent genes (value below 12) were set to 12. For the identification of changed transcripts between conditions, Microsoft Excel running the software package Significance Analysis of Microarrays (SAM; v1.12) (330) was used on all probe sets that were present in at least one condition. Statistical significance of observed differences was assessed by SAM using an expected false discovery rate (FDR) of 1% and a two-fold-change cut-off. For visualization of significantly changed genes, TreeView v1.60 was used. For clustering of genes with similar transcriptional profiles with NCR 'marker' genes, Genespring v6.02 was used. A confidence level of 0.80 was applied for all nine analyses (one analysis for each 'marker' gene) and genes were selected that met this criterion in at least five of the nine analyses. To calculate the significance of the differences in NCR-strength between conditions, a Students' t-test was used with n-1 degrees of freedom to calculate the p values. To represent the variation in the triplicate measurements, the average coefficient of variation was calculated as described previously for all conditions (32).

Results

Physiology of S. cerevisiae during growth on ammonium or amino acids

To verify our choice of good, intermediate and poor nitrogen sources, *S. cerevisiae* CEN.PK113-7D was grown in shake flasks on glucose with the six nitrogen sources selected for this study (Table 1). The specific growth rate was highest on asparagine, followed by ammonium. Phenylalanine and leucine gave significantly lower rates, while proline and methionine supported the lowest specific growth rates. These results are consistent with earlier studies in other *S. cerevisiae* strains (47, 57, 357).

To study quantitative physiology and genome-wide transcriptional regulation, *S. cerevisiae* CEN.PK113-7D was grown in aerobic glucose-limited chemostat cultures with either ammonium sulfate or one of the five amino acids as the nitrogen source. Under all conditions, glucose metabolism was fully respiratory, as no ethanol

was detected. Consistent with the absence of alcoholic fermentation, all cultures except for those grown on asparagine as the nitrogen source showed a respiratory quotient (RQ) of 1.

Despite the absence of alcoholic fermentation, the biomass yield on glucose was remarkably different for the six nitrogen sources (Table 1). On ammonium, the biomass yield was 0.49 g·(g glucose)$^{-1}$, which is a well-established value for these conditions (205, 340, 345). During growth on proline and asparagine, the biomass yield on glucose was much higher (0.79 and 0.63 g·(g glucose)$^{-1}$, respectively; Table 1). This increased biomass yield is probably due to the assimilation of the carbon skeletons of these amino acids into biomass and/or their use as an additional energy source. The high RQ of the asparagine-grown cultures (Table 1) is consistent with the fact that complete oxidation of oxaloacetate, a degradation product from asparagine, requires only 2.5 O_2 for the production of 4 CO_2. Conversely, the biomass yields on glucose of cultures grown on phenylalanine, leucine and methionine were much lower than the biomass yield found in ammonium-grown cultures (Table 1). This reduced biomass yield, as well as the increased respiration rates in these cultures (Table 1), implies a high-energy requirement associated with the use of these compounds as nitrogen sources.

In all cultures, the nitrogen source was present in excess (Table 1). In the ammonium- and asparagine-grown cultures free ammonium was detected in the culture supernatants (Table 1). In ammonium-grown cultures this is merely the excess ammonium due to the medium design. In asparagine-grown cultures, ammonium is formed due to a direct deamination reaction in the first step of asparagine degradation (297). Apparently, asparagine degradation leads to a surplus of ammonium, which is released from the cells.

Table 1: Specific growth rates in batch cultures and physiological parameters of chemostat cultures used in this study. (Unless indicated otherwise, data represent the average and S.D. of data from two independent batch cultivations or three independent steady-state chemostat cultivations).

	Batch	Chemostat							
N-source	μ[a]	Y_{sx}[b]	$q_{glucose}$[c]	q_{O2}[d]	q_{CO2}[e]	RQ[f]	Carbon recovery	Residual N	Residual NH_4^+
	hr^{-1}						%	mM	mM
Ammonium	0.37 ± 0.01	0.49 ± 0.01	1.1 ± 0.0	2.8 ± 0.3	2.8 ± 0.3	1.0 ± 0.0	98 ± 3	58 ± 1	58.2 ± 1.3
Asparagine	0.45 ± 0.00	0.63 ± 0.02	0.9 ± 0.0	2.7 ± 0.0	3.3 ± 0.0	1.2 ± 0.0	100 ± 2	43 ± 2	15.7 ± 2.3
Phenylalanine	0.28 ± 0.01	0.31 ± 0.03	1.7 ± 0.2	6.7 ± 0.7	6.7 ± 0.7	1.0 ± 0.0	101 ± 4	28 ± 2	< 1
Leucine	0.26 ± 0.00	0.28 ± 0.03	2.0 ± 0.2	8.0 ± 1.0	7.7 ± 1.0	1.0 ± 0.0	93 ± 1	69 ± 0	BD[g]
Methionine	0.20 ± 0.01	0.34 ± 0.01	1.6 ± 0.0	5.6 ± 0.1	5.5 ± 0.1	1.0 ± 0.0	95 ± 2	73 ± 2	BD
Proline	0.20 ± 0.01	0.79 ± 0.01	0.7 ± 0.0	3.0 ± 0.2	3.0 ± 0.2	1.0 ± 0.1	97 ± 5	47 ± 1	BD

[a] Specific growth rate.

[b] Biomass yield on glucose (g biomass/g of glucose consumed).

[c] mmol of glucose consumed/g of biomass/h.

[d] mmol of oxygen consumed/g of biomass/h.

[e] mmol of carbon dioxide produced/g of biomass/h.

[f] RQ, respiratory quotient (q_{CO2}/q_{O2}).

[g] BD, below detection.

Microarray reproducibility and global transcriptome responses

To control reproducibility and data quality, three independent chemostat cultivations were performed for each growth condition. The average coefficient of variation (ACV) for the triplicates of the six nitrogen sources was 0.21 or lower (Table 2). As an additional check the transcript levels of *ACT1* and *PDA1*, two commonly accepted Northern-blot loading standards (240, 360) were analysed. Levels of these transcripts were not notably different for cultures grown on the six different nitrogen sources (Table 2).

Table 2: Summary of microarray experiment quality parameters for each growth condition.

Nitrogen source	ACV[a]	*ACT1*[b]	*PDA1*[c]
Ammonium	0.18	2738 ± 392	352 ± 30
Asparagine	0.14	2418 ± 122	317 ± 19
Phenylalanine	0.21	2917 ± 575	441 ± 39
Leucine	0.14	2149 ± 204	413 ± 64
Methionine	0.11	2393 ± 143	490 ± 33
Proline	0.12	2295 ± 128	469 ± 53

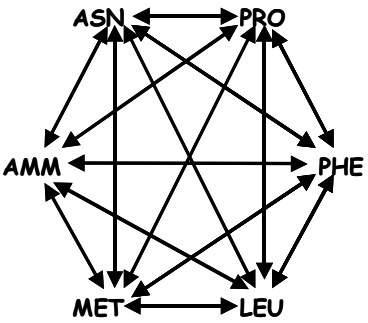
[a] Represents the average of the coefficient of variation (S.D. divided by the mean) for all genes except the 900 genes with the lowest mean expression.

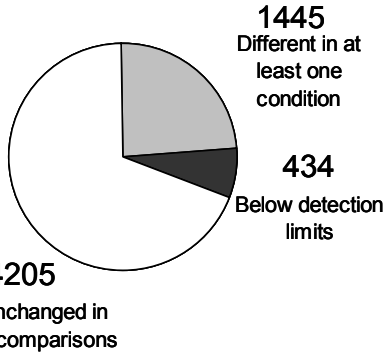
[b] Encoding actin; average signal and standard deviation from probe set "5392_at" comprised of 16 probe pairs found within 400 nucleotides of the 3' end of the open reading frame.

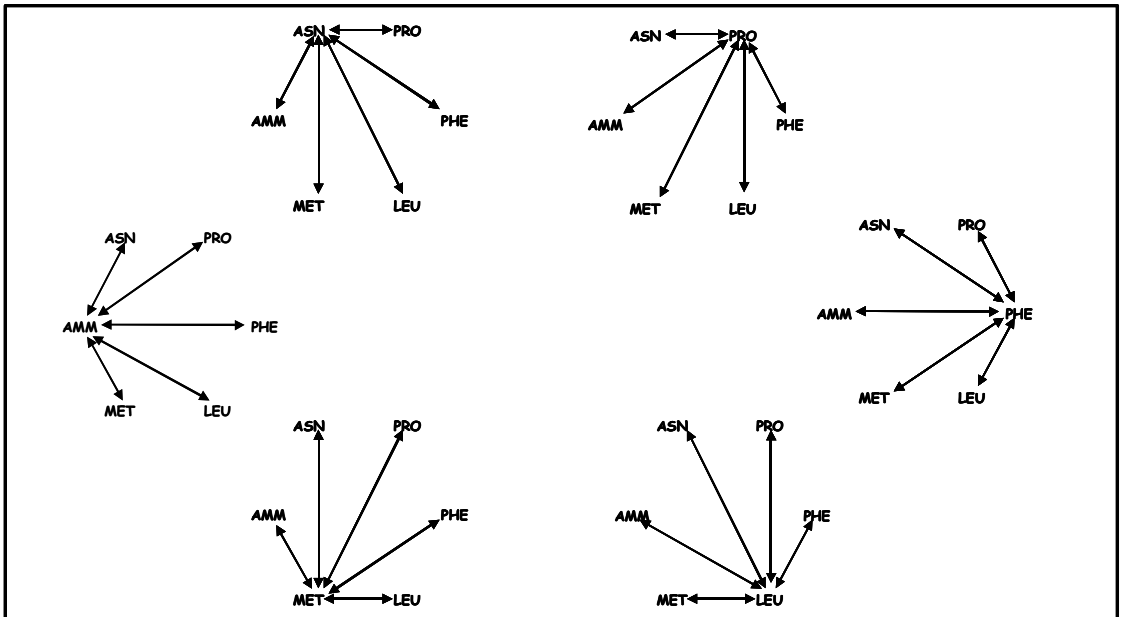
[c] Encoding pyruvate dehydrogenase; average signal and standard deviation from probe set "5526_at" comprised of 16 probe pairs found within 400 nucleotides of the 3' end of the open reading frame.

To analyse the transcriptome data, pair-wise comparisons were performed between the transcriptome data for the six nitrogen sources (Figure 2). In total, 30 pair-wise comparisons were carried out (reciprocal pair-wise comparisons, e.g. 'ammonium-grown cultures vs. asparagine-grown cultures' and 'asparagine-grown cultures vs. ammonium-grown cultures' were counted as two comparisons). 1445 genes (24 % of the genome) showed a different transcript level in at least one comparison (threshold of 2 fold-change with a 1 % false discovery rate), while 434 genes (representing 7 % of the genome) did not yield a detectable transcript level on any of the six nitrogen sources. The remaining 4205 transcripts differed by less than 2-fold in all pair-wise comparisons (Figure 2).

Figure 2: (opposing page). **Pair-wise comparisons of transcriptome data used in this study** (normal numeric denotes up-regulation and italic denotes down-regulation on the nitrogen source per column). [a] Transcripts that are consistently up- or down-regulated in each nitrogen source when compared with the other sources.







	$(NH_4)_2SO_4$	ASN	PHE	LEU	MET	PRO
$(NH_4)_2SO_4$		198 / *56*	143 / *184*	321 / *227*	225 / *218*	124 / *19*
Asparagine (ASN)	56 / *198*		92 / *250*	193 / *251*	219 / *430*	71 / *163*
Phenylalanine (PHE)	184 / *143*	250 / *92*		98 / *39*	174 / *129*	254 / *91*
Leucine (LEU)	227 / *321*	251 / *193*	39 / *98*		131 / *147*	252 / *177*
Methionine (MET)	218 / *225*	430 / *219*	129 / *174*	147 / *131*		273 / *147*
Proline (PRO)	19 / *124*	163 / *71*	91 / *254*	177 / *252*	147 / 273	
Signature transcripts[a]	2 / *3*	12 / *10*	2 / *3*	5 / *17*	17 / *34*	26 / *0*

Specific transcriptional response to growth on one out of the six nitrogen sources tested is extremely limited.

Out of the 1445 genes that showed differential expression between at least two cultivation conditions, only 131 genes were identified that yielded a unique response to one of the six nitrogen sources tested. Each of the six nitrogen sources tested yielded discrete sets of signature transcripts, although the number of signature transcripts for each nitrogen source differed (Figures 2 and 3).

During growth on ammonium as the sole nitrogen source, two genes were consistently up-regulated. *RPL7B* encodes a ribosomal protein and YLR211C a protein with unknown function. Three transcripts were lower in ammonium grown cultures than in cultures grown on the five amino acids (Figure 3). *CAR1* encodes the

		Promoter element analysis				
		Promoter element[a]		Putative-binding protein	Gene coverage %	Genome coverage[b] %
		Forward	Reverse			
NH_4 vs. ASN PHE LEU MET PRO	**Significantly up-regulated in ammonium (2)** Protein synthesis: *RPL7B* Unclassified: YLR211C	NS				
	Significantly down-regulated in ammonium (3) Metabolism: *CAR1* Transport: *DIP5* Cell fate: *ABP1*	NS				
ASN vs. NH_4 PHE LEU MET PRO	**Significantly up-regulated in asparagine (12)** Metabolism: YHR033W Transport: *ALP1* Cell wall maintenance: *CWH36* Cell fate and protein synthesis: *OPT1, RPL41A* Unclassified: *PRM3*, YDR396W, YLR101C, YMR141C, YOL150C, YOL155C, YPR150W	NS[c]				
	Significantly down-regulated in asparagine (10) Metabolism: *DAL3, GDH3, PUT1* Transport: *GAP1, MEP1, MEP2, PDR12* Transcription: *MSN4* Unclassified: *ICY1*, YPL264C	ATCTTATC	GATAAGAT	Gln3p/Gat1p/Dal80p/ Gzf3p	50	3
PHE vs. NH_4 ASN LEU MET PRO	**Significantly up-regulated in phenylalanine (2)** Transcription: *ARO80, MIP6*	NS				
	Significantly down-regulated in phenylalanine (3) Metabolism and energy: *GLY1*, YPL088W Unclassified: YLR108C	NS				
LEU vs. NH_4 ASN PHE MET PRO	**Significantly up-regulated in leucine (5)** Metabolism: *ECM40* Transport: *OPC2* Cell cycle: *CTF13* Unclassified: YOR135C, YOR203W	NS				
	Significantly down-regulated in leucine (17) Metabolism and energy: *HSP12, ISU2, LEU1, LEU2, MAL32, PGM2, SUC2* Transport: *OAC1* Cell wall/cycle/rescue: *DDR2, HSP30, MSC1, SPI1, SSA4, YGP1* Unclassified: YHR087W, YER067W, YMR206W	mAGGGGC	GCCCCTk	Msn2p/Msn4p	41	8
MET vs. NH_4 ASN PHE LEU PRO	**Significantly up-regulated in methionine (17)** Metabolism: *GIT1, SPE1*, YHL008C, YFR055W Transport: *HXT3, FUR4* Cell fate/rescue: *BAR1, SST2*, YIL121W Transcription: *HAC1*, YLL054C Protein fate: YNR069C Unclassified: *COS10, SKY1, YPS5*, YKL158W, YJL213W	NS				
	Significantly down-regulated in methionine (34) Metabolism: *ADH2, ADY2, ECI1, ECM17, FOX2, GRE2, MET1, MET10, MET14, MET16, MET17, MET2, MET28, MET3, MET32, MET6, MHT1, PCD1, STR3*, YNL335W, YPL113C Transport: *MMP1, PXA1, STL1, SUL1, SUL2* Cell cycle and rescue: *CHL4, CTA1, MXR1, RAD59* Unclassified: YLR364W, YLR194C, YDL218W, YMR009W	TCACGTG	CACGTGA	Cbf1p/Met4p/Met28p	44	6
		AAACTGTGG	CCACAGTTT	Met31p/Met32p	26	1
PRO vs. NH_4 ASN PHE LEU MET	**Significantly up-regulated in proline (26)** Metabolism: *TKL2* Transport: *AQY1, DAL4, MCH5, PHO84* Cell rescue and defense: *PAU1, PAU2, PAU3, PAU4, PAU5, PAU6, PAU7, PIR3, RTA1, TSA2* Unclassified: *DAN2, DAN3*, YLL064C, YIR041W, YMR325W, YKL224C, YAL068C, YGL261C, YGR294W, YDL218W, YIL176C	AGGCAC	GTGCCT		50	17
		CCGTTTAG	CTAAACGG		50	2
		TCGTTCA	TGAACGA		65	9
		ATACGA	TCGTAT		85	27

Figure 3: **Signature transcripts and the identities of the genes that were specifically up- or down-regulated in each of the nitrogen sources.** The three independent transcriptome datasets were averaged and then compared. The gene coverage of over-represented sequences from the promoters of co-regulated genes is also presented. Elements were counted present in a gene promoter if they occurred at least once.
[a] Redundant nucleotides are given by: m = A or C; k = G or T. [b] Relative to 6451 ORF upstream promoters in the yeast genome according to RSAT. [c] NS = no significant enrichment of motifs were identified from RSAT).

first enzyme in the arginine catabolic pathway, *ABP1* encodes an actin-binding protein and *DIP5* encodes an amino acid permease that transports Glu, Asp, Gln, Asn, Ser, Ala and Gly (271). The strongly elevated transcript level (average of 52-fold difference between ammonium and amino-acid grown cultures) suggests that *DIP5* is induced by a wide range of amino acids, even when these are not substrates for Dip5p. Repression of *DIP5* by ammonium ions is less likely in view of the high transcript levels in asparagine cultures, in which high concentrations of residual ammonium were observed. *DIP5* has recently been shown to be regulated by Grr1p, which is also involved in the transcriptional regulation of genes encoding the SPS-dependent permeases *AGP1, BAP2, BAP3, GNP1* and *TAT1* (85).

The 12 genes that showed an elevated transcript level in asparagine-grown cultures were scattered over several functional categories, and most of them lacked a clearly established biochemical function. Of the 10 genes that yielded a reduced transcript level on asparagine, six (*DAL3, GAP1, GDH3, PUT1, MEP1* and *MEP2*) are involved in nitrogen metabolism and have been demonstrated to be NCR-sensitive. Indeed, a motif containing the GATAA sequence was over-represented in this set of genes (Figure 3).

During growth on phenylalanine, two genes (*ARO80* and *MIP6*) showed an elevated transcript level relative to cultures grown on the other five nitrogen sources: *ARO80* encodes a positive regulator of *ARO9* and *ARO10*, both involved in phenylalanine degradation (146). The three genes with a reduced transcript level could not be directly linked to phenylalanine metabolism.

The five genes with specifically elevated transcript levels in leucine-grown cultures were scattered over several functional categories. Most of the 17 genes that were specifically down-regulated on leucine were related to either metabolism or cell rescue (Figure 3). Of these, *LEU1* and *LEU2* are directly linked to leucine metabolism and *ISU2* is linked to the iron-sulfur assembly cluster on *LEU1*. In silico promoter analysis of these 17 genes showed a five-fold overrepresentation of a promoter element that harboured the CCCCT sequence known to be bound by the “general” stress-related transcription factors Msn2p and Msn4p.

The 17 genes that yielded an increased transcript level with methionine as the nitrogen source belonged to a variety of functional categories and did not yield over-represented promoter elements. Of these genes, *SPE1*, encoding ornithine decarboxylase and YFR055W could be linked to methionine metabolism. YFR055W exhibits a strong sequence similarity to genes encoding β-cystathionases. Of the 34

down-regulated transcripts, 21 were related to metabolism, 5 to transport, 4 to cell rescue and cell cycle, while 4 transcripts were from genes with unknown function. Of the 21 genes involved in metabolism, 14 were directly related to methionine metabolism. In fact, all genes encoding enzymes involved in the synthesis of methionine from extracellular sulfate were down-regulated (*MET3*, *MET14*, *MET16*, *MET5/ECM17*, *MET10*, *MET25/MET17*, and *MET6*), as well as the transcriptional regulators of methionine and cysteine metabolism, *MET28* and *MET32*. In addition, permeases involved in transport of sulfur-containing compounds (*SUL1, SUL2* and *MMP1*) were down-regulated. In silico promoter analysis of the down-regulated genes showed an enrichment of two promoter elements corresponding to the binding sites for the Cbf1p/Met4p/Met28p complex and the Met31p/Met32p transcriptional regulators. Together, these transcriptional regulation complexes control sulfur fixation and biosynthesis of the sulfur-containing amino acids (324).

Growth on proline yielded 26 genes with a specifically elevated transcript level (Figure 3). Remarkably, 17 of these were members of the 23-member seripauperin protein family, characterized by the lack of a serine-rich C-terminus. It has been postulated that seripauperins contribute to the make-up of cell wall mannoproteins and stress resistance (214, 274, 349). However, these results have to be interpreted with care as high degree of sequence similarity of the members of the seripauperin family may have led to some cross hybridization on the arrays. The 9 remaining genes that responded uniquely to proline are mainly involved in transport and detoxification. Of the 26 genes specifically elevated during growth on proline, four over-represented promoter elements were found, all of which reside in half or more of the genes. The identity of possible binding proteins remains to be elucidated.

Genes with transcriptional co-responses to different nitrogen sources

Because of the involvement of common genes in transport, assimilation and dissimilation of different nitrogen sources, some of the genes involved can be expected to exhibit transcriptional co-responses to different nitrogen sources. To identify co-responsive genes, transcriptomes of cultures grown on the five amino acids were subjected to pair-wise comparisons with those of ammonium-grown cultures. Ammonium-grown cultures were used as a reference because ammonium is the only non-amino acid nitrogen source used in the present study. The 1001 genes (16 % of the genome) that were found to exhibit transcriptional co-responses were clustered based on nitrogen-source dependent expression patterns (Figure 4).

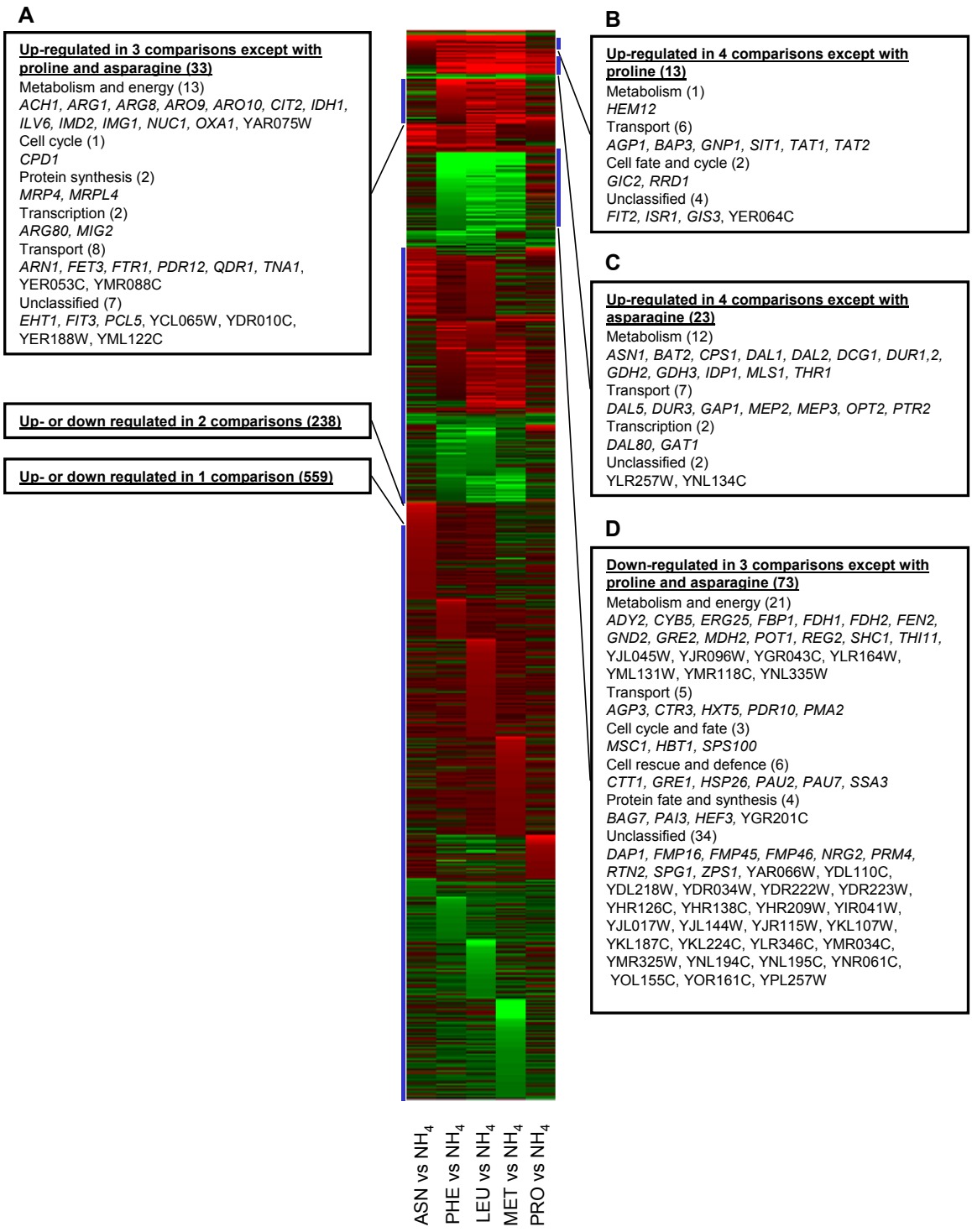


Figure 4: **Overall Eisen diagram and identities of the genes grown on different nitrogen sources that were cooperatively up- or down-regulated compared to ammonium.** The three independent transcriptome datasets were averaged and then compared. *Red* denotes a positive fold-change compared to ammonium while *green* squares denote a negative fold-change compared to ammonium grown cultures. List of all genes can be obtained on www.bt.tudelft.nl/nitrogen-source (Supplementary data Table 1).

Genes showing a similar upregulation on non preferred nitrogen sources (proline, phenylalanine, leucine and methionine)

A group of 23 genes was up-regulated during growth on every amino acid except on asparagine when each condition was compared to ammonia condition (Figure 4, panel A). This matches the classification of phenylalanine, leucine, methionine and proline as non-preferred nitrogen sources and of asparagine and ammonium as preferred nitrogen sources. The release of ammonium by asparagine-grown cultures (Table 1) may also contribute to the similar transcriptional response of these genes in asparagine- and ammonium-grown cultures. Of these 23 genes, 6 are involved in metabolism of the non-preferred nitrogen sources allantoin and urea (*DAL1*, *DAL2, DAL5, DUR1, DUR2*, *DUR3*) and 5 genes encoding transporters for nitrogen-containing compounds (*GAP1*, *PTR2*, *MEP2*, *MEP3* and *OPT2*). This group also includes the GATA-factor encoding genes *DAL80* and *GAT1*, and *GDH2* and *GDH3*, which encode NAD-dependent and NADP-linked glutamate dehydrogenases, respectively. 14 genes are established NCR-targets. An over-representation of the GATAAG motif was found within these 23 transcripts. This motif was present in 39 % of this gene cluster (with an occurrence of at least twice on the promoter region) compared to 3 % in the whole genome (not shown).

To analyse more in depth the NCR-mediated regulation in cultures grown at a fixed specific growth rate, two additional and complementary approaches were undertaken. First, a supervised approach, where the transcript levels of established GATA-factor regulated genes (as reported in Yeast Proteome Database (60)) were used to indicate NCR-strength (Table 3). To minimize secondary effects, genes from this list were excluded when (i) regulation by only one GATA factor was reported, (ii) interfering regulatory mechanisms (like Gcn4p or Put3p) could take effect, (iii) genes had a low expression (average below 80 units). This left us with nine NCR-sensitive 'marker' genes (Table 3). The average normalized signals of these nine transcripts were used to illustrate the trend over the six conditions (Figure 5, black bars).

In a second approach, we used the transcript levels of 32 ORFs previously identified to be specifically up-regulated by ammonium limitation (313) to indicate NCR-strength. Ammonium is regarded as a preferred nitrogen source, however when ammonium is limiting, this induces relief of NCR. The average normalized signals of these 32 transcripts were used to illustrate the trend over the six conditions (Figure 5, gray bars).

The normalized transcript levels in the two comparisons described above were also subjected to a Students' t-test to assess significant differences between the conditions. P-values below 0.01 were considered significant (Figure 5).

The two approaches reveal the same trend of NCR-sensitive transcript abundance over the six conditions. The average transcript levels in both approaches indicate that during growth on ammonium and asparagine, NCR-sensitive transcript abundance is lower compared to the other conditions ($p<0.01$). Growth on phenylalanine causes a lower NCR-sensitive transcript abundance than growth on

Table 3. Established and newly identified NCR sensitive genes. References can be obtained from online supplementary data Table 2 at www.bt.tudelft.nl/nitrogen-source

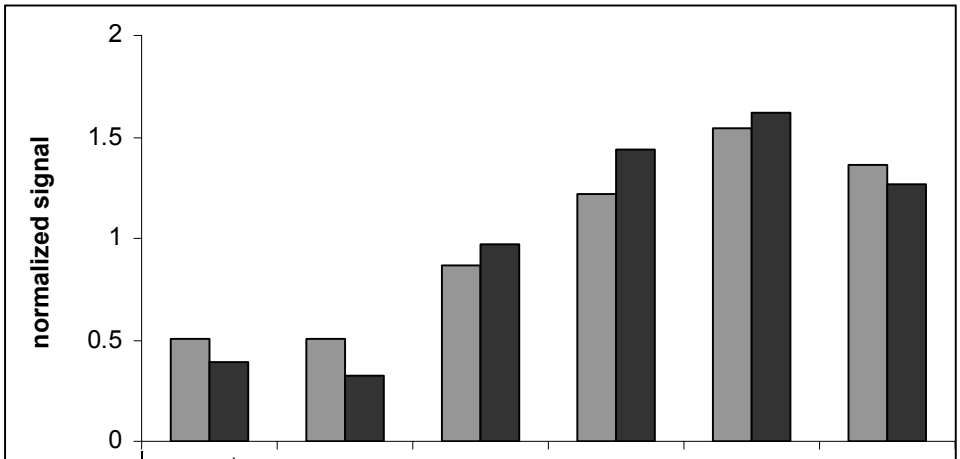
	GATA-Factor regulated[a]						
Gene	Gln3p	Gat1p	Dal80p	Gzf3p	Additional documented transcriptional regulators[b]	N-lim sig. trans.[c]	similar NCR profile[d]
CAN1[e]	✓		✓				✓
DAL2[e]	✓		✓		Dal82p	✓	✓
DAL3[e]	✓		✓			✓	✓
DAL80[e]	✓	✓	✓	✓		✓	✓
DUR1,2[e]	✓		✓		Dal82p	✓	✓
DUR3[e]	✓		✓		Dal82p	✓	
ECM38[e]	✓	✓				✓	✓
GAT1[e]	✓		✓				✓
GZF3[e]	✓	✓	✓	✓			✓
GDH2	✓						
GLT1	✓				Gcn4p		
MEP2	✓					✓	✓
PRB1	✓				Rim101p		
UGA1			✓		Dal82p, Uga3p		
UGA2			✓		Uga3p		
ASP3-1[f]	✓	✓	✓				
ASP3-2[g]	✓	✓	✓				
ASP3-3[g]	✓	✓	✓				
ASP3-4[g]	✓	✓	✓				
PUT1	✓	✓	✓		Put3p, Pho4p	✓	
PUT2	✓	✓	✓		Put3p, Pho4p	✓	
PUT4	✓		✓		Gcn4p	✓	
DAL4[h]	✓		✓		Dal82p	✓	
DAL7[h]	✓		✓		Dal82p	✓	✓
UGA4[h]	✓		✓	✓	Uga3p		
DAL1	✓		✓		Gcn4p, Dal82p	✓	
DAL5	✓		✓		Gcn4p	✓	✓
GAP1	✓	✓	✓	✓	Gcn4p	✓	✓
GDH1	✓	✓	✓		Gcn4p, Hap2p, Leu3p, Spt3p		
GLN1	✓	✓			Gcn4p, Pho2p		
ENA1	✓	✓			Cin5p, Crz1p, Hal9p, Rim101p, Skn7p, Yap6p, Nrg1p		
N-lim upregulated signature transcripts not included above							
OPT2						✓	✓
SDL1						✓	
MLS1						✓	
ALD2					Msn2p, Msn4p	✓	
CPS1						✓	✓
DCG1						✓	✓
SPS4					Ime1p	✓	
ADY3						✓	
YGK3						✓	
YMR088C						✓	
YBR139W						✓	
YGR190C[i]						✓	
YIL089W						✓	
YHR029C						✓	
YDR090C						✓	
YMR090W						✓	
YLR053C						✓	
Genes with a similar transcript profile to the nine marker genes not included above							
MEP1							✓
ARG1					Gcn2p, Arg80p, Gal11p, Hfi1p, Spt3p		✓
AVT4							✓
BSC6							✓
CPA1					Gcn4p		✓
GDH3					Msn2p, Msn4p		✓
IDP1							✓
LAP3							✓
MEP3							✓
SDS23							✓
YBR285W							✓
YOR093C							✓

[a] Regulated by these GATA-factors as documented by YPD (www.incyte.com) on March 4, 2005.
[b] Directly regulated by these transcription factors as given in YPD (www.incyte.com) on March 4, 2005.
[c] N-lim signature transcript (32 genes) as given in (Tai *et al*).

[d] Genes were identified that displayed the same transcript profile as at least five of the marker genes over all conditions. Genes that contained at least two GATAAG sequences in their promoter were included in this table (27 genes). For statistical criteria see Experimental procedures.
[e] These nine genes were selected as "marker genes" to analyse NCR-sensitive regulation. For criteria see text.
[f] non unique probeset
[g] not on array
[h] low signal
[i] below detection

leucine and methionine, however cannot be called significantly different to proline on both criteria (Figure 5). Growth on methionine, leucine and proline gave the highest average levels, and hence the lowest NCR-strength.

In order to find yet unidentified GATA-factor regulated genes, we used the transcript profile of the nine 'marker' genes to identify genes with a similar transcript profile (see Experimental procedures). Of the 76 genes thus identified, 27 genes (36 %) contained a GATAAG sequence (65, 211) in their promoter (at least twice), in comparison to only 7 % genome-wide. The 27 genes are included in Table 3. 15 of these genes have not yet been reported to be regulated by GATA-factors. However, the promoters of five of these; *ARG1, CPS1, DCG1, IDP1* and *OPT2* have recently been identified in chromatin imunoprecipitation (ChIP) studies, to be bound ($p<0.005$) by Gln3p, Gat1p or both (121).



	NH_4^+	ASN	PHE	LEU	MET	PRO
NH_4^+		0.5097	0.0016	<0.0001	<0.0001	0.0001
ASN	0.9763		0.0007	<0.0001	<0.0001	<0.0001
PHE	<0.0001	0.0003		0.0039	0.0021	0.0897
LEU	<0.0001	<0.0001	0.0013		0.1439	0.1824
MET	<0.0001	<0.0001	<0.0001	0.0102		0.0425
PRO	<0.0001	<0.0001	0.0005	0.3021	0.2069	

Figure 5: **Representation of the NCR-sensitive transcript levels.** Black bars, average normalized levels of established GATA-factor regulated genes; gray bars, average normalized levels of N-limitation signature transcripts (see text for details). Numbers represent p-values obtained with a two-tailed, unequal variance Students' t-test. Right of the diagonal gives the p-values of the Students' t-test of established GATA-factor sensitive transcript levels between conditions, and left of the diagonal gives p-values of the Students' t-test of N-limitation signature transcripts between conditions (see text for details).

13 genes were up-regulated relative to ammonium-grown cultures in all amino acid-grown cultures except for those grown on proline (Figure 4, panel B). Six of these are involved in transport: *AGP1*, a broad-substrate specificity permease; *GNP1*, a high-affinity glutamine permease; *BAP3*, a branched-chain amino acid permease; *TAT1*, a tyrosine and tryptophan permease; *TAT2*, encoding a high affinity typtophan permease and *SIT1* a heavy metal ion transporter. These genes, except *SIT1*, form a subset of related amino acid permeases (AAPs) that is induced by the availability of amino acids, except for proline and arginine (99, 239) and is dependent on the SPS (Ssy1p, Ptr3p, Ssy5p) sensor complex.

Co-responsive in 3 conditions. In the cluster of genes with significant changes in three amino acid-grown cultures, 33 out of 69 genes showed a consistent up-regulation in leucine-, phenylalanine- and methionine-grown cultures but not in cultures grown on asparagine and proline (Figure 4, panel A). Of these genes, *ARO9,* encoding an aromatic amino-acid aminotransferase and *ARO10,* encoding a broad-substrate specificity 2-oxoacid decarboxylase, showed the strongest up-regulation. Indeed, a functional analysis study based on these observations (350) demonstrated that the Aro10p-dependent decarboxylase activity also catalyses the decarboxylation of the 2-oxo acids derived from methionine and leucine. Further research is required to assess whether or not the *ARO9*-encoded transaminase has a similar broad substrate specificity. In silico promoter analysis of the 33 transcripts showed an over representation of a previously unidentified binding sequence GCACCC in 28 % of the transcripts, compared to 12 % in the whole genome (not shown).

89 genes showed a common reduction of transcript levels in three amino acid-grown cultures. Again, a large subset (73) of these genes showed a co-response in leucine-, phenylalanine-, and methionine-grown cultures (Figure 4, panel D). Promoter analysis of these 73 genes revealed that 33% contained the STRE motif CCCCT (Msn2p/Msn4p regulation) at least twice in their respective promoter regions, as compared to a 14 % coverage on a genome-wide basis. Several genes related to the seripauperin family (*PAU2, PAU7*, YIR041W, YMR325W, YKL224C), were also found in this category.

Glutamate and glutamine metabolism

Four reactions, catalysed by enzymes encoded by five genes make up the central nitrogen metabolism, that is the interconversion of α-ketoglutarate, ammonium, glutamate and glutamine. These are NADP-dependent glutamate dehydrogenase, encoded by *GDH1* and *GDH3*, NAD-dependent glutamate dehydrogenase, encoded by *GDH2*, glutamine synthetase, encoded by *GLN1,* and glutamate synthase, encoded by *GLT1*. The activity of these enzymes is mainly regulated at the transcriptional level, depending on the kind and concentration of the nitrogen source present (24, 231, 318, 320).

All genes except for *GDH2* display the lowest average transcript abundance during growth on asparagine (Table 4), which reflects the richness of this nitrogen source. *GDH2* is lowest under ammonium grown conditions, followed by asparagine grown conditions, which is in line with the function of Gdh2p in catalyzing the production of ammonium (231).

Table 4: Average mRNA levels with standard deviations (Affymetrix signal units) of genes involved in central nitrogen metabolism.

	Nitrogen source					
Gene	NH_4^+	ASN	PHE	LEU	MET	PRO
GDH1	1374 ± 154	710 ± 94	1502 ± 220	1514 ± 215	1428 ± 23	1366 ± 28
GDH3	222 ± 58	108 ± 11	1429 ± 262	1169 ± 116	1229 ± 102	531 ± 56
GDH2	306 ± 9	494 ± 38	635 ± 162	615 ± 26	1101 ± 77	901 ± 54
GLN1	1254 ± 185	698 ± 97	3578 ± 595	2490 ± 453	3053 ± 110	2430 ± 8
GLT1	606 ± 104	336 ± 51	432 ± 52	592 ± 50	696 ± 12	765 ± 104

It has been shown previously that conditions of low glutamate induces the activity of the transcriptional regulators Rtg1p and Rtg3p, by a mechanism referred to as retrograde regulation (151, 197, 201). These transcription factors induce transcription of target genes, particularly aimed at de novo synthesis of α-ketoglutarate. To investigate retrograde regulation, we have monitored transcript levels of established targets, *CIT2, DLD3, IDH1, IDH2, ACO1* and *CIT1* (51, 197). As can be seen in Figure 6, *CIT2* and *DLD3,* both exclusively regulated by Rtg1p and Rtg3p, are low in ammonium, asparagine and proline, all direct sources of ammonium or glutamate. *CIT2* and *DLD3* mRNA levels are much higher in phenylalanine, leucine and methionine, which all depend on dedicated transamination reactions to produce glutamate. The same profile of *CIT2* and *DLD3* expression, albeit less pronounced, is observed for other targets of retrograde regulation, *IDH1, IDH2, ACO1* and *CIT1*, which is in line with the relative retrograde-dependent expression of these genes (201).

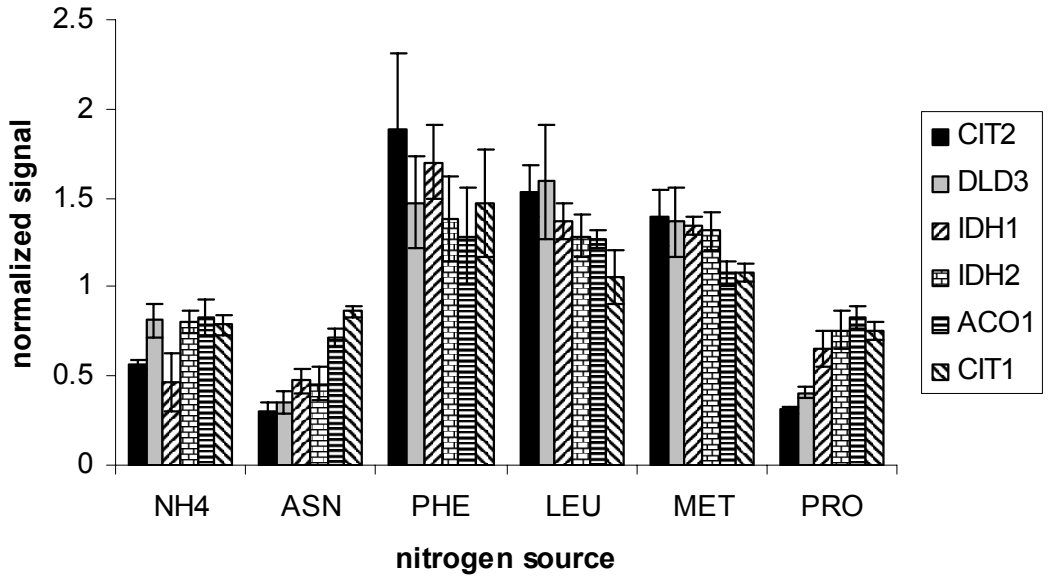


Figure 6: **Retrograde sensitive gene expression.** The average transcript level and standard deviation for each gene was average normalized over all conditions.

Discussion

Signature transcripts

One of the proposed applications of DNA-microarrays in biotechnology is to diagnose large-scale industrial fermentations. It has been proposed that the identification of signature transcripts, i.e. transcripts that show a unique response to change in a single environmental or nutritional parameter, may contribute to the development of small, cost-effective diagnostic arrays (32, 313, 368). The signature transcripts described in this study complement other sets of chemostat-based signature transcripts (32, 70, 262, 313, 316) that may facilitate the interpretation of studies with complex industrial media and dynamic cultivation conditions, such as transcriptome analysis of wine fermentations (216, 277).

Among the nitrogen sources analyzed in this study, methionine yielded the largest set of signature transcripts (Figure 3). This may be related to the fact that, in addition to functioning as a nitrogen source, methionine is a preferred sulfur source for *S. cerevisiae* as suggested by this study. The observed down-regulation of the entire biosynthetic pathway from extracellular sulfate (including sulfate transport) to intracellular methionine prevents the unnecessary expenditure of 2 ATP and 4 NADPH per methionine (324). Binding sites for two established regulators of sulfur amino acid metabolism, Cbf1p/Met4p/Met28p and Met31p/Met32p (324), were over-represented among the methionine-down-regulated genes. In contrast to the large set of signature transcripts identified for methionine, phenylalanine yielded only five signature transcripts. It is doubtful however whether any of these five 'phenylalanine signature transcripts' would retain this status when tyrosine or tryptophan were to be included in the analysis. Biosynthetic genes for phenylalanine, asparagine and

proline were not significantly repressed in the corresponding cultures, which may be attributed to cross-pathway control and shared biosynthetic pathways for amino acid families. Of the leucine biosynthesis pathway, only *LEU1* and *LEU2* were repressed in leucine-grown cultures, probably via the regulator of leucine biosynthesis, Leu3p (166). The other genes of the leucine biosynthetic pathway are predominantly controlled by other regulators under this condition, and hence not reduced (102, 131).

The present study illustrates that, in some respects, a 'signature-transcript-based approach' may be too simplistic. Depending on the cell's nutritional status and the identity of the amino acid, it may be used as nitrogen, carbon or, as illustrated by the methionine case, as sulfur source. Consequently, meaningful analysis of industrial fermentation with microarrays is likely to require interpretation of complex sets of genes, rather than simple 'indicator' genes for all relevant process parameters and nutrients.

New insights in amino acid metabolism

In addition to transcriptional responses that are unique for certain nitrogen sources, our study revealed co-responses to groups of nitrogen sources. The common up-regulation of the group of SPS-dependent transporters during growth on asparagine, phenylalanine, leucine and methionine, but not on proline and ammonium (Figure 4), can be directly linked to known regulation pathways. Proline and arginine are known to differ from other amino acids by their inability to elicit SPS-dependent transcription. Instead, proline is taken up by a specific transporter, Put4p. Proline's high specificity in transport may rely on the fact that proline is not required in large quantities in yeast, protein not utilized as nitrogen source under anaerobic conditions and that proline degradation increases the levels of reactive oxygen species which causes toxic effects to the cell (243). In addition, proline (like arginine) is a very abundant amino acid in must and wort (126, 127), and shared-signalling pathways could interfere with selective use of other amino acids.

A substantial number of genes exhibited transcriptional co-responses on phenylalanine, leucine and methionine. After these three amino acids are transaminated, the resulting two-oxo acids cannot be used for anabolic purposes (except to form the corresponding amino acids back), but are instead decarboxylated via the Ehrlich pathway, yielding fusel alcohols and acids (87, 350). Based on the common up-regulation of the *ARO10* gene on phenylalanine, leucine and methionine, we recently performed a detailed analysis of the Aro10p-dependent decarboxylase activity (350). Indeed, Aro10p was shown to be responsible for a broad-substrate-specificity decarboxylase activity involved in the catabolism of all three amino acids (350). This study provides a clear example of how transcriptome analysis can be used to guide functional analysis studies. The set of phenylalanine, leucine and methionine co-responsive genes provides additional leads for functional analysis.

The co-response of *ARO9*, encoding the aromatic amino acid transaminase, suggests that it may perhaps act as a broad-substrate transaminase. The up-regulation of *PDR12* on all three amino acids (Figure 4) has been recently correlated with ATP-driven transport (136) of fusel acids derived from catabolism of aromatic, branched chain amino acids and methionine (125). Together with passive diffusion of these compounds into the cells, this transport process is likely to contribute to uncoupling of the plasma membrane proton motive force, thus explaining the reduced biomass yields of cultures grown on methionine, leucine and phenylalanine as sole nitrogen sources (Table 1).

Nitrogen-source dependent transcriptional regulation

The conversion of α-ketoglutarate to glutamate is the link between carbon and nitrogen metabolism, and the biosynthesis of α-ketoglutarate is therefore also regulated by carbon- and nitrogen-specific factors. The genes encoding for the enzymes involved in the first three steps of the TCA-cycle, yielding α-ketoglutarate, are regulated by two principal regulators, heme activator protein complex Hap2,3,4,5p and retrograde regulator proteins Rtg1p/Rtg3p. Transcriptional induction by Rtg1p/Rtg3p is known to be triggered by low glutamate concentrations (201). For *CIT2*, which is directly regulated by Rtg1p/Rtg3p, and not by Hap2/3/4/5p or any other known regulatory regime, transcript levels are on average four-fold higher in phenylalanine, leucine and methionine grown cultures compared to ammonia, asparagine and proline. This implies a strong influence of retrograde regulation in these conditions. This also suggests that phenylalanine, leucine and methionine are poor sources of glutamate. Since Hap2/3/4/5p controlled genes are all up-regulated in glucose-limited aerobic chemostats (for review, see (292)), this retrograde effect is less dramatic for *CIT1, ACO1, IDH1* and *IDH2*.

Rtg-dependent activation of transcription has been proposed, just like Gln3p and Gat1p, to be regulated by the Tor1/2p signal transduction pathway (64, 168). However, mechanisms independent of Tor, with an important role for glutamate or ammonia as a signal molecule have also been postulated (201, 314, 315). Recent work describes a mechanism which includes both Tor signalling and glutamate based signals, in which Mks1p, a negative regulator of Rtg1p/Rtg3p, plays a central role (82, 202). Based on the transcript levels over the six conditions of genes strongly influenced by retrograde regulation, like *CIT2* and *DLD3* (Figure 6), in comparison with transcript levels of genes strongly influenced by nitrogen catabolite repression (and thus Tor, Figure 5), it seems that glutamate based signals play the more important role in retrograde regulation in our conditions.

The research described here provides the unique opportunity to quantify NCR-strength at a fixed growth rate, thus isolating GATA-factor regulated gene expression from growth rate dependent regulation (*e.g.* by TOR). Corroborating previous results obtained in shake flasks, asparagine and ammonium are found to elicit the strongest NCR. This can partly be explained by the presence of ammonium

in the medium, as ammonium has been described to be a strong effector of NCR regulation (318). However, other signals like glutamate and glutamine concentrations are likely to play important roles as well (21, 25, 304), which can be seen by the specifically lower transcript abundance of some GATA-factor regulated genes in asparagine grown cultures.

The NCR-sensitive transcript levels for the six nitrogen sources, analysed at a fixed specific growth rate, shows a similar trend as the respective doubling times in shake flasks. Asparagine and ammonium have the lowest values, followed by phenylalanine. Although the doubling time on leucine was considerably lower compared to both methionine and proline, NCR-sensitive transcript levels were comparable. Methionine and proline show comparable (high) NCR sensitive transcript levels in the chemostat cultures, and the doubling time of *S. cerevisiae* on these two amino acids was also the highest. These observations indicate that the many previous studies in which NCR has been studied in batch cultures grown on different nitrogen sources, indeed primarily reflect direct responses to the nitrogen source rather than indirect ‘artefacts’ caused by different specific growth rates.

Acknowledgments

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Chapter 5

New insight in temperature-dependent transcriptional response of *Saccharomyces cerevisiae*: Chemostat-based transcriptomics on low temperature cultivation under anaerobic carbon and nitrogen limitations

Siew Leng Tai, Michael C. Walsh, Jack T. Pronk, Jean-Marc Daran

Abstract

The global transcriptional response of *Saccharomyces cerevisiae* was investigated in low temperature chemostat cultures grown in carbon (glucose) or nitrogen (ammonium) limitation. During steady state chemostats, the growth rates and *in vivo* fluxes were kept constant however the growth limiting nutrient was significantly higher at 12 ^{o}C than at 30 ^{o}C and had significant effects on transcriptional responses. Growth at 12 ^{o}C resulted in a rearrangement of transporters for the limiting nutrient, where hexose transporters (HXTs) and ammonium permeases (MEPs) were differentially expressed in cultures grown at 30 ^{o}C in carbon and nitrogen limitations, respectively. In addition, we found repression of genes encoding proteins in reserve carbohydrate metabolism (trehalose and glycogen) and metabolism of alternative carbon or nitrogen sources other than glucose or ammonia. However, there were also similar responses when the transcriptional response was evaluated regardless of the growth limiting nutrient. In particular, induction of ribosome biogenesis genes emphasizes the significance of transcription and translational adaptation at low temperature. In contrast, genes encoding proteins during stress response were down-regulated. This down-regulation of stress elements better known as environmental stress response (ESR) is in contradiction with previous low temperature transcriptome analyses. During continuous steady state low temperature cultivation, ESR no longer plays an integral role in *S. cerevisiae's* response to temperature change. Similarly, trehalose accumulation, consistent with its gene expression, was not indispensable for growth at 12 ^{o}C. This response, however, does not exclude that ESR may be required for transition phase in low temperature growth when cells are transferred from one temperature to another.

Introduction

The yeast *Saccharomyces cerevisiae* exhibits an array of responses when encountered with conditions that are less than physiologically ideal. Since *S. cerevisiae* is a mesophilic microorganism, large deviation from moderate temperatures (25 – 40 ^{o}C) causes adverse effects primarily on the ability to growth fast. The response to low temperature varies from the changes in metabolic fluxes to changes in cellular factors like growth phase (361), respiratory mechanisms (195), lipid composition of the membrane (111) and accumulation of trehalose (194, 275, 334). A genome-wide transcriptome analysis may therefore provide a quick overall outlook on primary control of gene expression during cold adaptation due to possible changes to cellular homeostasis (137, 234).

Several cold-inducible genes have previous been identified, mainly in transcriptome studies carried out with freeze-tolerance treatments and/or cold-cultivation in batch fermentations. These genes include *NSR1*, encoding a nucleolar protein required for pre-rRNA processing and ribosome biogenesis (191, 192, 372) and *TIP1* (temperature inducible protein) encoding a protein cell wall mannoprotein and lipase activity mainly induced during heat- and cold-shocks (103, 169). Along side these genes, the seripauperin family genes

also related to *TIP1*, *TIR1* and *TIR2* are also well known cold-inducible genes (55, 171). The fatty acid desaturase *OLE1* is also induced upon cold treatment (237). This gene is thought to respond to the changes in membrane fluidity brought about by the increased viscosity caused by low temperatures.

Reserve carbohydrates, trehalose and glycogen, accumulate upon cold-shock and reduced temperature treatments (158, 234). Trehalose has been long regarded as a hallmark for general stress response and has been regarded as a thermoregulator during heat- and cold-shocks (256, 258). It has been observed that trehalose synthesis enzymes are up-regulated even at 0 ^{o}C, which in yeast is considered a very undesirable growth condition (158). At this close to freezing temperatures, heat shock protein *HSP104* was also induced, but it is only known to play a key role in cell survival at high temperatures (158). This cross-regulation of transcriptional control shows the complexity and the tightly interactive network of the stress responsive transcriptional regulation. It is generally known that transcriptional control during stress is activated by the (i) heat shock elements (HSE) (ii) stress response element (STREs) and (iii) AP-1-responsive elements (APEs) (279).

Most transcriptome analysis done on low temperature has mainly been dealt with in batch fermentations. Since the maximum specific growth rates, μ_{max}, is proportionally affected by the lowering of temperature, comparing transcriptome data with varying growth rates can lead to false interpretation of the results. Growth with varying specific growth rates, μ, (h^{-1}) such as in dynamic batch cultivations are known to affect the reproducibility of RNA sampling for microarrays (117, 262). Therefore, chemostat cultures were used in this study to tackle the manifestation of growth rate issues by controlling it by means of controlling the dilution rate. The dilution rate, D is defined as the ratio of the ingoing flowrate (dV/dt) and the culture volume (V). When the culture volume, V is kept constant, the growth rate is then dependent on the ingoing flowrate, in which growth is limited by one substrate (e.g. carbon- or nitrogen-source) that the culture medium was specifically designed for. Therefore, the growth rate is equal to the dilution rate set by the user. In steady-state chemostat cultures where the cultures are relatively young to avoid evolution changes (10-14 generations), metabolite and substrate profiles, enzyme specificities and other growth-related dependencies stay constant over time. This is important during sampling of mRNA as these parameters that can easily influence the outcome of transcriptome studies.

To this end, we describe, for the first time, the results of the transcriptome data analysis for changes in growth temperature from 12 ^{o}C to 30 ^{o}C in steady state carbon- or nitrogen-limited anaerobic chemostats. Since transcriptional responses is known to be highly context dependent (313), gene expression were analyzed in their respective limitations and also regardless of the growth limitation. Common functional classes and regulatory transcriptional control were sought after to identify clusters of genes that had significant presence during low temperature growth. Furthermore, since reserve carbohydrates glycogen and trehalose have been implicated with low temperature response, these compounds were also measured. Given that the change of temperature has many drastic

effects on the cell's homeostasis, we also addressed the possible differences of batch growth cultures (23, 234, 280, 286, 313) versus chemostat grown ones. In particular we looked into the effects of the environmental stress response (ESR) that has been frequently associated with drop in growth temperature.

Experimental procedures

Strain and growth conditions

The *S. cerevisiae* prototrophic haploid reference strain, CEN.PK113-7D (MAT***a***) (335), was grown at a dilution rate (D) of 0.03 h^{-1} at both 12°C or 30°C in 2 .0 liter chemostats (Applikon, Schiedam, The Netherlands) with a working volume of 1.0 liter as described in (313, 333). A temperature probe connected to a cryostat controlled cultures grown at 12°C. Cultures were grown in a defined synthetic medium that was limited by carbon with all other growth requirements in excess as described by (313). The dilution rate was set at 0.03 hr^{-1} with pH measured online and kept constant at 5.0 by automatic addition of 2 M KOH using an Applikon ADI 1030 Biocontroller. The stirrer speed was set to 600 rpm. Anaerobic growth and steady state conditions were maintained as described (313). Biomass dry weight, metabolite, dissolved oxygen, and gas profiles were constant for at least three volume changes prior to sampling.

Analytical methods

Culture supernatants were obtained with the method described in (218). For the purpose of glucose determination and carbon recovery, culture supernatants and media were analyzed by high performance liquid chromatography on an AMINEX HPX-87H ion exchange column using 5 mM H_2SO_4 as the mobile phase. Ethanol evaporation from cultures was determined as described in (177). Residual ammonium concentrations were determined using cuvette tests from DRLANGE (Dusseldorf, Germany). Culture dry weights were determined as described in (264) while whole cell protein determination was carried out as described in (344).

Trehalose & glycogen

Trehalose and glycogen measurements were adapted according to (257). Trehalose was determined in triplicate measurements for each chemostat. Glycogen was determined in duplicate for each chemostat. Glucose was determined using the UV-method based on Roche kit no. 0716251.

Microarray analysis

Sampling of cells from chemostats, probe preparation, and hybridization to Affymetrix Genechip® microarrays were preformed as described previously (262). RNA quality was determined using the Agilent 2100 Bioanalyser. The results for each growth condition were derived from three independently cultured replicates.

Data acquisition, quantification of array images and data filtering were performed as described previously (313). For additional statistical analyses, Microsoft Excel running the significance analysis of microarrays (SAM Version 1.12) (330) add-in was used for pairwise comparisons. Genes were considered changed in expression if they were called significantly different using SAM (median false discovery rate of 1%) by at least 2-fold from each other condition.

Promoter analysis was performed using the web-based softwares Regulatory Sequence Analysis (RSA) Tools (336) as described in (313).

For the statistical assessment of over-representation GO biological processes categories (http://www.geneontology.org/) (88) in the SAM-identified transcripts, Fisher's Exact test employing hypergeometric distribution was used with a *p*-value cut-off of 0.01 with a Bonferroni correction. The probability was calculated as follows: the *p*-value of observing z genes, belonging to the same functional category is:

$P = \sum_{x=z}^{\max(N,M)} \frac{\binom{N}{x}\cdot\binom{G-N}{M-x}}{\binom{G}{M}}$ where N is the total number of genes in a functional category , M is the total number of genes in the cluster (Upregulated clusters A,B, C and downregulated clusters D, E) and G is the total number of gene features on the YG98S array (6383).

The up and down regulated data inspection for overrepresentation of transcription factors as defined by ChIP on chip analysis (http://jura.wi.mit.edu/fraenkel/download/release_v24/bound_by_factor/ORFs_bound_by_factor_v24.0.p005b_041213.txt) was also performed employing an in-house version of the hypergeometric distribution test (172). Applying the same formula, the probability was calculated as follows: where N is the total number of genes where the TF can bind upstream (Harbison et al, 2004), M is the total number of genes in the cluster (Upregulated clusters A,B, C and downregulated clusters D, E) and G is the total number of gene features on the YG98S array (6383).

The gene annotation was made according to GO terms (88), the *Saccharomyces* Genome Database (available at www.yeastgenome.org) (84) and the Yeast Proteome Database (available at www.proteome.com). Data visualization was done using Treeview v1.60.

Comparison with other S. cerevisiae low temperature data

The expression datasets that were used for comparison in this study can be downloaded from (23) (http://biopuce.insa-toulouse.fr/jmflab/winegenomics), (234) (http://kasumi.nibh.jp/~iwahashi/), (280) (http://staff.aist.go.jp/t-sahara/), (286) (http://cbr-rbc.nrc-cnrc.gc.ca/genetics/cold/), (107) (http://genome-www.standford.edu/yeast_stress/). For the following low temperature datasets (23, 234, 280), the complete datasets were downloaded and since only average ratios were provided, statistical test could not be carried out and therefore genes that had a fold change of 2 or more were denoted as significantly changed. This amounted to 2287 genes for (23), 1609 genes for (280) and 2339 genes for (234). As for (286), only the statistically significant changed genes were provided and hence 634 genes were used. These resulting datasets were used as described in Figure 4. The characterized environmental stress response genes (107), were downloaded as provided from the website. Genome-wide transcriptome supplemental data can be obtained online on www.bt.tudelft.nl.

Results

Physiological and biochemical data of low temperature chemostat cultures

The biomass yield on glucose (Y_{Glu-X}) and fluxes observed from the uptake of glucose (q_{Glu}) to the ethanol production (q_{EtOH}) rates were not significantly different at 12 ^{o}C and 30 ^{o}C in both anaerobic carbon- or nitrogen-limitation indicating that the growth energetics were not drastically affected by temperature and thus provided an unique platform for comparing transcriptome response at 12 ^{o}C and 30 ^{o}C (Table 1). The concentration of the limiting nutrient, however, was in both limiting situations higher at 12 ^{o}C than at 30 ^{o}C. The residual ammonium salt concentration was 7.5-fold higher while the glucose concentration was around ten-fold (Table 1).

In carbon-limited cultures, the whole cell protein (WCP) was slightly lower at 12 ^{o}C than at 30 ^{o}C (0.40 g.g^{-1} vs 0.43 g.g^{-1}), while in nitrogen-limitation one noticed an increase of approximately 38% of the WCP content at 12 ^{o}C (0.47 g.g^{-1} vs 0.34 g.g^{-1}). In contrast to what happens at 30 ^{o}C, where the WCP of the cell was higher in carbon-limited cultures than in nitrogen-limited ones (187, 339), at 12 ^{o}C this ratio was reversed. On another note, reserve

carbohydrates (trehalose and glycogen), important markers for low temperature fermentation, were measured. In contrast to several previous reports on cold shock (105, 158, 234, 248) trehalose did not accumulate at all at 12 °C. Glycogen, on the other hand, was only slightly higher at 12 °C during carbon limitation while under nitrogen limitation glycogen was found 2.5 fold lower compared to a 30 °C culture (Table 1).

Table 1: Residual glucose/ammonia, whole cell protein and reserve carbohydrate concentrations. Unless state otherwise, values represent the mean ± standard deviation of data from three independent steady-state chemostat cultivations. DW: dry weight; C: Carbon

Limitation	Culture temperature	Physiological parameters				
		Y_{Glu-X}[b]	q_{Glu}	q_{EtOH}	q_{CO2}	C-recovery
	°C	$G_{glucose}.g_{DW-1}$	$mmol.g_{DW}^{-1}.h^{-1}$			%
Carbon	12	0.07 ± 0.01	- 2.5 ± 0.2	3.8 ± 0.3	4.4 ± 0.3	100 ± 3
Carbon	30	0.07 ± 0.00	- 2.3 ± 0.0	3.5 ± 0.0	3.8 ± 0.2	95 ± 1
Nitrogen	12	0.05 ± 0.00	-3.6 ± 0.2	6.1 ± 0.3	6.0 ± 0.6	97 ± 4
Nitrogen	30	0.04 ± 0.00	-4.0 ± 0.1	6.8 ± 0.2	7.4 ± 0.2	97 ± 2

Limitation	Culture temperature	Residual concentration		Whole cell protein	Reserve carbohydrate[a]	
		Glucose	Ammonia		Trehalose	Glycogen
	°C	g/liter	mM	$g_{proteins}.g_{DW}^{-1}$	$g_{equivalent\ glucose}.g_{DW}^{-1}$	
Carbon	12	0.5 ± 0.2	65.2 ± 2.2	0.40 ± 0.01	< 0.005	0.06 ± 0.01
Carbon	30	<0.05	61.3 ± 4.5	0.43 ± 0.01	0.02 ± 0.00	0.04 ± 0.00
Nitrogen	12	16.2 ± 3.0	1.5 ± 0.2	0.47 ± 0.03	< 0.005	0.02 ± 0.00
Nitrogen	30	15.3 ± 1.3	0.2 ± 0.1	0.34 ± 0.01	0.04 ± 0.00	0.05 ± 0.01

[a] trehalose and glycogen are expressed in units of glucose. See experimental procedures for details

[b] biomass yield on glucose (g of biomass/g glucose consumed)

Global transcriptome data analysis and functional categorization

Genome-wide transcriptome analysis was carried out on cells grown in anaerobic carbon and nitrogen limited chemostat cultures at both 12 °C and 30 °C. Each growth condition was performed in independent triplicates (262). To analyze the effects of the temperature on gene expression, DNA microarrays derived from carbon- and nitrogen-limited chemostats at 12 °C were compared to those grown at 30 °C. In terms of reproducibility, the average coefficient of variation for the triplicate transcriptome analyses for each of the four growth conditions was below 0.20. In addition, the level of the *ACT1* transcript, a common loading standard for conventional Northern analysis, varied less than 12% over the four growth conditions.

The two pair-wise comparisons of 12 °C against 30 °C in their respective limitations yielded a total of 1026 (representing 16 % of the *S.cerevisiae* genome) transcripts which were differentially expressed in at least one of the comparisons in accordance to the statistical evaluation described (fold change of > 2 with a false discovery rate of 1%, see experimental procedures) (330) (Figure 1A, Supplementary data Table 1 online). Meanwhile, 422 transcripts (7 % of the genome) remained below the detection limits of all the four conditions investigated and about 75% of the genome (4935 genes) did not show significant changes in expression levels in any of the comparisons.

Under carbon-limitation, 221 transcripts showed a significantly higher expression level at 12 °C while 275 transcripts were significantly downregulated (Figure 1B). On the other hand, 762 transcripts showed significant changes under nitrogen-limited conditions. Of these, 261 transcripts were upregulated at 12 °C while 501 transcripts were downregulated. Further analysis of these data identified that a large portion of the transcriptional response related to the temperature change was still context dependent. Only 227 genes showed consistent up or downregulation under both nutrient-limitations. Eighty-eight transcripts were found upregulated while 139 transcripts were seen downregulated at lower cultivation temperatures (Figure 1B).

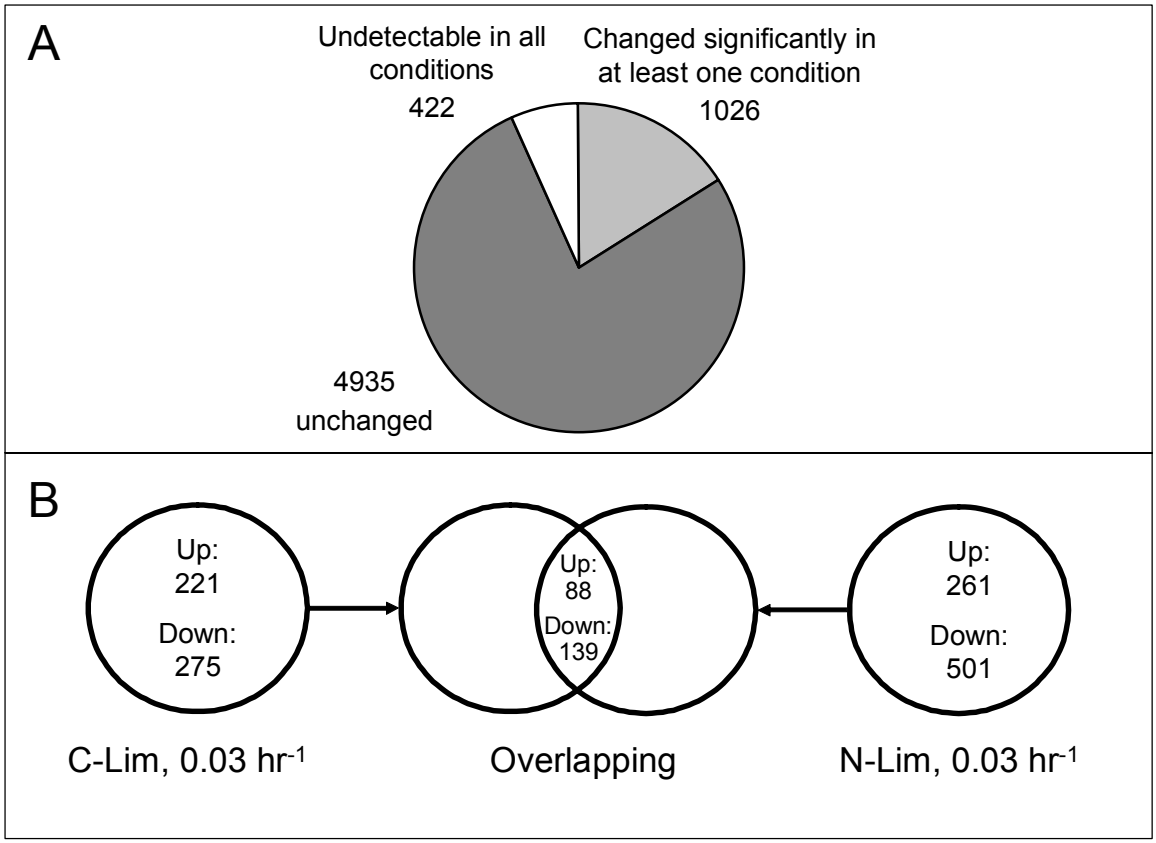


Figure 1: **Global transcriptome responses to anaerobic growth at 12 °C and 30 °C under limitations of carbon- and nitrogen. A** Pie chart of overall changes of carbon- and nitrogen- limited culture when 12 °C cultures were compared to 30 °C. **B** Pairwise comparisons of 12 °C versus 30 °C for cultures grown in carbon- and nitrogen-limitation. Up denotes induction of genes at 12 °C and Down denoted repression of genes at 12 °C compared to 30 °C.

Temperature responsive transcript clusters (carbon, nitrogen and co-responsive genes) were subsequently analyzed to assess the enrichment of functional categories according to GO (2) directories (Table 2, Supplemental data Table 2 online). To identify the regulatory network acting at low temperature, the different clusters were also compared to the 'location analysis' dataset available for 102 *Saccharomyces cerevisiae* transcription

factors (TF) (121) (Table 3, Supplemental data Table 3 online). To complete the analysis a systematic search for protein binding motives in promoter sequences was performed on the different clusters (Table 4).

Temperature-dependent transcriptome response in C-limited chemostat cultures

Only the GO category 'ribosome biogenesis' (GO: 0007046) was found enriched in the gene cluster exhibiting higher expression at 12 ^{o}C than at 30 ^{o}C (Table 2). Out of the 221 genes of this clusters, 20 belonged to this GO category (Table 5). Nine of these genes (*EBP2, IPI3, KRR1, MTR4, MND3, NOG1, NOG2, NOP4* and *RPF2*) were also associated to the nuclear Bud20p complex (133). In relation to this, the expression level of *BUD20* (+2.3) was also higher at 12 ^{o}C than at 30 ^{o}C. Along with the latter mentioned changes, the expression level of *RRN3* (+2.2), *RRN6* (+2.2) and *RRN7* (+2.7) genes that encode transcription factor subunits of the RNA polymerase I that participate in rRNA transcription was also higher at 12 ^{o}C than at 30 ^{o}C. With exception of *ECM1, NOP6, NSR1* and *UTP30,* all the remaining 20 genes exhibit lethal phenotype (Table 5). A protein binding motif search in promoter sequences of the genes comprising this cluster revealed the significant presence of a cis-regulatory PAC motif (GATGAG) characteristics of rRNA transcription (310) (Table 4).

The transcript data also showed that the temperature influenced the expression of hexose transporter (*HXT*) genes. Out of the seven *HXT* genes that were significantly expressed in at least one condition, five were differentially expressed at 12 ^{o}C and 30 ^{o}C (Table 5). *HXT2* (+2.6-fold), *HXT3* (+3.7), and especially *HXT4* (+33.5) were expressed at higher levels at 12 ^{o}C while *HXT5* (-40.4) and *HXT16* (-10.0) were repressed at 12 ^{o}C. Transcription of high affinity transporter genes, *HXT6* and *7* (273) was not significantly affected by the culture temperature. In addition, during carbon limitation, *CCT7* (+2.4) encoding a cytoplasmic chaperonin that is essential for yeast growth was upregulated. *CCT7* is a known cold-shock protein in *S. cerevisiae* (300). .

Meanwhile, the group of 275 genes with lower expression in C-limited chemostat cultures at 12 ^{o}C than at 30 ^{o}C (Figure 1) exhibited enrichment in GO categories that could be separated into two groups: i) categories related to carbon metabolism (GO:0006091, GO:0015980) and ii) categories related to transport (Table 2). Genes encoding enzymes participating in metabolism and utilization of alternative C-source like maltose (*MAL11*, *MAL13*, *MAL31*, *MAL32*, YGR287C), galactose (*GAL10*) and glycerol (*GUT1*, *GUT2*) displayed lower expression at 12 ^{o}C than at 30 ^{o}C. Furthermore, genes encoding enzymes of the glycolytic pathway, (*GPM2, PYK2, PDC6, PFK26, PFK27*), TCA cycle (*CIT2, SDH1*), pentose phosphate pathway (*TKL2, GND2*) and the electron transport chain (GO:0006118) (*COX4, COX5A, COX5B, COX6, CYC3, CYC7, QCR2, QCR10*) were also downregulated at 12 ^{o}C (Table 6). *MIG1*, a transcriptional regulator that participates to glucose repression was downregulated (-2.2) at 12 ^{o}C while concomitantly its homologue *MIG2* was inversely 2.9-fold upregulated.

Table 2: Over-representation of GO term category in the genes in up- and down-regulated gene clusters at low temperature (LT) under limitations of carbon (C-Lim) and nitrogen (N-Lim).

GO term	GO_complete category	*p*-value	k[a]	f[b]
LT C-lim UP				
GO:0007046	ribosome biogenesis	1.4E-05	20	198
LT C-lim DWN				
GO:0006091	generation of precursor metabolites and energy	4.7E-06	26	229
GO:0015980	energy derivation by oxidation of organic compounds	1.1E-06	25	199
GO:0000041	transition metal ion transport	4.6E-05	9	41
GO:0015849	organic acid transport	2.8E-05	10	48
GO:0046942	carboxylic acid transport	2.3E-05	10	47
GO:0015837	amine transport	2.8E-05	10	48
GO:0006118	electron transport	7.3E-05	8	34
GO:0006865	amino acid transport	1.3E-06	10	35
GO:0046688	response to copper ion	7.9E-05	3	3
LT N-lim UP				
GO:0007582	physiological process	3.0E-05	203	4243
GO:0008150	biological_process	5.8E-05	204	4299
GO:0009987	cellular process	6.1E-06	203	4173
GO:0016043	cell organization and biogenesis	1.6E-06	78	1158
GO:0050875	cellular physiological process	4.1E-06	202	4130
GO:0044249	cellular biosynthesis	4.4E-05	55	796
GO:0006996	organelle organization and biogenesis	1.0E-06	71	1007
GO:0006396	RNA processing	4.7E-11	41	325
GO:0016070	RNA metabolism	4.3E-11	49	439
GO:0009451	RNA modification	4.6E-06	14	82
GO:0000027	ribosomal large subunit assembly and maintenance	3.4E-08	12	40
GO:0006461	protein complex assembly	7.1E-06	19	147
GO:0007028	cytoplasm organization and biogenesis	9.3E-29	56	236
GO:0042254	ribosome biogenesis and assembly	9.3E-29	56	236
GO:0042255	ribosome assembly	2.7E-10	17	63
GO:0042257	ribosomal subunit assembly	1.2E-07	13	53
GO:0006364	rRNA processing	4.3E-20	39	164
GO:0006365	35S primary transcript processing	3.2E-08	14	56
GO:0007046	ribosome biogenesis	5.5E-28	51	198
GO:0016072	rRNA metabolism	1.7E-19	39	170
GO:0030490	processing of 20S pre-rRNA	3.4E-11	17	56
GO:0000154	rRNA modification	3.5E-07	8	19
GO:0042273	ribosomal large subunit biogenesis	3.5E-07	8	19
GO:0000054	ribosome export from nucleus	1.4E-06	7	16
GO:0006183	GTP biosynthesis	2.7E-06	4	4
GO:0046039	GTP metabolism	2.7E-06	4	4
LT N-lim DWN				
GO:0050896	response to stimulus	1.1E-05	67	510
GO:0009056	Catabolism	4.2E-06	54	370
GO:0044248	cellular catabolism	7.1E-05	48	349
GO:0016052	carbohydrate catabolism	6.0E-05	14	55
GO:0044275	cellular carbohydrate catabolism	6.0E-05	14	55
GO:0015837	amine transport	5.4E-05	13	48
GO:0005984	disaccharide metabolism	5.5E-07	7	9
GO:0046352	disaccharide catabolism	1.4E-06	6	7
GO:0000255	allantoin metabolism	1.6E-05	5	6
GO:0000256	allantoin catabolism	1.6E-05	5	6
GO:0046700	heterocycle catabolism	1.6E-05	5	6
LT C- & N-lim UP				
GO:0007046	ribosome biogenesis	7.6E-05	12	198
GO:0000054	ribosome export from nucleus	5.4E-05	4	16
LT C- & N-lim DWN				
GO:0006950	response to stress	1.7E-05	9	70

[a] Number of genes in category of cluster
[b] Number of genes in category over genome

Table 3: Over-representation of transcription factors (TF) that can bind the upstream promoter elements of genes in up and down-regulated gene clusters (according to the ChiP on chip analysis (121)) at low temperature (LT) under limitations of carbon (C-Lim) and nitrogen (N-Lim). The probability (p-value) that the representation of each factor occurred by chance was assessed by hypergeometric distribution. The table displays significant factors that returned a p-value lower than 0.05.

	T F	p-value	k[a]	f[b]
LT C-lim UP				
LT C-lim DOWN				
	Gln3p	5.3E-04	12	92
	Hsf1p	8.2E-07	20	133
	Nrg1p	4.3E-07	20	128
	Phd1p	4.0E-06	16	99
	Rcs1p	1.0E-03	11	86
	Rox1p	1.6E-06	13	62
	Skn7p	1.0E-03	16	156
	Sok2p	2.7E-05	13	79
	Hap3-Hap1	7.9E-05	3	3
	Phd1-Nrg1	2.3E-06	10	37
	Rox1-Phd1	1.7E-05	7	21
	Sok2-Nrg1	6.4E-08	11	33
LT N-lim UP				
	Fhl1p	5.9E-04	19	203
LT N-lim DOWN				
	Aft2p	3.4E-05	25	134
	Cin5p	4.2E-05	29	169
	Dal82p	1.38E-03	14	73
	Gln3p	4.0E-09	26	92
	Hsf1p	3.2E-06	27	133
	Nrg1p	4.3E-07	28	128
	Phd1p	6.5E-05	20	99
	Sok2p	7.3E-06	19	79
	Gln3-Dal82	1.7E-08	10	15
	Hap2-Dal82	1.5E-05	6	9
	Phd1-Nrg1	1.4E-05	12	37
	Sok2-Nrg1	2.4E-05	11	33
LT C- & N-lim UP				
	Dig1p	7.7E-04	8	144
LT C- & N -lim DOWN				
	Aft2p	6.3E-04	10	134
	Hsf1p	2.2E-08	16	133
	Nrg1p	5.8E-07	14	128
	Phd1p	2.7E-03	9	99
	Rcs1p	9.4E-05	9	86
	Rox1p	5.1E-05	8	62
	Sok2p	4.8E-05	9	79
	Nrg1-Aft2	5.4E-05	5	20
	Phd1-Nrg1	1.1E-05	7	37
	Rox1-Phd1	7.0E-05	5	21
	Sok2-Nrg1	3.6E-07	8	33

[a] Number of genes in category of cluster, [b] Number of genes in category over genome

Table 4: Gene coverage of over-represented sequences retrieved from promoters of co-regulated genes using regulatory sequence analysis tools (http://rsat.scmbb.ulb.ac.be/rsat/) (336) during low temperature (LT) in anaerobic chemostats of carbon (C-Lim) and nitrogen (N-Lim) limitation

Regulatory cluster	Motif	Putative-binding protein	Promoter element	occ[a]	expect occ[b]	occ E[c]
LT C-lim UP	PAC		CGATGAG	47	19.76	1.1E-03
	PAC		GATGAGC	40	15.57	1.4E-03
LT C-lim DWN			TGACACA	44	19.03	5.7E-03
LT N-lim UP			AAAAATTT	155	58.48	3.2E-21
	PAC		CGATGAG	53	12.47	4.7E-13
			GTGAAAAA	66	32.16	3.7E-03
LT N-lim DWN	GATA	Gln3/Gat1/Dal80/Gzf3	AAGATTAG	56	23.11	1.7E-04
	STRE	Msn2/Msn4	ACCCCTTA	29	8.73	1.6E-03
LT C- & N-lim UP	PAC		CGATGAG	26	7.75	1.6E-03
LT C- & N-lim DWN			CGTCCAC	13	2.85	7.8E-03

[a] number of occurrence of promoter element in the regulatory cluster
[b] expected number of occurrences of the promoter element in a randomly chosen cluster of genes of the same regulatory cluster size
[c] the probability of finding the number of patterns with the same level of overrepresentation, which would be expected by chance alone

Table 5: List of genes with gene expression data during low temperature (LT) growth in anaerobic carbon- (C-Lim) and nitrogen- (N-Lim) limited chemostats at 12 °C and 30 °C. Genes marked with an asterisk (*) are essential for growth.

Gene	Function	Gene expression data (Intensities)			
		C-Lim 12 °C	C-Lim 30 °C	N-Lim 12 °C	N-Lim 30 °C
Up-regulated LT C-Lim 12 °C					
Ribosomal biogenesis (GO:0007046)					
*CGR1**	Processing of pre-RNA for the 60S	168 ± 40	69 ± 3	122 ± 75	60 ± 12
*EBP2**	Maturation of 25S rRNA and 60S	255 ± 41	112 ± 20	230 ± 135	104 ± 19
ECM1	Interacts with *MTR2* in 60S	279 ± 22	85 ± 14	253 ± 38	56 ± 4
*EMG1**	maturation of the 18S rRNA and for 40S	451 ± 83	190 ± 43	392 ± 51	135 ± 16
*IPI3**	Possible in assembly of the ribosomal large subunit	68 ± 12	32 ± 5	56 ± 8	24 ± 4
*KRR1**	synthesis of 18S rRNA and the assembly of 40S	170 ± 22	67 ± 0	164 ± 37	66 ± 15
*MAK16**	constituent of 66S pre-ribosomal particles	87 ± 11	37 ± 13	69 ± 21	27 ± 3
*MTR4**	Dead-box family ATP dependent helicase	80 ± 5	40 ± 5	71 ± 2	39 ± 3
*NMD3**	nuclear export of the large ribosomal subunit	257 ± 79	127 ± 25	265 ± 47	89 ± 8
*NOG1**	Putative GTPase	368 ± 85	182 ± 16	383 ± 50	128 ± 14
*NOG2**	Putative GTPase	619 ± 57	263 ± 36	471 ± 68	88 ± 20
*NOP4**	Nucleolar protein	117 ± 17	48 ± 4	61 ± 15	31 ± 3
NOP6	Protein with similarity to hydrophilins	162 ± 42	79 ± 14	139 ± 39	73 ± 15
NSR1	Nucleolar protein in processing 20S to 18S rRNA	757 ± 169	341 ± 91	843 ± 193	74 ± 1
*RNT1**	RNAase III	134 ± 25	66 ± 9	93 ± 37	64 ± 7
*RPF2**	processing of pre-rRNA and assembly of the 60S	522 ± 50	245 ± 61	458 ± 116	150 ± 27
*URB1**	Nucleolar protein required for 25S and 5.8S rRNAs	80 ± 12	33 ± 6	51 ± 6	29 ± 5
*UTP13**	Nucleolar protein involved in pre-18S rRNA	114 ± 19	51 ± 9	107 ± 14	30 ± 7
UTP30	maturation of pre-18S rRNA	81 ± 16	38 ± 6	69 ± 14	48 ± 3
YDR412W*	Protein required for cell viability	68 ± 6	24 ± 4	44 ± 16	21 ± 4
Down-regulated LT C-Lim and N-Lim 12 °C					
Heat shock response protein family					
AHA1	Co-chaperone that binds to Hsp82p	193 ± 22	621 ± 88	205 ± 22	527 ± 40
APJ1	Putative chaperone of the HSP40	13 ± 1	39 ± 8	13 ± 1	44 ± 9
*HSF1**	Trimeric heat shock transcription factor	47 ± 20	99 ± 8	62 ± 7	177 ± 13
HSP26	Small heat shock protein	55 ± 11	1124 ± 78	113 ± 40	754 ± 14
HSP30	negatively regulates the H(+)-ATPase Pma1p	26 ± 13	922 ± 172	61 ± 22	446 ± 71
HSP42	Small cytosolic stress-induced chaperone	103 ± 25	246 ± 43	152 ± 47	529 ± 2
*HSP60**	Tetradecameric mitochondrial chaperonin	725 ± 172	1661 ± 22	772 ± 43	1217 ± 105
SSA3	Member of *HSP70* family	30 ± 4	108 ± 28	24 ± 6	73 ± 6
SSE2	Member of *HSP70* family	123 ± 20	261 ± 13	106 ± 19	247 ± 62
Down-regulated LT N-Lim 12°C					
Reserved carbohydrate metabolism					
ATH1	Acid trehalase	37 ± 2	84 ± 4	18 ± 3	58 ± 2
NTH1	Neutral trehalase	93 ± 0	124 ± 17	91 ± 16	221 ± 29
NTH2	Putative neutral trehalase	52 ± 14	111 ± 26	48 ± 10	169 ± 24
TPS1	trehalose-6-phosphate synthase	437 ± 99	433 ± 48	287 ± 69	612 ± 54
TPS2	trehalose-6-phosphate synthase	191 ± 64	232 ± 48	213 ± 69	434 ± 21
TSL1	trehalose 6-phosphate synthase	215 ± 25	167 ± 25	96 ± 42	353 ± 33
GSY1	Glycogen synthase	162 ± 20	122 ± 34	70 ± 13	212 ± 27
GSY2	Glycogen synthase	105 ± 50	154 ± 39	105 ± 15	230 ± 20

Consistent with previous reports that cited transport activities are influenced by temperature (41, 236, 362), four redundant GO categories (GO:0015849, GO0046942, GO:0015837 and GO:0006865) (Table 2) were overrepresented in the set of genes exhibiting a lower expression at 12 °C than at 30 °C. These categories were comprised of 10 genes of which the function was associated with the transport of amino acids (*MMP1, BTN2, MUP1, GNP1, BAP3, AGP1 BAP2, TAT1, DIP5, TAT2*). Some genes on the other hand, did

not belong to any enriched GO categories, but a group of nine genes including *AHA1* (-3.2), *APJ1* (-2.9), *HSF1* (-2.1), *HSP26* (-20.6), *HSP30* (-35.0), *HSP42* (-2.4), *HSP60* (-2.3), *SSA3* (-3.6) and *SSE2* (-2.1) associated with thermal shock stress were found downregulated in contrast to several previous low temperature transcriptome studies (Table 5) (137, 158, 234). The presence of these genes was consistent with the significant overrepresentation in this cluster of targets of Hsf1, the yeast heat shock transcription factor (363) (Table 4). To test the consistency of the transcriptional response to a downshift of the cultivation temperature, a comparison of the transcriptome response at 12 ^{o}C and 30 ^{o}C was also carried out on nitrogen-limited grown yeast cultures.

Temperature-dependent transcriptome response in N-limited chemostat cultures

The group of 261 genes that showed a higher expression at 12 ^{o}C than at 30 ^{o}C under nitrogen limitation (Figure 1) were substantially enriched in genes belonging to GO categories related to RNA metabolism and ribosome biogenesis (Table 2). As seen in C-limited conditions, some gene transcripts encoding subunits of the polymerase I (*RPA12*, *RPA34*, *RPA49*, *RPA135*) exhibited higher hybridization signal at 12 ^{o}C than at 30 ^{o}C. Three subunits of the RNA polymerase III (*RPC11*, *RPC31*, *RPC40*) that participates in tRNA transcription were likewise induced at 12 ^{o}C. A total of 56 genes involved in 17 GO categories covering the complete ribosome biogenesis as ribosome assembly (GO:0042255), 35S primary transcript processes (GO:0006365), 20S pre-rRNA processing (GO:0030490), ribosome export from nucleus (GO:0000054) were upregulated at 12 ^{o}C (Tables 2, Supplementary data Table 2). The promoter search revealed the significant presence of a cis-regulatory PAC motif (GATGAG) characteristics of this rRNA transcription (310). In addition to the re-formatting ribosome biogenesis genes, the expression of 21 ribosomal proteins was increased at 12 ^{o}C that further supports an increase of the whole translational machinery (Table 2). In good agreement with this upregulation, this cluster exhibited a significant enrichment of targets of Fhl1, a factor involved in ribosomal protein transcription (217, 278) (Table 3).

Under nitrogen-limitation, a large set of 501 genes exhibited a lower expression signal at 12 ^{o}C than at 30 ^{o}C (Figure 1). This cluster exhibited an enrichment in GO categories related to response to environmental stimulus (GO:0050896) and catabolism of both carbon and nitrogen sources (GO:0009056, GO:0044248, GO:0016052, GO:0044275, GO:0015837, GO:0005984, GO:0046352, GO:0000255, GO:0000256, GO:0046700) (Table 2, Supplementary data Table 2). This included genes encoding enzymes implicated in metabolism of arginine (*ARG1*, *CAR1*, *CAR2*), asparagine (*ASP3*), glutamate and glutamine (*GDH3*, *GLN1, GLT1*), proline (*PUT1*, *PUT4*), serine/threonine (*CHA1*, *CHA4*, YIL167w), aromatic amino acids (*ARO10*), sulfur amino acids (*MET4*, *MUP1*, *SUL1*, YFR055w), branch chain amino acids (*BAT2, LAP4*) and nitrogen source transporters (*AGP1*, *CAN1*, *GAP1*, *DIP5, DUR3*). The GO lists ‘allantoin metabolism’ and ‘catabolism’ (GO:0000255 and GO:0000256) found significantly enriched in the N-limited downregulated genes comprised

of five genes (*DAL1, DAL2, DAL7, DAL3*, *DUR1,2)*, key genetic markers of the nitrogen-catabolite-response (NCR). The products of these genes participate in nitrogen assimilation from allantoate, a product of purine catabolism specifically induced in nitrogen limitation conditions, and in the presence of a less favored nitrogen source (e.g. phenylalanine, leucine, proline, methionine). The occurrence of downregulation of genes that are subjected to NCR was supported twice i) by enrichment of Gln3 and Dal82 targets in this set (Table 3) and ii) by overrepresentation of protein binding motifs (AAGATAAG) similar to the GATAAG factor binding motif involved in NCR (58, 65) (Table 4).

In contrast to C-limitation where only *ATH1* and *NTH2* genes that encode an acidic and neutral trehalase, respectively, were expressed to a lower level at 12 ^{o}C, the genes encoding for the complete trehalose biosynthesis (*TPS1*, *TPS2*, *TSL1*) and degradation (*ATH1*, *NTH1*, *NTH2*) pathways were all downregulated at 12 ^{o}C (Table 5). Both the glycogen synthase genes (*GSY1*, *GSY2*) also exhibited lower expression level at 12 ^{o}C than at 30 ^{o}C (Table 5). These expression data were in strong agreement with the amount of reserve carbohydrate measured at 12 ^{o}C that was lower than at 30 ^{o}C (Table 1). Moreover, this cluster showed significant overrepresentation of STR (STRess) element known to be bound by Msn2/Msn4 (Table 4) which have been implicated in the regulation of the reserve carbohydrate metabolism (101). In addition, as already shown in C-limited downregulated genes, a set of heat shock proteins including *HSF1* that encodes the main heat shock transcription factor were repressed at 12 ^{o}C (Table 5). The presence of these genes was eventually supported by the enrichment in this cluster of Hsf1 targets as shown in Table 3.

Low temperature co-responsive genes

Out of the 1026 genes differentially expressed in at least one of the two pairwise comparisons, a subset of 227 genes exhibited a similar temperature dependent regulation irrespective of the nutrient that is in limitation (Figures 1 and 2). Hypergeometric distribution analysis using Fisher's Exact revealed overrepresentation of 'ribosome biogenesis' (GO:0007046) and 'ribosome export from nucleus' (GO0000054) categories in the 88 gene cluster that exhibited a higher expression at 12 ^{o}C than at 30 ^{o}C in both growth limitation conditions. Twelve out of the 88 genes were represented in these two GO categories (Table 2, Figure 2). As it was already true for the nutrient specific sets, the promoter sequence analysis revealed the overrepresentation of the PAC cis-regulatory sequence (Table 4). Close inspection of this set also revealed the presence of some genes previously described as cold temperature markers, in particular *NSR1*, a nucleolar protein involved in processing 20S to 18S rRNA (170, 192). *DBP2*, an ATP-dependent RNA helicase of DEAD box family that might participate in increasing translation efficiency, by analogy with plants and bacteria (155, 323) was also co-induced. However, several key genes considered as markers for low temperature showed a transcriptional profile in opposite expression as with all previously published yeast low temperature transcriptome studies. Among those, the genes related to

stress response (*HSF1*, *HSP26*, *HSP30*, *HSP42*, *HSP60*, *SSA3*, *SSE2, AHA1*, *NTH2, ATH1)* were consistently lower at 12 °C than at 30 °C.

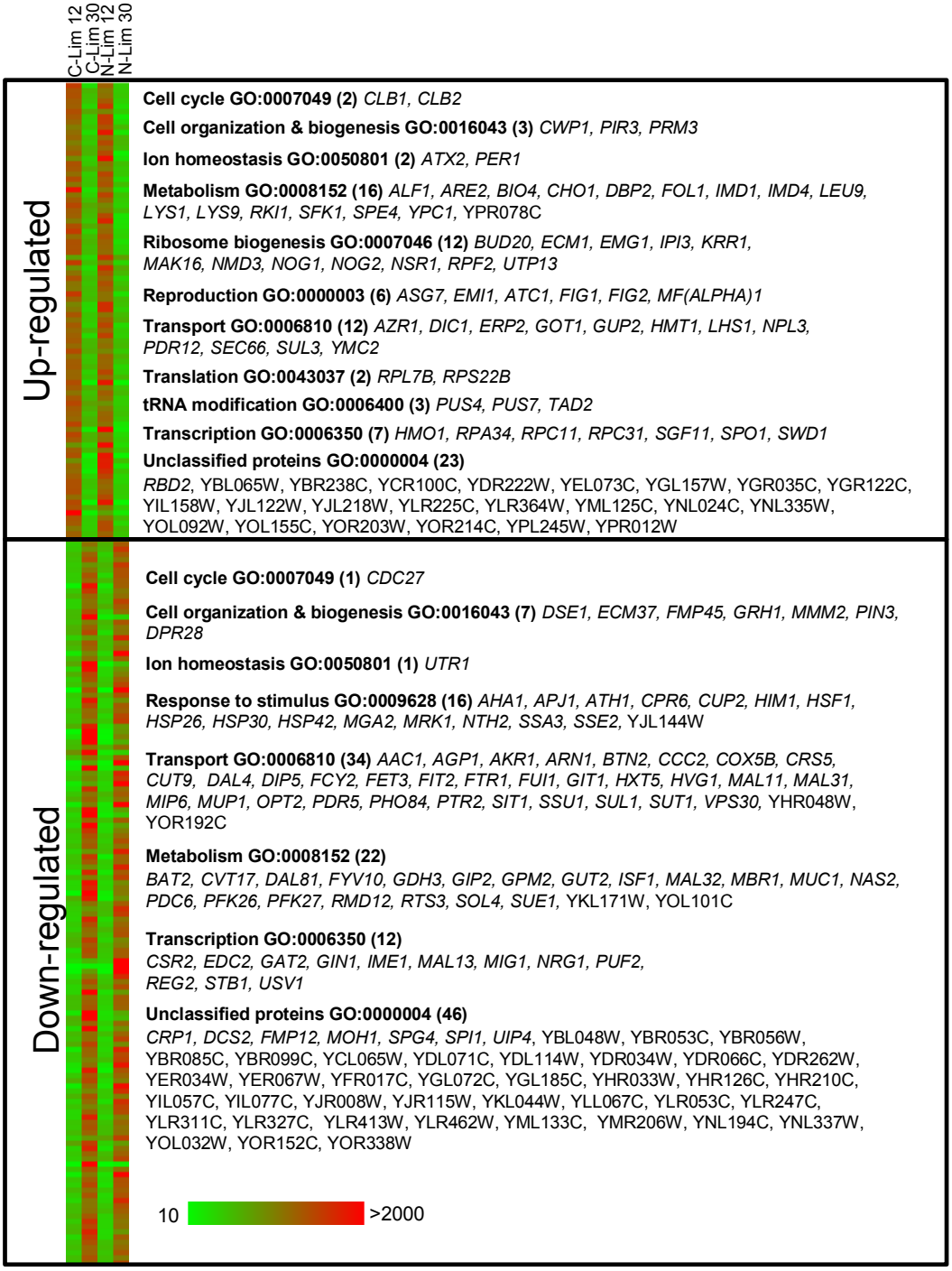


Figure 2 **Expression profiles of differentially expressed genes in both anaerobic carbon and nitrogen limited cultures of 12 °C compared to 30 °C.** The three independent transcriptome datasets for each temperature condition were averaged and then compared. Green (relatively low expression) and red (relatively high expression) squares are used to represent the transcriptome profiles of genes deemed significantly changed. These genes were sorted by functional categories according to the GO Biological process functions.

Out of the downregulated genes in both limitations, six were encoding macronutrient transporters of sulfur (*SUL1*, *MUP1*), phosphorus (*PHO84*), nitrogen (*DAL4*) and carbon (*HXT5*, *MAL31).* The genes encoding for five metal carriers (*FTR1*, *FET3*, *CRS5 CCC2*, *UTR3*) were also consistently repressed at 12 ^{o}C and at least three *(FET3, FTR1* and *CRS5)* are under the control of *CUP2* that showed a similar downregulated profile.

Low temperature literature comparison

Several transcriptome reports dealing with the low temperature adaptation of *Saccharomyces cerevisiae* have already been published (23, 234, 280, 286). In contrast to the approach described here, these studies have been performed in batch (shake flask experiments) cultures. The data set corresponding to four previously mentioned batch-based transcriptome studies were downloaded from their respective websites (see experimental procedures for data processing) and compared with the data generated in this study (1026 genes). These systematic comparisons revealed that no global positive correlation between the chemostat and the batch data could be drawn. Conversely, a substantial fraction of the genes exhibiting either up or downregulation in this study was inversely regulated in most of the other batch studies (Figure 3). To point out the heterogeneity of the cold temperature transcriptome responses, we determined how many genes were found similarly regulated in all the studies (Figure 4A) by comparing the genes coregulated in C- and N-limitation cultures with the differentially expressed genes in the other studies (Figure 4B). Only 6 genes (*DBP2, FCY2, HMT1, HSP26, MOH1* and *NOG2*) were over-represented. This limited set was even more reduced as none of these genes showed consistent profiles over the studies. *DBP2, NOG2* and *HTM1* that exhibited a clear increased expression at 12 ^{o}C in C- and N-limited chemostat displayed a lower expression at 10 or 13 ^{o}C in batch cultivations as reported by Sahara *et al.* (2002) and Beltran *et al.* (2006) (Figure 4C). *HSP26* on the other hand which was strongly repressed in chemostat cultures but was highly induced notably in the final stages of all the batch cultivations (Figure 4C).

It has generally been accepted from previous low temperature transcriptome experiments that the reduction in temperature leads to an induction of a set of genes commonly associated with environmental stress response (ESR) (158, 286). These genes are regulated by transcription factors Msn2p and/or Msn4p via STR elements in their promoter regions (37, 233). When the transcript levels of the characterized ESR genes (107, 108) (Figure 3, Supplementary data Table 5) obtained in C-and N-limited cultures at 12 ^{o}C and 30 ^{o}C were compared with the three other sets (23, 234, 280), the response was surprisingly different. In low temperature chemostat only two genes, *FAA1* encoding protein involved in fatty acid metabolism and *GRE1* encoding a NADPH-dependent methylglyoxal reductase, were upregulated under carbon limitations (Figure 3, Supplementary data Table 5). With exception of the two latter genes all ESR genes were significantly repressed during chemostat cultivations at 12 ^{o}C (compared to 30 ^{o}C).

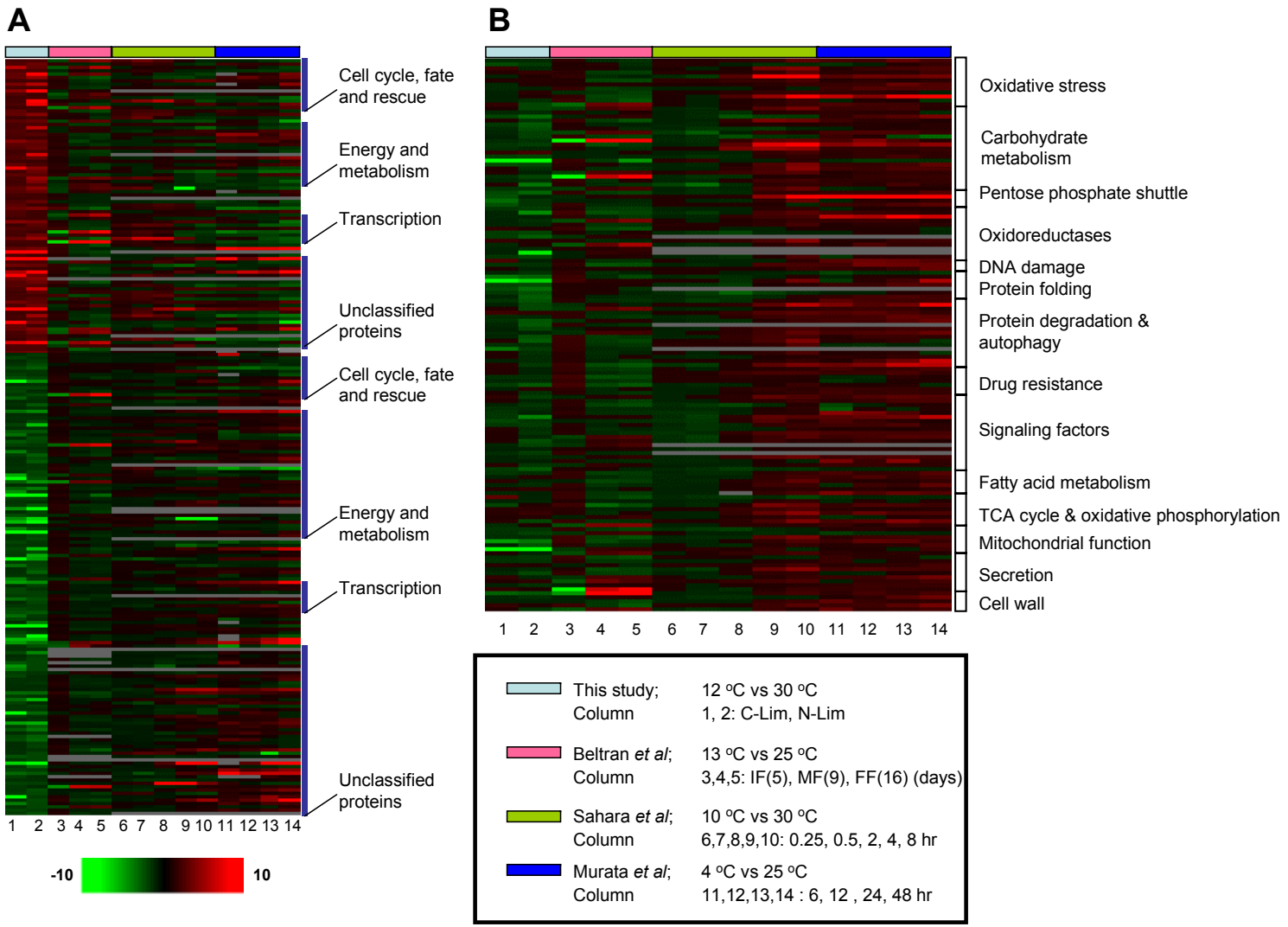
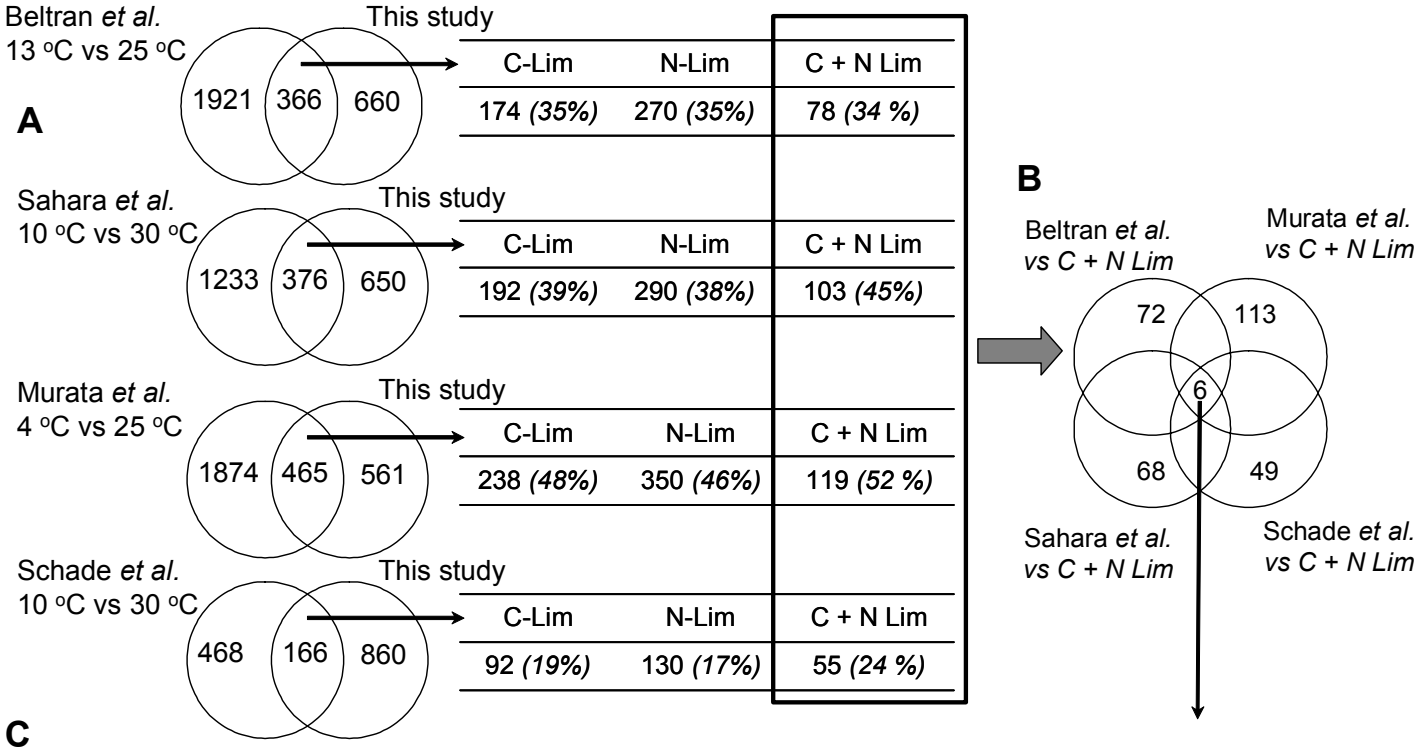


Figure 3: **Fold change comparisons with other genome-wide low temperature studies. A** Fold change of the 88 genes up-regulated and 139 genes down-regulated (> 2-folds, 1 % False discovery rate (see methods)) in both carbon- (C-Lim) and nitrogen-limited (N-Lim) chemostat cultures at 12 ^{o}C compared to 30 ^{o}C. These genes were compared to expression data of Beltran *et al*. (23), Sahara *et al.* (280) and Murata *et al.* (234). **B** Fold change of characterized Environmental Stress Response (ESR) genes from Gasch et al (107). Genes denoted in grey were not available. IF: Initial fermentation, MF: Middle fermentation, FF: Final fermentation.



C

	This study		Beltran *et al.* (days)			Sahara *et al.* (hours)					Murata *et al.* (hours)				Schade *et al.* (hours)					
Gene	C	N	5	9	16	0.25	0.5	2	4	8	6	12	24	48	0	0.17	0.5	2	12	60
DBP2	4.0	6.1	1.8	-2.2	-3.5	4.0	6.9	6.8	3.2	-1.3	1.1	1.0	-2.3	-2.0	-1.0	2.1	2.4	2.9	1.9	-1.3
FCY2	-2.5	-2.4	2.2	-1.6	-1.7	2.6	3.3	1.4	-3.1	-4.5	-7.6	-5.4	-7.6	-6.4	1.0	1.3	1.4	1.7	-3.5	-1.9
HMT1	3.1	4.6	-1.8	2.2	2.9	2.6	3.1	-1.2	n/a	-1.4	-1.2	-1.3	-2.1	-1.9	1.0	1.8	1.0	2.7	1.7	1.1
HSP26	-21	-6.7	2.0	1.3	1.3	1.1	1.0	-1.5	1.4	3.6	-1.8	1.3	2.0	5.5	1.1	-1.0	-1.1	-1.1	-1.1	2.1
MOH1	-7.3	-5.4	-3.4	4.4	5.6	1.0	1.1	1.3	2.7	4.5	8.3	3.6	8.5	30	-1.0	-1.1	-1.1	1.1	2.6	1.3
NOG2	2.4	5.3	1.6	-1.4	-2.1	2.4	3.9	3.0	-1.0	-1.4	-3.5	-2.6	-3.0	-2.6	1.1	1.5	2.0	1.7	-1.0	-1.1

Figure 4 (previous page): A Venn diagrams comparing genes that showed at least two fold changes in each batch study (data obtained from online supplementary data. See Experimental procedures) with those found significantly changed in this study (1026 genes). The overlapping region is further broken down to the different clusters of genes found in this study. C-Lim represents the genes significantly up- and down-regulated in carbon-limited chemostats grown at 12 ^{o}C compared to 30 ^{o}C. N-Lim (nitrogen-limited), C + N Lim (consistently in both carbon and nitrogen limited). % indicate the percentage of overlapping genes (batch study vs this study) in each cluster over the total number of genes in the cluster; C-Lim (496 genes), N-Lim (762 genes), C + N Lim (227 genes) (Figure 1). **B** Venn diagram of genes overlapping with the genes what were found in the comparison of the 'batch vs this study' with C + N Lim in Figure 4A. **C** 6 genes that were found consistently different in all the comparisons of the batch studies against the C + N Lim set. Values shown are the fold changes of the expression intensities at low temperature compared to a reference temperature base. C = Carbon limitation, N = Nitrogen limitation, IF = Initial fermentation, MF = Middle fermentation, FF = Final fermentation.

Discussion

In this study we have investigated how the genome wide expression of *Saccharomyces cerevisiae* was reprogrammed at the suboptimal temperature of 12 ^{o}C. Some of the key genes (*TIP1, TIR1, TIR2, OLE1*) purportedly induced during a shift to low temperature in *S. cerevisiae* where not changed persistently in chemostats as previously seen in batch and shake flask cultures (169, 171, 234, 286). The transcript data discussed in this study were performed under anaerobic conditions, in which *TIP1, TIR1, TIR2* and other seripauperin genes were already known to be upregulated (313). Consequently, no discrepancy between 30 and 12 ^{o}C could be recorded. To sustain growth in absence of oxygen, essential exogenous ergosterol and unsaturated fatty acid, oleate (in the form of Tween 80) (8, 9) were added to the culture medium to compensate repression of *OLE1* a stearoyl-CoA desaturase (delta-9 fatty acid desaturase) that is required for synthesis of unsaturated fatty acids (309) under anaerobic conditions. Addition of Tween 80 consequently resulted in repression of *MGA2* positive regulator of the expression of *OLE1* (153).

The yeast transcriptome response at 12 ^{o}C was recorded on both C- and N-limitation. This comparison illustrated how the experimental setup, in which an environmental parameter (e.g. the temperature) is studied, may influence the genome wide expression response. Although, chemostat cultures allowed for controlling and maintaining of the cultured cells in a steady state where all physiological characteristics (growth rate, consumption and production rates) remained constant, a complete homogeneity between the environmental conditions could not be achieved between 12 ^{o}C and 30 ^{o}C. This resulted in a transcriptional response specific to the environmental conditions used. Consistently, the residual concentrations of the limiting nutrient were higher at 12 ^{o}C than at 30 ^{o}C. This increase is likely to participate to a higher degree of substrate saturation of the limiting nutrient transport system (glucose or ammonium) and thereby compensate for a reduced capacity of the transporters at low temperature. In the case of C-limitation, the residual glucose concentration was accompanied by a differential

regulation of five HXT genes. Whether these differential expressions are the direct consequence of the increased extracellular glucose or vice versa, remain unsolved.

Intuitively, the increase of the extracellular ammonium concentration at 12 °C was accompanied by the differential expression of the ammonia permease genes (*MEP1, MEP2* and *MEP3*). Both high affinity permeases *MEP1* (K_m=5-10 μM) and *MEP2* (K_m1-2 μM) were downregulated -2.8-fold and –4.0-fold respectively at 12 °C. Meanwhile *MEP3*, the low affinity permease (K_m=1.4-2.1 mM) exhibited higher expression (+ 1.8) at 12 °C at 30 °C (215). In contrast to what happened on glucose transport, the changes affecting the ammonia transport seems to be in good agreement with the literature (215). Besides, the downregulation of Nitrogen Catabolite Repression (NCR) responsive genes in N-limitation, the increased residual ammonium concentration in the fermentation broth is likely to reflect this response as well.

Furthermore, cells grown in N-limitation showed a reliable correlation between the whole cell protein content and the transcription of large sets of genes involved in the biogenesis and assembly of the translational machinery. According to Warner (1999) (356) ribosomal proteins represent around 50% of the translated mRNA pool in yeast. Up to now, there is no estimation of the ribosomal protein representation over the whole cell, however it would be tempting to translate the increase in whole cell protein with an increase in ribosomal protein as 21 ribosomal protein genes were upregulated at 12 °C under nitrogen limitation (Supplemental data Table 1).

In both nutrient limitations, 55 to 70% of the transcriptome response was the result of combinatorial effects indirectly related to the culture temperature. The integrity of the transcription and translation processes is shown to be essential for the cell survival, as the concentration of genes involved in ribosome biogenesis exhibited a higher expression at 12 °C than at 30 °C. In particular, temperature decrease affects RNA secondary structures stability leading to a rate-limiting step of translation initiation (91, 155). Consistent with a previous phenotypic characterization and low temperature transcriptome study (286), *NSR1* that encodes a nucleolar protein required for pre-rRNA processing and ribosome biogenesis, and *DED1* (73) and *DBP2* that encode ATP-dependent RNA helicases of DEAD box family were induced at 12 °C irrespective of the limitation applied. RNA helicases are known to respond to abiotic stresses (254) and in particular to temperature by unwinding cold-stabilized mRNA secondary structures to increase translation efficiency in bacteria (155, 323). Cold-inducible RNA helicases have been reported in plants as well (293) broadening the essential role of RNA helicases in cold adaptation.

On the other hand, genes with function related to response to stimulus (stress) were clearly downregulated at 12 °C in both limitation conditions. Among them, a subset of *HSP* genes and their transcription factor *HSF1* were exhibiting a consistent lower expression at 12 °C than at 30 °C. Hsp proteins (e.g. Hsp42 and Hsp26) have been implicated in chaperone activities participating to cytosolic protein folding and suppress the aggregation of cytosolic proteins under heat shock

conditions (122, 123). This mechanism seems to play a less crucial role at low than at high temperatures. However, so far these genes (*HSP42* and *HSP26*) have been systematically shown to be upregulated at low temperature (137, 286). Increased expression of *HSP* genes is often accompanied by higher expression of genes encoding reserve carbohydrate pathways followed by concomitant augmentation of their intracellular concentrations. This remains in total contrast to what has been measured in cells grown at a constant growth rate. So what then could explain such a difference in expression regulation?

The presence of STR elements (115) in the gene cluster exhibiting a lower expression at 12 ^{o}C than at 30 ^{o}C under N-limitation, suggested overrepresentation of Msn/Msn4 targets (Table 4). Msn2/Msn4 regulatory complex have been shown to govern to some extent the expression of reserve carbohydrates metabolism genes (37, 101, 373)} and in cooperation with Hsf1 the expression of *HSP* genes (e.g. HSP26, HSp104) (7). In more general terms, Msn2/Msn4 complex have been assigned to a broader response named Environmental Stress response (ESR) (107). No ESR genes (with exception of two genes (*FAA1*, *GRE2*)) showed higher expression at 12 ^{o}C than at 30 ^{o}C as already claimed (286). The undetectable intracellular trehalose concentration was also a supporting evidence of the absence of participation of Msn2/Msn4 regulation in long-term adaptation at low temperature.

The expression of the ESR genes is also tightly related to the specific growth rate of the yeast cell. ESR genes increase as specific growth rate decreases (117). Most of the conditions that induce ESR inhibit cellular growth as well. Thus, we can speculate that growth rate, more than the nature of the stress applied, is sensed and serves as a transduction signal. This would be very similar to what have been reported in *E.coli*, where the specific growth rate is known to control the general stress response, that is mediated by the concentration of the RNA polymerase subunit RpoS (145). This would explain to some extent the differences between the batch and chemostat low temperature transcriptome studies. The application of chemostat cultures allowed the identification of a temperature dependent transcriptional response by neglecting the effects of growth rate that remains constant between the fermentation conditions tested. However, the data presented here could not rule out the role of the ESR response in transition phase where the cells are transferred from one temperature to the other. But they strongly support that ESR does not contribute to cellular growth of low temperature adapted yeast cells. Similarly, trehalose is not indispensable to yeast cell to grow at 12 ^{o}C.

In summary, the transcriptional response to prolonged cold cultivation at 12 ^{o}C of *S. cerevisiae* in chemostat culture showed that in contrast to many cold-shock batch-based related studies, the response to low temperature is not mediated by stress signals and subsequently allowed the identification of unbiased sets of genes. Among the changes recorded, transcription and translational functions seemed to be the most important of all.

Acknowledgements

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Chapter 6

Control of glycolytic flux in *Saccharomyces cerevisiae* grown at low temperature: a multi-level analysis in anaerobic chemostat cultures

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Abstract

Growth temperature has a profound impact on the kinetic properties of enzymes in microbial metabolic networks. Activities of glycolytic enzymes in *Saccharomyces cerevisiae* were up to 7.5-fold lower when assayed at 12 ^{o}C than at 30 ^{o}C. Nevertheless, the *in vivo* glycolytic flux in chemostat cultures (dilution rate; 0.03 h^{-1}) grown at these two temperatures was essentially the same. To investigate how the yeast maintained a constant glycolytic flux despite the kinetic challenge imposed by a lower growth temperature, a systems approach was applied that involved metabolic flux analysis, transcript analysis, enzyme activity assays and metabolite analysis. Expression of hexose-transporter genes was affected by the growth temperature, as indicated by differential transcription of five *HXT* genes and changed zero-trans-influx kinetics of ^{14}C glucose transport. No such significant changes in gene expression were observed for any of the glycolytic enzymes. Fermentative capacity (assayed off-line at 30 ^{o}C), which was two-fold higher in cells grown at 12 ^{o}C, was therefore probably controlled predominantly by glucose transport. Massive differences in the intracellular concentrations of nucleotides (resulting in an increased adenylate energy charge at low temperature) and glycolytic intermediates indicated a dominant role of metabolic control as opposed to gene expression in the adaptation of glycolytic enzyme activity to different temperatures. In evolutionary terms, this predominant reliance on metabolic control of a central pathway, which represents a significant fraction of the organism's cellular protein, may be advantageous to limit the need for protein synthesis and degradation during adaptation to diurnal temperature cycles.

Introduction

Changing ambient temperature, for example as a result of diurnal temperature cycling, is among the most common environmental changes that micro-organisms have to contend with in nature. Temperature effects on microbial physiology are also relevant for the industrial exploitation of micro-organisms. For example, the temperature in industrial processes for production of alcoholic beverages with the yeast *Saccharomyces cerevisiae* (commonly 8 ^{o}C to 20 ^{o}C) is much lower than the optimum temperature range for growth (25 – 30 ^{o}C) (17).

Much of the current knowledge on adaptation of the model eukaryote *S. cerevisiae* to suboptimal temperatures has been derived from studies on cold shock. These studies have identified cold-induced changes in membrane lipid composition (23, 142), transport functions, translational efficiency, protein folding and nucleic acid structure (for a review see (5)). Transcriptome analysis of cold adaptation in *S. cerevisiae* has revealed several types of responses that depend on the temperature range applied, namely "cold shock" (between 20 and 10 ^{o}C) (286) and "near freezing" (< 10 ^{o}C) (137, 234, 280) conditions. Responses to low temperature are also affected by the exposure period to low temperature (early phase and late phase responses,

(23, 137, 286). The early response in cold shock experiments encompasses upregulation of low-temperature marker genes such as *NSR1* (170), *TIR1*, *TIR2* (171), and *TIP1* (169), which are involved in *de novo* ribosome biogenesis, transcription and translation. The late phase is characterized by upregulation of genes involved in protein folding, trehalose synthesis and stress responses (e.g. *HSP12* and *HSP26* (158, 234)), which suggests involvement of *MSN2* and *MSN4* in the regulatory circuit for cold adaptation (158, 286). Recent reports have implicated the HOG pathway in the transduction of the low temperature signal, resulting in the production of glycerol and trehalose required for resistance to freezing but not for growth at 12 ^{o}C (255).

Temperature also has a drastic effect on the catalytic properties of enzymes. The temperature dependency of enzyme kinetics on the catalytic rate constant, k_{cat} of a reaction is partially governed by the Arrhenius equation [1]

[1] $k_{cat} = Ae^{\left(\frac{-E_a}{RT}\right)}$. This equation dictates that increasing the temperature or decreasing the activation energy (for instance through the use of a catalyst) will result in an increase of k_{cat} (10). Each enzyme has unique catalytic properties, including temperature optimum and specific binding to allosteric effectors that contribute to regulation of its *in vivo* activity (these effects are not taken into consideration by the equation [1]).

In natural environments, both specific growth rate and the fermentation rate are key parameters in determining evolutionary fitness of microorganisms. To optimize rates at lower temperatures, micro-organisms can, in principle, resort to different strategies. Firstly, the synthesis of rate controlling enzymes may be increased. This 'vertical' regulation may be effected at the level of transcription, mRNA degradation, protein synthesis and degradation and/or post-translational modification. Alternatively or additionally, temperature-compensatory shifts of *in vivo* enzyme activity may be accomplished by metabolic regulation altering intracellular and extracellular concentrations of metabolites and effectors. The impact of metabolic control may also result from the temperature-dependent expression of (iso)enzymes with different kinetic properties (301).

The aim of the present study is to assess the contribution of vertical and metabolic regulation in the physiological adaptation of *S. cerevisiae* to lower temperature. In batch cultures, the tight coupling of temperature and specific growth rate makes it exceedingly difficult to dissect effects of these two parameters. For example, it is well documented that, even at a fixed growth temperature, different specific growth rates result in different gene expression patterns (117) as well as different intracellular metabolite concentrations (172, 370). In the present study, we circumvent this problem by comparing growth of *S. cerevisiae* at 12 and 30 ^{o}C in glucose-limited chemostat cultures. Since, in chemostat cultures, the specific growth rate is equal to the dilution rate, this cultivation technique enables investigation of the impact of temperature at a fixed specific growth rate. Our study is focused on

glycolysis and alcoholic fermentation, a pathway that plays a central role in anaerobic growth and represents a substantial fraction of the yeast's cellular protein (337). To dissect the regulation of *in vivo* glycolytic flux, data from physiological studies are integrated with *in silico* metabolic flux analysis, transcriptome analysis, enzyme-activity assays and intracellular metabolite analyses.

Experimental procedures

Strain and growth conditions

The *S. cerevisiae* prototrophic haploid reference strain CEN.PK113-7D (MAT***a***) (335) was grown at a dilution rate (D) of 0.03 h^{-1} at both 12 ^{o}C or 30 ^{o}C in 2-liter chemostats (Applikon, Schiedam, The Netherlands) with a working volume of 1.0 liter as described in (313, 333). A temperature probe connected to a cryostat controlled cultures grown at 12 ^{o}C. Cultures were grown in a defined synthetic medium that was limited by carbon with all other growth requirements in excess as described by (313). The dilution rate was set at 0.03 hr^{-1} with pH measured online and kept constant at 5.0 by automatic addition of 2 M KOH using an Applikon ADI 1030 Biocontroller. The stirrer speed was set to 600 rpm. Anaerobic growth and steady state conditions were maintained as described (313). Biomass dry weight, metabolite, dissolved oxygen, and gas profiles were constant at least three volume changes prior to sampling.

Analytical methods

Culture supernatants were obtained with the method described in (218). For the purpose of glucose determination and carbon recovery, culture supernatants and media were analyzed by high performance liquid chromatography on an AMINEX HPX-87H ion exchange column using 5 mM H_2SO_4 as the mobile phase. Ethanol evaporation from cultures was determined as described in (177). Residual ammonium concentrations were determined using cuvette tests from DRLANGE (Dusseldorf, Germany). Culture dry weights were determined as described in (264) while whole cell protein determination was carried out as described in (344).

Trehalose & glycogen

Trehalose and glycogen concentrations measurement were as according to Parrou et al. (1997) (257). Trehalose was determined in triplicate measurements for each chemostat. Glycogen was determined in duplicate for each chemostat. Glucose was determined using the UV-method based on Roche kit no. 0716251.

Fermentative capacity assay

Fermentative capacity assays were as previously described in (149).

Microarray analysis

Sampling of cells from chemostats, probe preparation, and hybridization to Affymetrix Genechip® microarrays were preformed as described previously in (262). RNA quality was determined using the Agilent 2100 Bioanalyser. The results for each growth condition were derived from three independently cultured replicates. Data acquisition and analysis as well as statistical analysis were performed as described previously in (313). The transcriptome have been deposited at Genome Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under the serie number GSE6190.

In vitro *enzyme assays*

Each in-vitro enzyme assays for the glycolytic pathway were performed as previously described in (149). All enzyme assays were performed at 12 and 30 ^{o}C (with exception of enolase that has not been measured). Protein determinations of cell extracts were as previously described in (344).

Metabolic flux distribution

Intracellular metabolic fluxes were calculated through metabolic flux balancing using compartmented stoichiometric model derived from the model developed in (184). The setup for the model was performed using the dedicated software (SPAD it, Nijmegen, The Netherlands). For both 12 and 30^{o}C chemostats, the specific rates of growth, substrate consumption, carbon dioxide and ethanol production during steady-state cultivation were calculated from the measured concentrations and flow rates from three independent chemostats. Assumptions and the theory of the model were previously described in (70).

Zero trans-influx uptake assays with labeled ^{14}C-glucose

For both cultures from different temperatures, cells were harvested from duplicate fermentations by centrifugation at 4^{o}C (5 min at 5000 rpm) and zero trans-influx uptake of labeled ^{14}C-glucose was determined at 30^{o}C according to (355). The parameters of sugar transport were derived according to single component Michaelis-Menten kinetics.

Intracellular metabolite determination

The procedure and equipment for rapid sampling of intracellular metabolites were based on (218) using the cold methanol quenching method. Two independent chemostats for each culture temperature was ran and metabolite measurements were done in triplicate for each chemostat. Glycolytic intracellular metabolites were analyzed by ESI-LC-MS/MS according to (332) and the quantification was preformed following the IDMS concept (369). Nucleotide concentration in the cell extract was analyzed by an ion pairing ESI-LC-MS/MS method and quantified following the IDMS concept (369). The adenylate charge (AC) was calculated as shown in equation [2]:

$$[2]\ AC = \frac{([ATP] + {}^{1}/_{2}[ADP])}{([ATP] + [ADP] + [AMP])}$$

Results

Physiological and biochemical analysis of chemostat cultures grown at different temperatures

Biomass yields, as well as specific rates of glucose consumption, ethanol and carbon-dioxide production in anaerobic, glucose-limited chemostat cultures of *S.cerevisiae* differed by less than 15% at 12 ^{o}C and 30 ^{o}C (Table 1a), indicating that growth energetics were not drastically affected by temperature. The only physiological characteristic that clearly distinguished the cultures was a 10-fold higher residual glucose concentration at 12 ^{o}C compared to 30 ^{o}C (Table 1a).

While trehalose accumulation is a commonly observed phenomenon in studies on transient cold stress (5, 158), the trehalose concentration during steady-state growth at 12 ^{o}C was much lower than at 30 ^{o}C. This is consistent with previous studies indicating that trehalose is essential for freezing tolerance, but not for growth at temperatures above 10 ^{o}C (255, 286). The cellular glycogen content was 1.5-fold higher at 12 ^{o}C than at 30 ^{o}C (Table1a).

In vivo fluxes in glycolysis were calculated *via* flux balancing using a stoichiometric model (Table 1b) (70, 184). To calculate *in vivo* fluxes, the model was fed with quantitative data on the biomass composition of *S. cerevisiae* (308) and with

the substrate consumption and product formation rates measured in the carbon-limited chemostat cultures (Table 1a). Consistent with the similar culture kinetics (Table 1a), the glycolytic flux was not substantially different in cultures grown at 12 and 30 ^{o}C (Table 1b). Conversely, off-line measurements of the fermentative capacity in glucose-rich medium at 30 ^{o}C yielded values that were 70% higher for cells grown at 12 ^{o}C than for cells grown at 30 ^{o}C (Table 1a, Supplemental Table S1).

The increased fermentative capacity of cells grown at 12 ^{o}C suggested that an upregulation of key rate-controlling enzymes involved in fermentative glucose metabolism and/or glucose transport is part of the mechanism by which *S.cerevisiae* maintained a constant glycolytic flux in the chemostat cultures grown at 12 ^{o}C and 30 ^{o}C. We subsequently analyzed the contribution of different levels of cellular control to the regulation of glycolytic flux in more detail.

Table 1: A-Physiological and biochemical characteristics of glucose-limited anaerobic chemostats grown at 12 °C and 30 °C Values represent the mean ± S.D. of data from three independent steady-state chemostat cultures. B- *In silico* glycolytic fluxes

A

Culture temp (°C)	Physiological data							Biochemical data		
	Y_{Glc-X}	q_{Glu}	$q_{ethanol}$	q_{CO2}	Carbon recovery	Residual glucose	Fermentative capacity	Trehalose	Glycogen	Whole cell proteins
°C	$g_{glucose}.g_{DW}^{-1}$	*mmol.*$g_{DW}^{-1}.h^{-1}$			*%*	*g/l*	*mmol.*$g_{DW}^{-1}.h^{-1}$	$g_{equivalent\ glucose}.g_{DW}^{-1}$		$g_{proteins}.g_{DW}^{-1}$
12	0.07 ± 0.01	- 2.5 ± 0.2	3.8 ± 0.3	4.4 ± 0.3	100 ± 3	0.5 ± 0.2	19.3 ± 1.6[i]	< 0.005	0.06 ± 0.0	0.40 ± 0.0
30	0.07 ± 0.00	- 2.3 ± 0.0	3.5 ± 0.0	3.8 ± 0.2	95 ± 1	0.05	11.4 ± 0.6[i]	0.02 ± 0.0	0.04 ± 0.0	0.43 ± 0.0

B

Reaction		***In silico* fluxes analysis (mmol.$g^{-1}.h^{-1}$)**		12 °C vs 30 °C
number	Enzymes	12°C	30°C	
1	Hexokinase	2.4±0.1	2.1 ±0.1	1.1
2	Glucose-6P isomerase	2.3±0.1	2.0 ±0.1	1.1
3	Phosphofructokinase	2.3±0.1	2.0 ±0.1	1.1
4	Fructose –1,6 P aldolase	2.3±0.1	2.0 ±0.1	1.1
5	Triose-P isomerase	1.9±0.1	1.7 ±0.1	1.1
6	G3P dehydrogenase	4.3±0.2	3.8 ±0.1	1.1
7	3P-glycerate kinase	4.3±0.2	3.8 ±0.1	1.1
8	3P-glycerate mutase	4.3±0.2	3.8 ±0.1	1.1
9	Enolase	4.3±0.2	3.8 ±0.1	1.1
10	Pyruvate kinase	4.3±0.2	3.8 ±0.1	1.1
11	Pyruvate decarboxylase	4.1±0.2	3.6±0.1	1.1
12	Alcohol dehydrganase	4.1±0.2	3.6 ±0.1	1.1

Transcriptome reprogramming at low temperature cultivation

DNA-microarrays were used for a genome-wide comparison of transcript levels in anaerobic, glucose-limited chemostat cultures grown at 12 ^{o}C and 30 ^{o}C. A full analysis of the data (also including data on nitrogen-limited cultures) was presented in Chapter 5. Here, we focus on transcripts that encode key enzymes involved in glucose transport, glycolysis and alcoholic fermentation (Figure 1).

The most striking differences were observed in the expression of several hexose-transporter (*HXT*) genes. Out of the seven *HXT* genes that were significantly expressed in at least one condition, five were differentially expressed at 12 ^{o}C and 30 ^{o}C (Table 2). *HXT2* (+2.6-fold), *HXT3* (+3.7), and especially *HXT4* (+33.5) were expressed at higher levels at 12 ^{o}C while *HXT5* (-40.4) and *HXT16* (-10.0) were expressed at a lower level at 12 ^{o}C. Interestingly, these changes did not seem to follow a simple logic that could be deduced from previous classification of the encoded hexose tranporters as either low (*HXT3*), high (*HXT2*) or moderate affinity carriers (*HXT4*) (212) were coordinately changed. Transcription of high affinity transporter genes, *HXT6* and *7* (273) was not significantly affected by the culture temperature.

Only four genes encoding enzymes of the glycolytic pathway were differentially expressed at 12 ^{o}C compared to 30 ^{o}C (Table 2). Three were downregulated (*GPM2, PYK2, PDC6*) while *PDC5* was upregulated. Despite their significant change in expression, all four genes encode minor isoforms of the corresponding enzyme activities and were transcribed at low level compared to the genes encoding the main isoforms (*GPM1, PYK1* and *PDC1*) (Table 2).

Glucose uptake assay and glycolytic enzyme activities in low temperature chemostat cultivations

To investigate whether the different transcript levels of the five *HXT* genes were reflected in glucose transport kinetics, zero trans-influx uptake assays with ^{14}C-glucose were performed at 30 ^{o}C. Cells grown at 12 ^{o}C and 30 ^{o}C exhibited high-affinity transport with substrate-saturation constants (K_m) of 1.1 mM and 0.8 mM, respectively (Figure 2a). The maximum transport capacity (V_{max}) was 60% higher at low temperature (Figure 2a), probably as a consequence of the increased transcript levels of several *HXT* genes (Table 2).

In anaerobic carbon-limited chemostat cultures, substrate-level phosphorylation *via* glycolysis is the main metabolic route for ATP supply. One mechanism to compensate for the decreased turnover numbers of glycolytic enzymes at low temperature might be an increased abundance (expression) of glycolytic enzymes. Hence, i*n vitro* assays of the glycolytic and fermentative enzymes were carried out with cell extracts from chemostats cultures at 12 ^{o}C and 30 ^{o}C (Table 2). To gain insight in the effect of the temperature on enzymes, the assays were performed at both 12 ^{o}C and 30 ^{o}C.

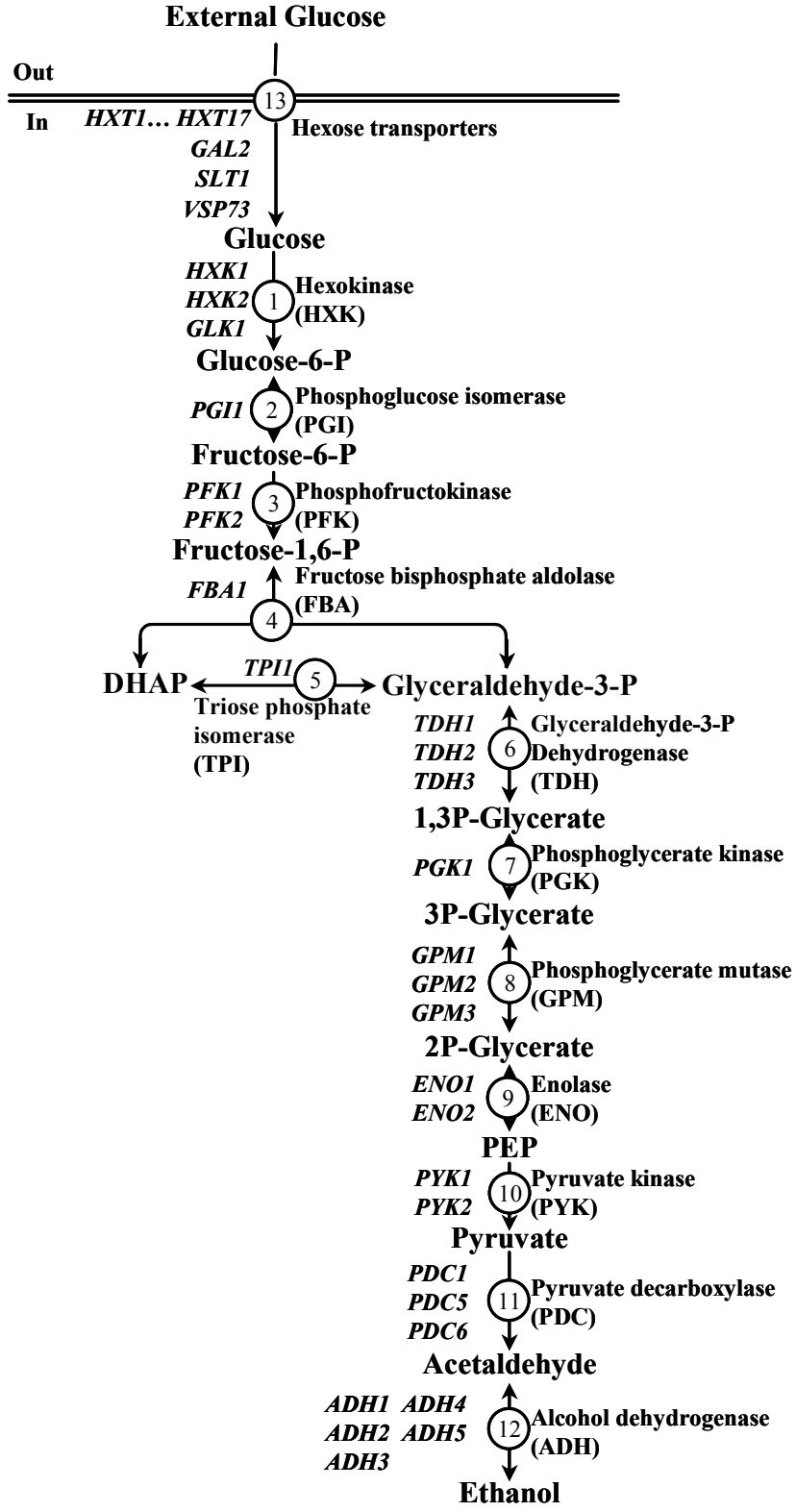

Figure 1: **Central carbon metabolism in *S. cerevisiae* from external glucose to the production of ethanol**

Table 2: Hexose transporters and glycolytic pathway transcript levels in *S. cerevisiae* grown in glucose-limited anaerobic chemostat cultivations at 12 °C and 30 °C.
Values represent the mean ± S.D. of data from three independent steady-state chemostat cultivations. Numbers at the last column correspond to the glycolytic pathway displayed in Figure 1.
[a] Fold change of transcription intensities of 12 °C over 30 °C.
[b] ORF not available on Affymetrix Genechip® YG-S98
n/a not applicable

Activity	Gene	Expression levels Culture temperature 12 °C	30 °C	FC[a]	Reaction number
Glucose transport	*HXT1*	17 ± 8	12 ± 0	1.4	**13**
	HXT2	**557 ± 160**	**214 ± 29**	**2.6**	
	HXT3	**840 ± 240**	**230 ± 27**	**3.7**	
	HXT4	**1022 ± 290**	**31 ± 2**	**33.5**	
	HXT5	**12 ± 0**	**485 ± 104**	**-40.4**	
	HXT6	3317 ± 513	3028 ± 485	1.1	
	HXT7	2593 ± 313	2097 ± 361	1.2	
	HXT8	19 ± 8	13 ± 1	1.4	
	HXT9	23 ± 2	12 ± 0	1.9	
	HXT10	12 ± 0	19 ±6	-1.6	
	HXT11[b]	n/a	n/a	-	
	HXT12	43 ± 22	45 ± 10	1.0	
	HXT13[b]	n/a	n/a	-	
	HXT14	12 ± 0	22 ± 3	-1.8	
	HXT15[b]	n/a	n/a	-	
	HXT16	**34 ± 8**	**337 ± 96**	**-10.0**	
	HXT17[b]	n/a	n/a	-	
	SLT1	25 ± 6	28 ± 4	-1.1	
	GAL2	15 ± 3	12 ± 0	1.3	
	VSP73	82 ± 31	52 ± 2	1.6	
Hexokinase	*HXK1*	2052 ± 473	2968 ± 237	-1.4	**1**
	HXK2	1662 ± 183	979 ± 132	1.7	
	GLK1	1281 ± 37	1704 ± 174	-1.3	
Phosphoglucomutase	*PGI1*	3123 ± 180	2846 ± 283	1.1	**2**
Phosphofructo-kinase	*PFK1*	1271 ± 145	1098 ± 94	1.2	**3**
	PFK2	1353 ± 40	1232 ± 91	1.1	
Fructose-bispho- aldolase	*FBA1*	3482 ± 844	2704 ± 411	1.3	**4**
Triose-p isomerase	*TPI1*	4181 ± 537	3702 ± 379	1.1	**5**
Glyceraldehyde 3P	*TDH1*	2528 ± 118	1812 ± 297	1.4	**6**
Dehydrogenase	*TDH2*	3673 ± 166	2758 ± 385	1.3	
	TDH3	4189 ± 868	5412 ± 1219	-1.3	
Phosphoglycerate kinase	*PGK1*	4310 ± 240	3753 ± 356	1.1	**7**
Phosphoglycerate mutase	*GPM1*	3358 ± 100	3055 ± 232	1.1	**8**
	GPM2	**91 ± 24**	**196 ± 35**	**-2.1**	
	GPM3	127 ± 2	87 ± 8	1.5	
Enolase	*ENO1*	3427 ± 377	2888 ± 290	1.2	**9**
	ENO2	3499 ± 760	2848 ± 429	1.2	
Pyruvate kinase	*PYK1*	2828 ± 217	2045 ± 157	1.4	**10**
	PYK2	**47 ± 24**	**133 ± 34**	**-2.8**	
Pyruvate decarboxylase	*PDC1*	2196 ± 45	1410 ± 198	1.6	**11**
	PDC5	**130 ± 12**	**56 ± 8**	**2.3**	
	PDC6	**15 ± 2**	**66 ± 13**	**-4.4**	
Alcohol dehydrogenase	*ADH1*	4743 ± 183	3983 ± 595	1.2	**12**
	ADH2	40 ± 7	57 ± 17	-1.4	
	ADH3	749 ± 55	871 ± 113	-1.2	
	ADH4	237 ± 80	252 ± 35	-1.1	
	ADH5	937 ± 184	830 ± 137	1.1	

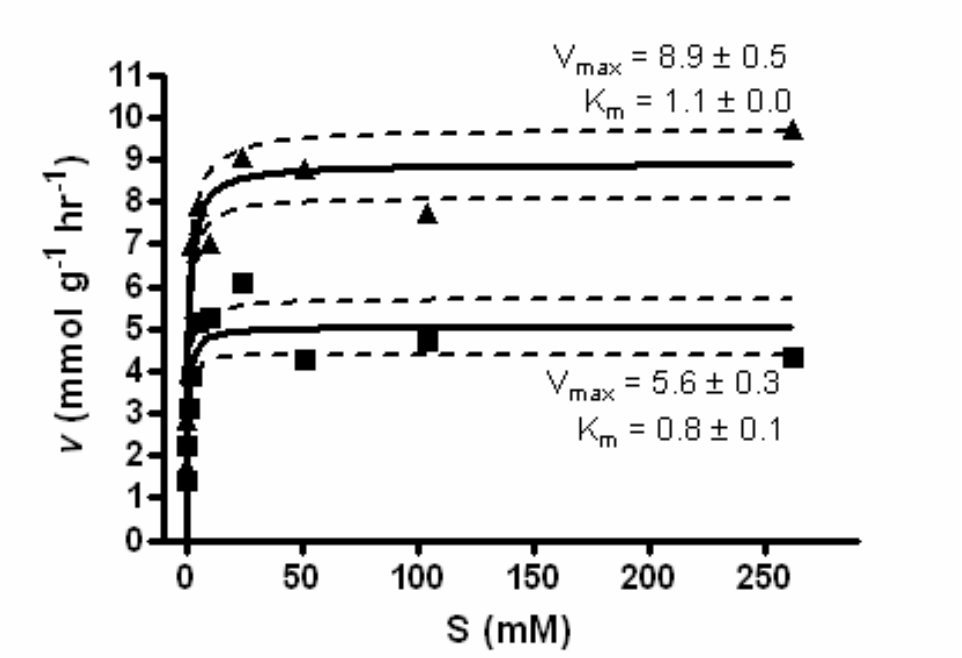

Figure 2A: **Zero trans-influx uptake kinetics of labeled ^{14}C-glucose of strain CEN.PK 113-7D measured at 30 °C with chemostat cultures grown at 12 °C (▲) and 30 °C (■).** Data and mean ± S.D. result from single component Michaelis-Menten kits of the averaged triplicates of two independent chemostat cultures, with K_m in mM and Vmax in mmol.(g dwt)$^{-1}$.hr^{-1}. The dotted lines indicate the 95 % confidence interval.

Irrespective of the culture temperature, activities measured in cell extracts at 12 °C were strongly reduced (2.1 to 7.5-fold) when comparing with *in vitro* measurements at 30 °C, (Figure 2b, Supplemental table S2). Fructose-1,6P aldolase (FBA) was most significantly affected by temperature with a seven-fold reduction for both growth temperatures. Hexokinase (HXK), Glucose-6P isomerase (PGI), G3P dehydrogenase (TDH) and 3P-glycerate kinase (PGK) showed a two to three-fold lower activity when measured at 12 °C. Meanwhile phosphofructokinase (PFK), Triose-P isomerase (TPI), P-glycerate mutase (PGM), pyruvate kinase (PYK) and pyruvate decarboxylase (PDC) activities were three to seven-fold lower (Fig 2b). These observations show that the glycolytic enzymes have different temperature/activity relationships. To our surprise, the alcohol dehydrogenase activity did not show any difference in activity when assayed at 12 °C and 30 °C and this irrespective of the cultivation temperature. Addition of Zn^{2+}, Cu^{2+} or concentration of the cell extract by filtration on membrane with a cut-off of 10kDa did not yield any significant differences higher than 20% between 12 °C and 30 °C *in vitro* ADH activity.

A comparison of the in vitro enzymatic activities done at the same temperature revealed only minor differences between yeast cultures grown at 12 °C and 30 °C. Only the pyruvate decarboxylase activity was significantly higher (p-value<0.01) after growth at 12 °C than after growth at 30 °C (Figure 2b). Surprisingly, four activities were significantly lower at the low cultivation temperature (HXK, PGI,

PFK and PGK) (Figure 2b), irrespective of the assay temperature. The capacity of the glycolytic and fermentative enzymes was thus generally kept constant or even decreased at low temperature. The observations are in good agreement with the transcript levels (Table 2).

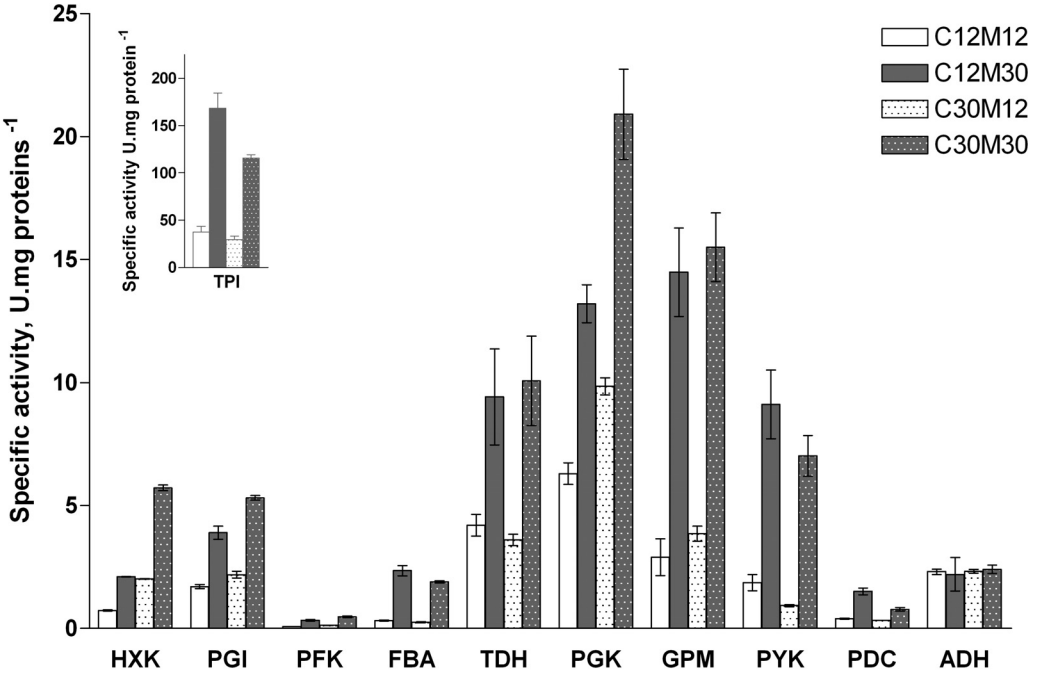

Figure 2B: ***In vitro* enzyme assays in *S. cerevisiae* grown in glucose-limited anaerobic chemostat cultivations at 12 °C and 30 °C.** Assay conditions were carried out at both 12 °C and 30 °C. Mean ± S.D. of data represented are from duplicate measurements from three independent chemostat cultures.

The glycolytic enzyme activities of yeast cultures grown at 12 °C and assayed at 12 °C were much lower than those of cultures grown at 30 °C and assayed at 30 °C (Figure 2b). A comparison of estimated *in vivo* fluxes (Table1b) with the *in vitro* enzyme activities (Figure 2b) showed that for all reactions, except PFK, the enzyme capacity exceeded the *in vivo* fluxes (2 to 1000-fold, Figure 3). The degree of *in vivo* saturation of PFK was well above 100 % (Figure 3), indicating that this enzyme activity measured *in vitro* could not account for the estimated in vivo fluxes.

Since *m*RNA and enzyme activity measurements did not provide an indication for regulation of the *in vivo* activity of glycolytic enzymes at the level of enzyme synthesis (Vertical control), we subsequently analyzed the intracellular concentration of metabolites of central carbon metabolism.

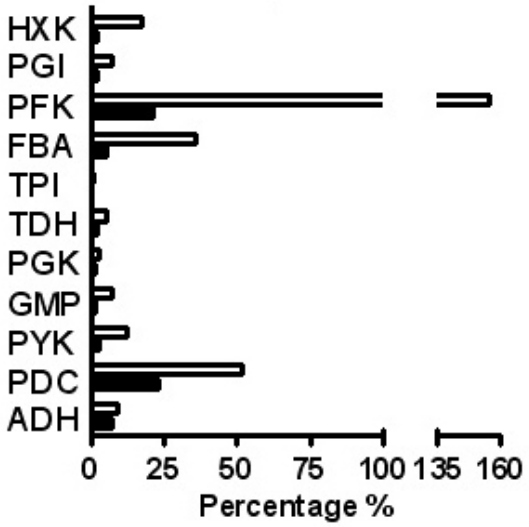

Figure 3: **Degree of *in vivo* saturation of glycolytic enzymes, calculated from estimated *in vivo* fluxes and enzyme-capacity estimates from enzyme assays in cell extracts. □) 12 °C - ■) 30 °C.** *In vitro* enzyme activities were expressed in g per dry weight by assuming a soluble protein content of 33 % in dry biomass (345).

Intracellular Metabolite concentrations at 12 °C and 30 °C

The activities of glycolytic and fermentative enzymes are affected by their substrate and product concentrations and moreover regulated by allosteric effectors such as adenosine nucleotides (e.g. ATP, ADP and AMP), glycolytic intermediates (e.g. fructose-1,6 biphosphate, PEP) and pyridine nucleotide cofactors. The intracellular concentrations of most of the measured compounds were significantly and markedly different in the glucose-limited chemostat grown at 12 °C and 30 °C. The concentrations of the intermediates of both upper and lower glycolysis were concertedly increased by 1.5 to 5.7-fold (G6P, F6P, FBP, G3P, 2PG/3PG, PEP, PYR) (Table 3).

Adenine nucleotides act as allosteric effectors on several glycolytic enzymes (PFK (48), PYK (120, 128)) but are also involved as substrates and products of glycolytic reactions (HXK, PFK, PGK, PYK). The intracellular ATP concentration was 2-fold higher at 12 °C, while ADP and AMP levels were much lower (2.3 and 6.9-fold respectively), resulting in a higher adenylate (∑AXP) and adenylate charge (AC) at 12 °C than at 30 °C (Table 3).

The concentration of trehalose-6-phosphate (T6P) dropped 5.7-fold at 12 °C, concomitantly with the concentration of intracellular trehalose (Table 3). Besides it role in trehalose biosynthesis, T6P is a potent inhibitor of the hexokinase activity (29, 105). Its lower concentration may participate in controlling the glucose phosphorylation and consequently the glycolytic flux (321, 322). The increased in UTP and glucose-1-phosphate (G1P) concentrations were consistent with the coordinated increased concentration of glycogen at 12 °C (Table 3) (67, 68).

Table 3: Intracellular metabolite concentrations. Values represent the mean ± S.D. of data from two independent steady-state chemostat cultivations measured in triplicates.

	Metabolite concentration μmol (g dwt)$^{-1}$ Culture temp		Student – test	FC[a]
	12°C	30 °C	*p* value	
Glycolysis				
G6P	21.6 ± 0.6	9.6 ± 0.2	*1.1E-07*	2.3
F6P	2.5 ± 0.4	1.1 ± 0.1	*1.8E-04*	2.3
FBP	69.8 ± 5.5	30.3 ± 1.5	*1.5E-06*	2.3
G3P	3.9 ± 0.1	1.0 ± 0.1	*1.5E-10*	3.8
2PG/3PG	1.1 ± 0.1	0.4 ± 0.0	*1.9E-06*	2.5
PEP	0.6 ± 0.1	0.1 ± 0.0	*2.9E-06*	5.7
PYR	3.0 ± 0.1	2.1 ± 0.2	*6.8E-06*	1.5
Reserve carbohydrate				
T6P	0.1 ± 0.0	0.4 ± 0.0	2.8E-09	-5.7
G1P	2.6 ± 0.1	1.2 ± 0.0	*3.7E-09*	2.2
Nucleotides[b]				
ATP	51.0 ± 1.1	26.7 ± 1.5	*1.1E-10*	1.9
ADP	4.6 ± 0.7	10.4 ± 2.5	*4.6E-04*	-2.3
AMP	0.9 ± 0.2	6.5 ± 1.3	*1.0E-04*	-6.9
AC[c]	0.94	0.73	-	1.3
UTP	10.3 ± 0.5	4.1 ± 0.1	*3.7E-05*	2.5
UDP	0.5 ± 0.1	1.4 ± 0.6	*9.4E-03*	-2.7

[a] Fold change of metabolite concentrations of 12°C over 30°C

[b] UMP, CXP and GXP were also measured, however the concentration were too low to be accurately quantify

[c] Adenylate charge of the cell (ATP + 0.5ADP)/(ATP + ADP + AMP)

Discussion

This study investigates how *S. cerevisiae* deals with the strong temperature dependence of key enzymes in glycolysis and alcoholic fermentation when it is grown at a sub-optimal temperature of 12 °C. The activity assays of glycolytic enzymes, performed at 12 °C and 30 °C (Figure 2B, Supplemental table S2), indicate that this temperature dependence is very strong. It should be realised that these assays were performed at a single concentration of substrates and effectors. Consequently, effects of temperature on the affinity for substrates and effectors may further increase the kinetic challenges posed by a low cultivation temperature (4, 207, 209).

Glucose transport across the plasma membrane was shown to be regulated at different levels. The higher residual glucose concentration at low temperature (Table 1) is likely to contribute to a higher degree of substrate saturation of hexose transporters and thereby compensate for a reduced capacity of transporters at 12 °C. An additional level of regulation was observed by an analysis of mRNA levels for the *HXT* (hexose transporter) genes. While levels of the dominant *HXT* transcripts *HXT6* and *HXT7* (which encode transporters that, at 30 °C, catalyse high-affinity glucose transport (212, 273)) were not affected by temperature, other *HXT* genes, which exhibit lower affinities and as such would be less sensitive to an increased

intracellular glucose concentration, showed clear transcriptional regulation. Since no kinetic data are available for the individual Hxt transporters at low temperature, it is not possible to interpret the significance of these changed transport levels. In addition to adjusting the overall capacity and/or affinity of hexose transport, this altered expression of *HXT* genes may represent an adjustment to temperature-dependent changes in membrane composition and/or fluidity (23, 327). Kinetic analysis of glucose transport by cells pre-grown at 12 ^{o}C and 30 ^{o}C in chemostat cultures showed a clear increase of the capacity of transport. A plausible explanation for this increased capacity can be found in the differential expression of transcription of *HXT2, 3, 4, 5* and *16*. The fact that the maximum specific rates of glucose consumption (measured at 30 ^{o}C) for cells pre-grown at 12 ^{o}C and 30 ^{o}C closely corresponded to the fermentative capacity of the same cultures (Table 1, Figure 2a and Supplemental Table S1) would support this hypothesis. However, the possibility cannot be excluded that glucose-transport activity was increased as a result of changes outside the hexose transporters, such as membrane composition. The change in glucose transport capacity, together with the minor changes in the levels of glycolytic enzymes (see below), strongly suggests that glucose transport also controls fermentative capacity in these slowly growing chemostat cultures as well as at 30 ^{o}C.

In contrast to sugar transport, there was virtually no evidence that regulation of glycolysis and alcoholic fermentation at the enzyme synthesis level (vertical regulation) contributed to the maintenance of *in vivo* glycolytic flux at low temperature. Indeed, the few glycolytic enzymes that did show a clear change at transcript levels and/or enzyme-activity level appear to show a lower level at low temperature, thus augmenting the effect of temperature on enzyme activity rather than compensating for it. The absence of a clear upregulation of the synthesis of glycolytic enzymes at low temperature is perhaps less surprising when it is taken into account that, in fermenting yeast cultures grown at 30 ^{o}C, glycolytic enzymes already make up a significant fraction of the total cell protein (up to 21 % estimation extrapolated from (337)). It would take very significant increases in the concentrations of these glycolytic enzymes resulting in a significantly increased energetic demand to counteract the effects of the reduction in temperature.

The minor role of vertical control in the regulation of glycolytic flux at low temperature was in marked contrast to drastic differences in the intracellular concentrations of glycolytic intermediates and effectors. Several of the observed changes may contribute to a higher degree of saturation of the glycolytic capacity in the low-temperature cultures, thus compensating for the reduction of enzyme activity at low temperature. The lower intracellular concentration of T6P may have a similar effect, as this compound is a well-documented inhibitor of the *S. cerevisiae* hexokinases (97, 105, 261) that prevents “glucose-accelerated death” (29, 101). The lower intracellular T6P concentration, an intermediate in trehalose biosynthesis, is

also consistent with the lower trehalose concentrations in the cultures grown at 12 ^{o}C.

Extreme changes were observed in the intracellular concentrations of adenine nucleotides. While the ATP and ADP concentrations were higher at low temperature, the AMP concentration was much lower, thus leading to an adenylate charge (AC) (see methods section equation [2]) of 0.94 in the chemostat cultures grown at 12 ^{o}C. This change seems counter-intuitive in a situation where the *in vivo* activity of glycolytic enzymes has to be boosted to compensate for low-temperature-induced reduction of their turnover numbers. An increased AC is generally correlated with a decrease of the activity of enzymes and/or pathways involved in ATP production (12, 354) and in *S. cerevisiae*, intracellular ATP concentration is negatively correlated with glycolytic flux (186). This unexpected relation between growth temperature and AC may be related to strong changes of the kinetic and/or allosteric properties of enzymes at low temperature and to the changes of other relevant metabolites. For example, inhibition of PYK by the increased intracellular ATP concentration at 12 ^{o}C may be compensated by the strongly increased intracellular concentration of its activator F1,6P (33, 35, 48) (Table 3). Similarly, a negative effect of increased ATP and decreased AMP on PFK activity (48) may be compensated for by changes of the positive allosteric regulator F2,6P (164, 172), which could not be accurately measured in our experimental set-up.

An important factor in the interpretation of the intracellular metabolite data is that the kinetics of glycolytic enzymes and, in particular, the impact of low-molecular-weight effectors, has been extensively studied at 30 ^{o}C, but not at 12 ^{o}C. For example, if the kinetics of allosteric regulation are strongly temperature dependent, this may affect the impact of the AC on glycolytic flux by removing or reducing bottlenecks at the lower temperature. Indeed, several studies show that the affinity for allosteric effectors is reduced at low temperature (i.e. inhibition and activation constants are increased) (4, 207-209). The apparent discrepancy between in vivo and in vitro PFK activities 12 ^{o}C represents a case in point (Figure 3). In previous studies at 30 ^{o}C, the maximum capacity of PFK estimated in cell extracts was close to the *in vivo* flux through this enzyme (338). The difference between these parameters in cultures grown at 12 ^{o}C may reflect suboptimal conditions in the enzyme assays due to temperature dependent changes in the complex allosteric regulation of this enzyme (176, 289). A deeper understanding of the *in vivo* kinetics of glycolysis at low temperature, involving the application of kinetic modelling, will require quantitative data on the impact of temperature on the kinetics and allosteric regulation of the entire glycolytic pathway in *S. cerevisiae*.

In addition to providing ATP for anaerobic growth, glycolysis also is a key cellular supply line for biosynthesis. The temperature-dependent changes of the intracellular concentrations of glycolytic intermediates are therefore also likely to affect biosynthetic pathways. Clearly the relative demands placed on anaerobic glycolysis in terms of energetics and biosynthetic supply are not the same at 12 ^{o}C and 30 ^{o}C.

In addition to vital biomass components, the products derived from these biosynthetic pathways include important flavour compounds (77-79, 350, 351). Therefore, a further analysis of the mechanisms by which the entire yeast metabolic network adapts to temperature is highly relevant for yeast-based industrial fermentations that are performed at low temperature. Further insights will be gained from a similar study to this one using an industrial yeast strain, which is adapted to the low temperature conditions as opposed to a lab strain, which is probably adapted to the higher temperature conditions,

The present study demonstrates that, at low specific growth rate, the *in vivo* glycolytic flux at low temperature is maintained by a combination of vertical and metabolic regulation. Glucose transport represents the clearest case of vertical (transcriptional) regulation through the synthesis of additional hexose transporters. However, the increased extracellular glucose concentration at low temperature is likely to be just as decisive in maintaining a constant rate of glucose import into the cell. Vertical regulation did not appear to contribute to compensating for sub-optimal kinetics of glycolytic enzymes at low temperature. Instead, metabolic control, reflected by massive changes of intracellular concentrations of glycolytic metabolites and effectors, was dominant.

From an evolutionary perspective, a dominant role of metabolic regulation seems logical for a pathway that represents a substantial fraction of the cellular protein, in an organism that is subjected to circadian temperature cycles. In nature, *S. cerevisiae* is likely to encounter relatively exposed, and sugar-rich niches such as fruits and nectar. Moreover, growth is probably slow due to the low availability of nitrogen. In such environments, a preliminary vertical regulation of glycolytic activity would require wasteful cycles of massive synthesis (as temperature decreases in the evening) and degradation (in the morning) of glycolytic enzymes. This interpretation suggests that the high fermentative capacity of wild-type *S. cerevisiae* strains (assayed at 30 ^{o}C) may – at least in part – reflect an evolutionary adaptation to fluctuating temperatures in its natural environment.

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Supplemental Table S1: **Fermentative capacity assays.** Samples containing exactly 200 mg dry weight of biomass were harvested from a steady-state chemostat culture by centrifugation (5000 g, 3 min), and resuspended in 10 ml fivefold-concentrated synthetic medium (pH 5.6). Subsequently, these cell suspensions were introduced into a thermostat-controlled (30 ^{o}C) vessel. The volume was adjusted to 40 ml with demineralized water.

After 10 min incubation, 10 ml glucose solution (100 g.l^{-1}) was added, and samples (1 ml) were taken at appropriate time intervals for 30 min. The 10 ml headspace was continuously flushed with water-saturated CO2 at a flow rate of approximately 30 ml min^{-1}.

The ethanol concentration in the supernatant was analyzed using a colorimetric assay (346). Fermentative capacity can be calculated from the linear increase in ethanol concentration and is expressed as mmol ethanol produced (g dry yeast biomass)$^{-1}$ h^{-1} (338). Glucose, pyruvate and acetate concentrations were measured by HPLC and as calculated for ethanol, consumption and production specific consumption and production rates (mmol/g/h) were measured. Growth during these assays can be neglected, as no significant change in biomass concentration was observed.

Temp.	Fermentative capacity mmol/g/h	q_{Gluc}	q_{Gly}	q_{Pyr}	q_{Ace}
30 ^{o}C	11.37 ± 0.63	7.29 ± 0.00	1.91 ± 0.07	0.11 ± 0.00	0.39 ± 0.03
12 ^{o}C	19.29 ± 1.6	11.63 ± 1.1	2.15 ±0.22	0.13 ± 0.00	0.36 ± 0.07

Supplemental Table S2: ***In vitro* enzyme assays**

In vitro enzyme assays in *S. cerevisiae* grown in glucose-limited anaerobic chemostat cultivations at 12°C and 30°C. Assay conditions were carried out at both 12°C and 30°C. Mean ± S.D. of data represented are from duplicate measurements from three independent chemostat cultures. Fold change (FC) of glycolytic enzymes with enzyme assays measured at 12 °C and 30 °C. FC shown, are the ratios of enzyme activities (experiment vs base) in the respective culture and assay temperature. *p*-values shown are from standard student T-test. In vitro enzyme activity of enolase was not measured. Numbers at the last column correspond to the glycolytic pathway displayed in Figure 1.

	Enzyme activities (umol/mg protein/min)				A vs B		C vs D		B vs D		A vs C		
Culture T	**12 °C**	**12 °C**	**30 °C**	**30 °C**	**12**	**12**	**30**	**30**	**12**	**30**	**12**	**30**	
Assay T	*12 °C*	*30 °C*	*12 °C*	*30 °C*	*12*	*30*	*12*	*30*	*30*	*30*	*12*	*12*	
Enzyme	***A***	***B***	***C***	***D***	FC	p-value	FC	p-value	FC	p-value	FC	p-value	
HXK	0.7 ± 0.0	2.1 ± 0.0	2.0 ± 0.0	5.7 ± 0.1	-2.9	*1.5E-05*	-2.8	*1.6E-04*	-2.7	*1.6E-04*	-2.7	*3.5E-05*	**1**
PGI	1.7 ± 0.1	3.9 ± 0.3	2.2 ± 0.1	5.3 ± 0.1	-2.3	*1.4E-03*	-2.4	*1.1E-05*	-1.4	*3.5E-03*	-1.3	*5.1E-03*	**2**
PFK	0.1 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.5 ± 0.0	-4.3	*2.3E-03*	-3.7	*3.5E-04*	-1.4	*2.1E-03*	-1.7	*8.1E-03*	**3**
FBA	0.3 ± 0.0	2.4 ± 0.2	0.3 ± 0.0	1.9 ± 0.0	-7.3	*1.6E-03*	-7.4	*1.4E-05*	1.2	*2.9E-02*	1.3	*9.5E-03*	**4**
TPI	37.6 ± 5.7	168.4 ± 16.0	29.3 ± 3.8	115.9 ± 3.5	-4.5	*1.1E-03*	-4.0	*4.5E-06*	1.5	*1.2E-02*	1.3	*5.6E-02*	**5**
TDH	4.2 ± 0.4	9.4 ± 2.0	3.6 ± 0.2	10.1 ± 1.8	-2.2	*1.9E-02*	-2.8	*1.2E-02*	-1.1	*3.5E-01*	1.2	*6.7E-02*	**6**
PGK	6.3 ± 0.4	13.2 ± 0.8	9.9 ± 0.4	20.9 ± 1.8	-2.1	*3.3E-04*	-2.1	*3.6E-03*	-1.6	*4.7E-03*	-1.6	*2.5E-04*	**7**
GMP	2.9 ± 0.8	14.5 ± 1.8	3.9 ± 0.3	15.5 ± 1.4	-5.0	*1.6E-03*	-4.0	*1.7E-03*	-1.1	*2.4E-01*	-1.3	*7.2E-02*	**8**
PYK	1.9 ± 0.3	9.1 ± 1.4	0.9 ± 0.1	7.0 ± 0.8	-4.9	*4.6E-03*	-7.5	*3.0E-03*	1.3	*5.2E-02*	2.0	*1.8E-02*	**10**
PDC	0.4 ± 0.0	1.5 ± 0.1	0.3 ± 0.0	0.8 ± 0.1	-3.8	*2.2E-03*	-2.4	*3.7E-03*	1.9	*2.1E-03*	1.2	*9.5E-03*	**11**
ADH	2.3 ± 0.1	2.2 ± 0.7	2.3 ± 0.1	2.4 ± 0.2	1.1	*4.0E-01*	1.0	*2.5E-01*	1.1	*3.2E-01*	1.0	*4.1E-01*	**12**

P<0.01 *P*<0.05

Reference List

1. 2004. Expression profiling--best practices for data generation and interpretation in clinical trials. Nat.Rev.Genet. **5**:229-237.
2. 2005. Get ready to GO! A biologist's guide to the Gene Ontology. Brief.Bioinform. **6**:298-304.
3. **Abramova, N., O. Sertil, S. Mehta, and C. V. Lowry**. 2001. Reciprocal regulation of anaerobic and aerobic cell wall mannoprotein gene expression in *Saccharomyces cerevisiae*. J.Bacteriol. **183**:2881-2887.
4. **Ahlers, J.** 1981. Temperature effects on kinetic properties of plasma membrane ATPase from the yeast *Saccharomyces cerevisiae*. Biochim.Biophys.Acta **649**:550-556.
5. **Al Fageeh, M. B. and C. M. Smales**. 2006. Control and regulation of the cellular responses to cold shock: the responses in yeast and mammalian systems. Biochem.J. **397**:247-259.
6. **Almaguer, C., W. Cheng, C. Nolder, and J. Patton-Vogt**. 2004. Glycerophosphoinositol, a novel phosphate source whose transport is regulated by multiple factors in *Saccharomyces cerevisiae*. J.Biol.Chem. M403648200.
7. **Amoros, M. and F. Estruch**. 2001. *Hsf1p* and *Msn2/4p* cooperate in the expression of *Saccharomyces cerevisiae* genes *HSP26* and *HSP104* in a gene- and stress type-dependent manner. Mol.Microbiol. **39**:1523-1532.
8. **Andreasen, A. A. and T. J. STIER**. 1953. Anaerobic nutrition of *Saccharomyces cerevisiae*. I. Ergosterol requirement for growth in a defined medium. J.Cell Physiol **41**:23-36.
9. **Andreasen, A. A. and T. J. STIER**. 1954. Anaerobic nutrition of *Saccharomyces cerevisiae*. II. Unsaturated fatty acid requirement for growth in a defined medium. J.Cell Physiol **43**:271-281.
10. **Arrhenius, S.** 1884. Recherches sur la conductivite galvanique des electrolytes, *In* Royal Publishing House, Stolkholm.
11. **Askwith, C. C., D. de Silva, and J. Kaplan**. 1996. Molecular biology of iron acquisition in *Saccharomyces cerevisiae*. Mol.Microbiol. **20**:27-34.
12. **Atkinson, D. E.** 1968. The energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifiers. Biochemistry **7**:4030-4034.
13. **Baganz, F., A. Hayes, R. Farquhar, P. R. Butler, D. C. Gardner, and S. G. Oliver**. 1998. Quantitative analysis of yeast gene function using competition experiments in continuous culture. Yeast **14**:1417-1427.
14. **Baganz, F., A. Hayes, D. Marren, D. C. Gardner, and S. G. Oliver**. 1997. Suitability of replacement markers for functional analysis studies in *Saccharomyces cerevisiae*. Yeast **13**:1563-1573.
15. **Barczak, A., M. W. Rodriguez, K. Hanspers, L. L. Koth, Y. C. Tai, B. M. Bolstad, T. P. Speed, and D. J. Erle**. 2003. Spotted long oligonucleotide arrays for human gene expression analysis. Genome Res. **13**:1775-1785.
16. **Barnett, J. A.** 2003. A history of research on yeasts 5: the fermentation pathway. Yeast **20**:509-543.
17. **Barnett, J. A., R. W. Payne, and D. Yarrow**. 1983. Yeasts: characteristics and identification, p. 467-469. Cambridge University Press, Cambridge.
18. **Barnett, V. and T. Lewis**. 1994. Outliers in statistical data. 3rd Edition Wiley and Sons, New York.
19. **Bartowsky, E. J. and P. A. Henschke**. 2004. The 'buttery' attribute of wine--diacetyl--desirability, spoilage and beyond. Int.J.Food Microbiol. **96**:235-252.
20. **Becerra, M., L. J. Lombardia-Ferreira, N. C. Hauser, J. D. Hoheisel, B. Tizon, and M. E. Cerdan**. 2002. The yeast transcriptome in aerobic and hypoxic conditions: effects of *hap1*, *rox1*, *rox3* and *srb10* deletions. Mol.Microbiol. **43**:545-555.
21. **Beck, T. and M. N. Hall**. 1999. The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. Nature **402**:689-692.
22. **Bell, P. J., V. J. Higgins, and P. V. Attfield**. 2001. Comparison of fermentative capacities of industrial baking and wild-type yeasts of the species *Saccharomyces cerevisiae* in different sugar media. Lett.Appl.Microbiol. **32**:224-229.
23. **Beltran, G., M. Novo, V. Leberre, S. Sokol, D. Labourdette, J. M. Guillamon, A. Mas, J. Francois, and N. Rozes**. 2006. Integration of transcriptomic and metabolic analyses for understanding the global responses of low-temperature winemaking fermentations. FEMS Yeast Res.
24. **Benjamin, P. M., J. I. Wu, A. P. Mitchell, and B. Magasanik**. 1989. Three regulatory systems control expression of glutamine synthetase in *Saccharomyces cerevisiae* at the level of transcription. Mol.Gen.Genet. **217**:370-377.
25. **Bertram, P. G., J. H. Choi, J. Carvalho, W. Ai, C. Zeng, T. F. Chan, and X. F. Zheng**. 2000. Tripartite regulation of Gln3p by *TOR*, Ure2p, and phosphatases. J.Biol.Chem. **275**:35727-35733.
26. **Birrell, G. W., J. A. Brown, H. I. Wu, G. Giaever, A. M. Chu, R. W. Davis, and J. M. Brown**. 2002. Transcriptional response of *Saccharomyces cerevisiae* to DNA-damaging agents does

not identify the genes that protect against these agents. Proc.Natl.Acad.Sci.U.S.A **99**:8778-8783.

27. **Bitter, G. A., K. M. Egan, W. N. Burnette, B. Samal, J. C. Fieschko, D. L. Peterson, M. R. Downing, J. Wypych, and K. E. Langley**. 1988. Hepatitis B vaccine produced in yeast. J.Med.Virol. **25**:123-140.
28. **Blanchard, A. P. and L. Hood**. 1996. Sequence to array: probing the genome's secrets. Nat.Biotechnol. **14**:1649.
29. **Blazquez, M. A., R. Lagunas, C. Gancedo, and J. M. Gancedo**. 1993. Trehalose-6-phosphate, a new regulator of yeast glycolysis that inhibits hexokinases. FEBS Lett. **329**:51-54.
30. **Blinder, D. and B. Magasanik**. 1995. Recognition of nitrogen-responsive upstream activation sequences of *Saccharomyces cerevisiae* by the product of the *GLN3* gene. J.Bacteriol. **177**:4190-4193.
31. **Blom, J., M. J. De Mattos, and L. A. Grivell**. 2000. Redirection of the respiro-fermentative flux distribution in *Saccharomyces cerevisiae* by overexpression of the transcription factor Hap4p. Appl.Environ.Microbiol. **66**:1970-1973.
32. **Boer, V. M., J. H. de Winde, J. T. Pronk, and M. D. Piper**. 2003. The genome-wide transcriptional responses of *Saccharomyces cerevisiae* grown on glucose in aerobic chemostat cultures limited for carbon, nitrogen, phosphorus, or sulfur. J.Biol.Chem. **278**:3265-3274.
33. **Boles, E., P. Jong-Gubbels, and J. T. Pronk**. 1998. Identification and characterization of *MAE1*, the *Saccharomyces cerevisiae* structural gene encoding mitochondrial malic enzyme. J.Bacteriol. **180**:2875-2882.
34. **Boles, E., F. Schulte, T. Miosga, K. Freidel, E. Schluter, F. K. Zimmermann, C. P. Hollenberg, and J. J. Heinisch**. 1997. Characterization of a glucose-repressed pyruvate kinase (Pyk2p) in *Saccharomyces cerevisiae* that is catalytically insensitive to fructose-1,6-bisphosphate. J.Bacteriol. **179**:2987-2993.
35. **Bond, C. J., M. S. Jurica, A. Mesecar, and B. L. Stoddard**. 2000. Determinants of allosteric activation of yeast pyruvate kinase and identification of novel effectors using computational screening. Biochemistry **39**:15333-15343.
36. **Bowtell, D. D.** 1999. Options available--from start to finish--for obtaining expression data by microarray. Nat.Genet. **21**:25-32.
37. **Boy-Marcotte, E., M. Perrot, F. Bussereau, H. Boucherie, and M. Jacquet**. 1998. Msn2p and Msn4p control a large number of genes induced at the diauxic transition which are repressed by cyclic AMP in *Saccharomyces cerevisiae*. J.Bacteriol. **180**:1044-1052.
38. **Brejning, J., N. Arneborg, and L. Jespersen**. 2005. Identification of genes and proteins induced during the lag and early exponential phase of lager brewing yeasts. J.Appl.Microbiol. **98**:261-271.
39. **Briggs, D. E., J. S. Hough, R. Stevens, and T. W. Young**. 1981. Malting and Brewing Science. Chapman and Hall, London .
40. **Bro, C., B. Regenberg, and J. Nielsen**. 2004. Genome-wide transcriptional response of a *Saccharomyces cerevisiae* strain with an altered redox metabolism. Biotechnol.Bioeng. **85**:269-276.
41. **Brot-Laroche, E., M. A. Serrano, B. Delhomme, and F. Alvarado**. 1986. Temperature sensitivity and substrate specificity of two distinct Na+-activated D-glucose transport systems in guinea pig jejunal brush border membrane vesicles. J.Biol.Chem. **261**:6168-6176.
42. **Brown, C. J., K. M. Todd, and R. F. Rosenzweig**. 1998. Multiple duplications of yeast hexose transport genes in response to selection in a glucose-limited environment. Mol.Biol.Evol. **15**:931-942.
43. **Burke, D., D. Dawson, and T. Stearn**. 2000. Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press, New York.
44. **Burnie, J. P., T. L. Carter, S. J. Hodgetts, and R. C. Matthews**. 2006. Fungal heat-shock proteins in human disease. FEMS Microbiol.Rev. **30**:53-88.
45. **Button, D. K.** 1991. Biochemical Basis for Whole-Cell Uptake Kinetics - Specific Affinity, Oligotrophic Capacity, and the Meaning of the Michaelis Constant. Applied and Environmental Microbiology **57**:2033-2038.
46. **Cartwright, J. L. and A. G. McLennan**. 1999. The *Saccharomyces cerevisiae* YOR163w gene encodes a diadenosine 5', 5"'-P1,P6-hexaphosphate (Ap6A) hydrolase member of the MutT motif (Nudix hydrolase) family. J.Biol.Chem. **274**:8604-8610.
47. **Castor, J. G. B.** 1953. The free amino acids of must and wines. II. the fate of amino acids of must during alcoholic fermentation. Journal of Food Research **19**:146-151.
48. **Caubet, R., B. Guerin, and M. Guerin**. 1988. Comparative studies on the glycolytic and hexose monophosphate pathways in Candida parapsilosis and *Saccharomyces cerevisiae*. Arch.Microbiol. **149**:324-329.
49. **Causton, H. C., B. Ren, S. S. Koh, C. T. Harbison, E. Kanin, E. G. Jennings, T. I. Lee, H. L. True, E. S. Lander, and R. A. Young**. 2001. Remodeling of yeast genome expression in response to environmental changes. Mol.Biol.Cell **12**:323-337.
50. **Chao, L. and S. M. McBroom**. 1985. Evolution of transposable elements: an IS10 insertion increases fitness in Escherichia coli. Mol.Biol.Evol. **2**:359-369.

51. **Chelstowska, A., Z. Liu, Y. Jia, D. Amberg, and R. A. Butow**. 1999. Signalling between mitochondria and the nucleus regulates the expression of a new D-lactate dehydrogenase activity in yeast. Yeast **15**:1377-1391.
52. **Cherry, J. M., C. Adler, C. Ball, S. A. Chervitz, S. S. Dwight, E. T. Hester, Y. Jia, G. Juvik, T. Roe, M. Schroeder, S. Weng, and D. Botstein**. 1998. SGD: Saccharomyces Genome Database. Nucleic Acids Res. **26**:73-79.
53. **Cliften, P., P. Sudarsanam, A. Desikan, L. Fulton, B. Fulton, J. Majors, R. Waterston, B. A. Cohen, and M. Johnston**. 2003. Finding functional features in Saccharomyces genomes by phylogenetic footprinting. Science **301**:71-76.
54. **Coffman, J. A. and T. G. Cooper**. 1997. Nitrogen GATA factors participate in transcriptional regulation of vacuolar protease genes in *Saccharomyces cerevisiae*. J Bacteriol **179**:5609-5613.
55. **Cohen, B. D., O. Sertil, N. E. Abramova, K. J. Davies, and C. V. Lowry**. 2001. 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. **29**:799-808.
56. **Colson, I., D. Delneri, and S. G. Oliver**. 2004. Effects of reciprocal chromosomal translocations on the fitness of *Saccharomyces cerevisiae*. EMBO Rep. **5**:392-398.
57. **Cooper, T. G.** 1982. Nitrogen metabolism in *Saccharomyces cerevisiae*, p. 39-99. *In* The molecular biology of the yeast *Saccharomyces*: metabolism and gene expression. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
58. **Cooper, T. G.** 2002. Transmitting the signal of excess nitrogen in *Saccharomyces cerevisiae* from the Tor proteins to the GATA factors: connecting the dots. FEMS Microbiol.Rev. **26**:223-238.
59. **Cooper, T. G. and R. A. Sumrada**. 1983. What is the function of nitrogen catabolite repression in *Saccharomyces cerevisiae*? J.Bacteriol. **155**:623-627.
60. **Costanzo, M. C., M. E. Crawford, J. E. Hirschman, J. E. Kranz, P. Olsen, L. S. Robertson, M. S. Skrzypek, B. R. Braun, K. L. Hopkins, P. Kondu, C. Lengieza, J. E. Lew-Smith, M. Tillberg, and J. I. Garrels**. 2001. YPD, PombePD and WormPD: model organism volumes of the BioKnowledge library, an integrated resource for protein information. Nucleic Acids Res 2001 Jan 1;29(1):75.-9. **29**:75-79.
61. **Cousens, L. S., J. R. Shuster, C. Gallegos, L. L. Ku, M. M. Stempien, M. S. Urdea, R. Sanchez-Pescador, A. Taylor, and P. Tekamp-Olson**. 1987. High level expression of proinsulin in the yeast, *Saccharomyces cerevisiae*. Gene **61**:265-275.
62. **Crespo, J. L. and M. N. Hall**. 2002. Elucidating TOR signaling and rapamycin action: lessons from *Saccharomyces cerevisiae*. Microbiol.Mol.Biol.Rev. **66**:579-91, table.
63. **Crespo, J. L., S. B. Helliwell, C. Wiederkehr, P. Demougin, B. Fowler, M. Primig, and M. N. Hall**. 2004. *NPR1* kinase and *RSP5-BUL1/2* ubiquitin ligase control *GLN3*-dependent transcription in *Saccharomyces cerevisiae*. J.Biol.Chem. **279**:37512-37517.
64. **Crespo, J. L., T. Powers, B. Fowler, and M. N. Hall**. 2002. The TOR-controlled transcription activators *GLN3*, *RTG1*, and *RTG3* are regulated in response to intracellular levels of glutamine. Proc.Natl.Acad.Sci.U.S.A **99**:6784-6789.
65. **Cunningham, T. S. and T. G. Cooper**. 1991. Expression of the *DAL80* gene, whose product is homologous to the GATA factors and is a negative regulator of multiple nitrogen catabolic genes in *Saccharomyces cerevisiae*, is sensitive to nitrogen catabolite repression. Mol.Cell Biol. **11**:6205-6215.
66. **Cunningham, T. S., V. V. Svetlov, R. Rai, W. Smart, and T. G. Cooper**. 1996. Gln3p is capable of binding to UAS(NTR) elements and activating transcription in *Saccharomyces cerevisiae*. J.Bacteriol. **178**:3470-3479.
67. **Daran, J. M., W. Bell, and J. Francois**. 1997. Physiological and morphological effects of genetic alterations leading to a reduced synthesis of UDP-glucose in *Saccharomyces cerevisiae*. FEMS Microbiol.Lett. **153**:89-96.
68. **Daran, J. M., N. Dallies, D. Thines-Sempoux, V. Paquet, and J. Francois**. 1995. Genetic and biochemical characterization of the *UGP1* gene encoding the UDP-glucose pyrophosphorylase from *Saccharomyces cerevisiae*. Eur.J.Biochem. **233**:520-530.
69. **Daran-Lapujade, P., J. M. Daran, P. Kotter, T. Petit, M. D. Piper, and J. T. Pronk**. 2003. Comparative genotyping of the *Saccharomyces cerevisiae* laboratory strains S288C and CEN.PK113-7D using oligonucleotide microarrays. FEMS Yeast Res. **4**:259-269.
70. **Daran-Lapujade, P., M. L. Jansen, J. M. Daran, W. Van Gulik, J. H. de Winde, and J. T. Pronk**. 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.
71. **Daran-Lapujade, P., S. Rossell, W. Van Gulik, M. A. Luttik, M. J. L. de Groot, M. Slijper, A. J. Heck, J. M. Daran, J. H. de Winde, H. V. Westerhoff, J. T. Pronk, and B. M. Bakker**. 2006. What regulates glycolysis in *Saccharomyces cerevisiae*? A systems biology approach. Plos Biology **Submitted for publication**.

72. **David, L., W. Huber, M. Granovskaia, J. Toedling, C. J. Palm, L. Bofkin, T. Jones, R. W. Davis, and L. M. Steinmetz**. 2006. A high-resolution map of transcription in the yeast genome. Proc.Natl.Acad.Sci.U.S.A **103**:5320-5325.
73. **de la, C. J., I. Iost, D. Kressler, and P. Linder**. 1997. The p20 and Ded1 proteins have antagonistic roles in eIF4E-dependent translation in *Saccharomyces cerevisiae*. Proc.Natl.Acad.Sci.U.S.A **94**:5201-5206.
74. **Dean, A. M.** 1989. Selection and neutrality in lactose operons of Escherichia coli. Genetics **123**:441-454.
75. **Dean, A. M., D. E. Dykhuizen, and D. L. Hartl**. 1988. Fitness effects of amino acid replacements in the beta-galactosidase of Escherichia coli. Mol.Biol.Evol. **5**:469-485.
76. **DeRisi, J. L., V. R. Iyer, and P. O. Brown**. 1997. Exploring the metabolic and genetic control of gene expression on a genomic scale. Science **278**:680-686.
77. **Dickinson, J. R., S. J. Harrison, J. A. Dickinson, and M. J. Hewlins**. 2000. An investigation of the metabolism of isoleucine to active Amyl alcohol in *Saccharomyces cerevisiae*. J.Biol.Chem. **275**:10937-10942.
78. **Dickinson, J. R., S. J. Harrison, and M. J. Hewlins**. 1998. An investigation of the metabolism of valine to isobutyl alcohol in *Saccharomyces cerevisiae*. J.Biol.Chem. **273**:25751-25756.
79. **Dickinson, J. R., M. M. Lanterman, D. J. Danner, B. M. Pearson, P. Sanz, S. J. Harrison, and M. J. Hewlins**. 1997. A 13C nuclear magnetic resonance investigation of the metabolism of leucine to isoamyl alcohol in *Saccharomyces cerevisiae*. J.Biol.Chem. **272**:26871-26878.
80. **Diehn, M., M. B. Eisen, D. Botstein, and P. O. Brown**. 2000. Large-scale identification of secreted and membrane-associated gene products using DNA microarrays. Nat.Genet. **25**:58-62.
81. **Diers, I. V., E. Rasmussen, P. H. Larsen, and I. L. Kjaersig**. 1991. Yeast fermentation processes for insulin production. Bioprocess.Technol. **13**:166-176.
82. **Dilova, I., S. Aronova, J. C. Chen, and T. Powers**. 2004. Tor signaling and nutrient-based signals converge on Mks1p phosphorylation to regulate expression of *RTG1*.Rtg3p-dependent target genes. J.Biol.Chem. **279**:46527-46535.
83. **Drgon, T., L. Sabova, G. Gavurnikova, and J. Kolarov**. 1992. Yeast ADP/ATP carrier (*AAC*) proteins exhibit similar enzymatic properties but their deletion produces different phenotypes. FEBS Lett. **304**:277-280.
84. **Dwight, S. S., R. Balakrishnan, K. R. Christie, M. C. Costanzo, K. Dolinski, S. R. Engel, B. Feierbach, D. G. Fisk, J. Hirschman, E. L. Hong, L. Issel-Tarver, R. S. Nash, A. Sethuraman, B. Starr, C. L. Theesfeld, R. Andrada, G. Binkley, Q. Dong, C. Lane, M. Schroeder, S. Weng, D. Botstein, and J. M. Cherry**. 2004. *Saccharomyces* genome database: underlying principles and organisation. Brief.Bioinform. **5**:9-22.
85. **Eckert-Boulet, N., B. Regenberg, and J. Nielsen**. 2005. Grr1p is required for transcriptional induction of amino acid permease genes and proper transcriptional regulation of genes in carbon metabolism of *Saccharomyces cerevisiae*. Curr.Genet. **47**:139-149.
86. **Edgar, R., M. Domrachev, and A. E. Lash**. 2002. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucl.Acids.Res. **30**:207-210.
87. **Ehrlich F.** 1907. Uber die Bedingungen der Fuselolbildung and uber ihren Zusammenhang mit dem Eiweissaufbau der Hefe. Ber.Deut.Chem.Gesellsch **40**:1027-1047.
88. **Eilbeck, K., S. E. Lewis, C. J. Mungall, M. Yandell, L. Stein, R. Durbin, and M. Ashburner**. 2005. The Sequence Ontology: a tool for the unification of genome annotations. Genome Biol. **6**:R44.
89. **Eisen, M. B., P. T. Spellman, P. O. Brown, and D. Botstein**. 1998. Cluster analysis and display of genome-wide expression patterns. Proc.Natl.Acad.Sci.U.S.A **95**:14863-14868.
90. **Ertugay, N. and H. Hamamci**. 1997. Continuous cultivation of bakers' yeast: change in cell composition at different dilution rates and effect of heat stress on trehalose level. Folia Microbiol.(Praha) **42**:463-467.
91. **Farewell, A. and F. C. Neidhardt**. 1998. Effect of temperature on in vivo protein synthetic capacity in *Escherichia coli*. J.Bacteriol. **180**:4704-4710.
92. **Ferea, T. L., D. Botstein, P. O. Brown, and R. F. Rosenzweig**. 1999. Systematic changes in gene expression patterns following adaptive evolution in yeast. Proc.Natl.Acad.Sci.U.S.A **96**:9721-9726.
93. **Ferguson, J. A., T. C. Boles, C. P. Adams, and D. R. Walt**. 1996. A fiber-optic DNA biosensor microarray for the analysis of gene expression. Nat.Biotechnol. **14**:1681-1684.
94. **Fisher, F. and C. R. Goding**. 1992. Single amino acid substitutions alter helix-loop-helix protein specificity for bases flanking the core CANNTG motif. EMBO J. **11**:4103-4109.
95. **Fix, G. J.** 1989. Principles of Brewing Science. Brewers Publications, Boulder, Colorado .
96. **Flick, J. S. and J. Thorner**. 1998. An Essential Function of a Phosphoinositide-Specific Phospholipase C Is Relieved by Inhibition of a Cyclin-Dependent Protein Kinase in the Yeast *Saccharomyces cerevisiae*. Genetics **148**:33-48.
97. **Flores, C. L., C. Rodriguez, T. Petit, and C. Gancedo**. 2000. Carbohydrate and energy-yielding metabolism in non-conventional yeasts. FEMS Microbiol.Rev. **24**:507-529.

98. **Fodor, S. P., J. L. Read, M. C. Pirrung, L. Stryer, A. T. Lu, and D. Solas**. 1991. Light-directed, spatially addressable parallel chemical synthesis. Science **251**:767-773.
99. **Forsberg, H., C. F. Gilstring, A. Zargari, P. Martinez, and P. O. Ljungdahl**. 2001. The role of the yeast plasma membrane SPS nutrient sensor in the metabolic response to extracellular amino acids. Mol.Microbiol. **42**:215-228.
100. **Forsburg, S. L. and L. Guarente**. 1989. Identification and characterization of *HAP4*: a third component of the CCAAT-bound *HAP2/HAP3* heteromer. Genes Dev. **3**:1166-1178.
101. **Francois, J. and J. L. Parrou**. 2001. Reserve carbohydrates metabolism in the yeast *Saccharomyces cerevisiae*. FEMS Microbiol.Rev. **25**:125-145.
102. **Friden P, S. P.** 1987. *LEU3* of *Saccharomyces cerevisiae* encodes a factor for control of RNA levels of a group of leucine-specific genes. Mol Cell Biol **7**:2708-17.
103. **Fujii, T., H. Shimoi, and Y. Iimura**. 1999. Structure of the glucan-binding sugar chain of Tip1p, a cell wall protein of *Saccharomyces cerevisiae*. Biochim.Biophys.Acta **1427**:133-144.
104. **Fyrst, H., B. Oskouian, F. A. Kuypers, and J. D. Saba**. 1999. The *PLB2* gene of *Saccharomyces cerevisiae* confers resistance to lysophosphatidylcholine and encodes a phospholipase B/lysophospholipase. Biochemistry **38**:5864-5871.
105. **Gancedo, C. and C. L. Flores**. 2004. The importance of a functional trehalose biosynthetic pathway for the life of yeasts and fungi. FEMS Yeast Res. **4**:351-359.
106. **Gancedo, J. M.** 1998. Yeast carbon catabolite repression. Microbiol.Mol.Biol.Rev. **62**:334-361.
107. **Gasch, A. P., P. T. Spellman, C. M. Kao, O. Carmel-Harel, M. B. Eisen, G. Storz, D. Botstein, and P. O. Brown**. 2000. Genomic expression programs in the response of yeast cells to environmental changes. Mol.Biol.Cell **11**:4241-4257.
108. **Gasch, A. P. and M. Werner-Washburne**. 2002. The genomics of yeast responses to environmental stress and starvation. Funct.Integr.Genomics **2**:181-192.
109. **Gatbonton, T., M. Imbesi, M. Nelson, J. M. Akey, D. M. Ruderfer, L. Kruglyak, J. A. Simon, and A. Bedalov**. 2006. Telomere length as a quantitative trait: genome-wide survey and genetic mapping of telomere length-control genes in yeast. PLoS.Genet. **2**:e35.
110. **Gavin, A. C., M. Bosche, R. Krause, P. Grandi, M. Marzioch, A. Bauer, J. Schultz, J. M. Rick, A. M. Michon, C. M. Cruciat, M. Remor, C. Hofert, M. Schelder, M. Brajenovic, H. Ruffner, A. Merino, K. Klein, M. Hudak, D. Dickson, T. Rudi, V. Gnau, A. Bauch, S. Bastuck, B. Huhse, C. Leutwein, M. A. Heurtier, R. R. Copley, A. Edelmann, E. Querfurth, V. Rybin, G. Drewes, M. Raida, T. Bouwmeester, P. Bork, B. Seraphin, B. Kuster, G. Neubauer, and G. Superti-Furga**. 2002. Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature **415**:141-147.
111. **Gelinas, P., G. Fiset, C. Willemot, and J. Goulet**. 1991. Lipid Content and Cryotolerance of Bakers' Yeast in Frozen Doughs. Appl.Environ.Microbiol. **57**:463-468.
112. **Giaever, G., A. M. Chu, L. Ni, C. Connelly, L. Riles, S. Veronneau, S. Dow, A. Lucau-Danila, K. Anderson, B. Andre, A. P. Arkin, A. Astromoff, M. El Bakkoury, R. Bangham, R. Benito, S. Brachat, S. Campanaro, M. Curtiss, K. Davis, A. Deutschbauer, K. D. Entian, P. Flaherty, F. Foury, D. J. Garfinkel, M. Gerstein, D. Gotte, U. Guldener, J. H. Hegemann, S. Hempel, Z. Herman, D. F. Jaramillo, D. E. Kelly, S. L. Kelly, P. Kotter, D. LaBonte, D. C. Lamb, N. Lan, H. Liang, H. Liao, L. Liu, C. Luo, M. Lussier, R. Mao, P. Menard, S. L. Ooi, J. L. Revuelta, C. J. Roberts, M. Rose, P. Ross-Macdonald, B. Scherens, G. Schimmack, B. Shafer, D. D. Shoemaker, S. Sookhai-Mahadeo, R. K. Storms, J. N. Strathern, G. Valle, M. Voet, G. Volckaert, C. Y. Wang, T. R. Ward, J. Wilhelmy, E. A. Winzeler, Y. Yang, G. Yen, E. Youngman, K. Yu, H. Bussey, J. D. Boeke, M. Snyder, P. Philippsen, R. W. Davis, and M. Johnston**. 2002. Functional profiling of the *Saccharomyces cerevisiae* genome. Nature **418**:387-391.
113. **Giaever, G., P. Flaherty, J. Kumm, M. Proctor, C. Nislow, D. F. Jaramillo, A. M. Chu, M. I. Jordan, A. P. Arkin, and R. W. Davis**. 2004. Chemogenomic profiling: identifying the functional interactions of small molecules in yeast. Proc.Natl.Acad.Sci.U.S.A **101**:793-798.
114. **Goffeau, A., B. G. Barrell, H. Bussey, R. W. Davis, B. Dujon, H. Feldmann, F. Galibert, J. D. Hoheisel, C. Jacq, M. Johnston, E. J. Louis, H. W. Mewes, Y. Murakami, P. Philippsen, H. Tettelin, and S. G. Oliver**. 1996. Life with 6000 genes. Science **274**:546, 563-546, 567.
115. **Gorner, W., E. Durchschlag, M. T. Martinez-Pastor, F. Estruch, G. Ammerer, B. Hamilton, H. Ruis, and C. Schuller**. 1998. Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. Genes Dev. **12**:586-597.
116. **Gresham, D., D. M. Ruderfer, S. C. Pratt, J. Schacherer, M. J. Dunham, D. Botstein, and L. Kruglyak**. 2006. Genome-wide detection of polymorphisms at nucleotide resolution with a single DNA microarray. Science **311**:1932-1936.
117. **Grotkjaer, T.** 2005. DTU, Denmark. Bioinformatics tools inmetabolic engineering of *Saccharomyces cerevisiae*.
118. **Gu, Z., L. M. Steinmetz, X. Gu, C. Scharfe, R. W. Davis, and W. H. Li**. 2003. Role of duplicate genes in genetic robustness against null mutations. Nature **421**:63-66.
119. **Guldener, U., S. Heck, T. Fielder, J. Beinhauer, and J. H. Hegemann**. 1996. A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Res. **24**:2519-2524.

120. **Haeckel, R., B. Hess, W. Lauterborn, and K. H. Wuster**. 1968. Purification and allosteric properties of yeast pyruvate kinase. Hoppe Seylers.Z.Physiol Chem. **349**:699-714.
121. **Harbison, C. T., D. B. Gordon, T. I. Lee, N. J. Rinaldi, K. D. Macisaac, T. W. Danford, N. M. Hannett, J. B. Tagne, D. B. Reynolds, J. Yoo, E. G. Jennings, J. Zeitlinger, D. K. Pokholok, M. Kellis, P. A. Rolfe, K. T. Takusagawa, E. S. Lander, D. K. Gifford, E. Fraenkel, and R. A. Young**. 2004. Transcriptional regulatory code of a eukaryotic genome. Nature **431**:99-104.
122. **Haslbeck, M., N. Braun, T. Stromer, B. Richter, N. Model, S. Weinkauf, and J. Buchner**. 2004. *Hsp42* is the general small heat shock protein in the cytosol of *Saccharomyces cerevisiae*. EMBO J. **23**:638-649.
123. **Haslbeck, M., S. Walke, T. Stromer, M. Ehrnsperger, H. E. White, S. Chen, H. R. Saibil, and J. Buchner**. 1999. Hsp26: a temperature-regulated chaperone. EMBO J. **18**:6744-6751.
124. **Hayes, A., N. Zhang, J. Wu, P. R. Butler, N. C. Hauser, J. D. Hoheisel, F. L. Lim, A. D. Sharrocks, and S. G. Oliver**. 2002. Hybridization array technology coupled with chemostat culture: Tools to interrogate gene expression in *Saccharomyces cerevisiae*. Methods **26**:281-290.
125. **Hazelwood, L. A., S. L. Tai, V. M. Boer, J. H. de Winde, J. T. Pronk, and J. M. Daran**. 2006. A new physiological role for Pdr12p in *Saccharomyces cerevisiae*: export of aromatic and branched-chain organic acids produced in amino acid catabolism. FEMS Yeast Res. **6**:937-945.
126. **Henschke, P. A. and V. Jiranek**. 1993. metabolism of nitrogen compounds, p. 77-164. *In* G. H. Fleet (ed.), Wine Microbiology and Biotechnology. Harwood Academic Publishers, Chur, Switzerland.
127. **Hernandez-Orte, P., J. F. Cacho, and V. Ferreira**. 2002. Relationship between varietal amino acid profile of grapes and wine aromatic composition. Experiments with model solutions and chemometric study. J.Agric.Food Chem. **50**:2891-2899.
128. **Hess, B., R. Haeckel, and K. Brand**. 1966. FDP-activation of yeast pyruvate kinase. Biochem.Biophys.Res.Commun. **24**:824-831.
129. **Higgins, V. J., A. G. Beckhouse, A. D. Oliver, P. J. Rogers, and I. W. Dawes**. 2003. Yeast genome-wide expression analysis identifies a strong ergosterol and oxidative stress response during the initial stages of an industrial lager fermentation. Appl.Environ.Microbiol. **69**:4777-4787.
130. **Higgins, V. J., P. J. Rogers, and I. W. Dawes**. 2003. Application of genome-wide expression analysis to identify molecular markers useful in monitoring industrial fermentations. Appl.Environ.Microbiol. **69**:7535-7540.
131. **Hinnebusch, A. G.** 1992. General and pathway specific regulatory mechanisms controlling the synthesis of amino acid biosynthetic enzymes in *Saccharomyces cerevisiae*, *In* The molecular and cellular biology of the yeast *Saccharomyces*: gene expression. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
132. **Hirschman, J. E., R. Balakrishnan, K. R. Christie, M. C. Costanzo, S. S. Dwight, S. R. Engel, D. G. Fisk, E. L. Hong, M. S. Livstone, R. Nash, J. Park, R. Oughtred, M. Skrzypek, B. Starr, C. L. Theesfeld, J. Williams, R. Andrada, G. Binkley, Q. Dong, C. Lane, S. Miyasato, A. Sethuraman, M. Schroeder, M. K. Thanawala, S. Weng, K. Dolinski, D. Botstein, and J. M. Cherry**. 2006. Genome Snapshot: a new resource at the *Saccharomyces* Genome Database (SGD) presenting an overview of the *Saccharomyces cerevisiae* genome. Nucleic Acids Res. **34**:D442-D445.
133. **Ho, Y., A. Gruhler, A. Heilbut, G. D. Bader, L. Moore, S. L. Adams, A. Millar, P. Taylor, K. Bennett, K. Boutilier, L. Yang, C. Wolting, I. Donaldson, S. Schandorff, J. Shewnarane, M. Vo, J. Taggart, M. Goudreault, B. Muskat, C. Alfarano, D. Dewar, Z. Lin, K. Michalickova, A. R. Willems, H. Sassi, P. A. Nielsen, K. J. Rasmussen, J. R. Andersen, L. E. Johansen, L. H. Hansen, H. Jespersen, A. Podtelejnikov, E. Nielsen, J. Crawford, V. Poulsen, B. D. Sorensen, J. Matthiesen, R. C. Hendrickson, F. Gleeson, T. Pawson, M. F. Moran, D. Durocher, M. Mann, C. W. Hogue, D. Figeys, and M. Tyers**. 2002. Systematic identification of protein complexes in *Saccharomyces cerevisiae* by mass spectrometry. Nature **415**:180-183.
134. **Hofman-Bang, J.** 1999. Nitrogen catabolite repression in *Saccharomyces cerevisiae*. Mol.Biotechnol. **12**:35-73.
135. **Hohmann, S. and H. Cederberg**. 1990. Autoregulation may control the expression of yeast pyruvate decarboxylase structural genes *PDC1* and *PDC5*. Eur.J.Biochem. **188**:615-621.
136. **Holyoak, C. D., D. Bracey, P. W. Piper, K. Kuchler, and P. J. Coote**. 1999. The *Saccharomyces cerevisiae* weak-acid-inducible ABC transporter Pdr12 transports fluorescein and preservative anions from the cytosol by an energy-dependent mechanism. J.Bacteriol. **181**:4644-4652.
137. **Homma, T., H. Iwahashi, and Y. Komatsu**. 2003. Yeast gene expression during growth at low temperature. Cryobiology **46**:230-237.
138. **Hoskisson, P. A. and G. Hobbs**. 2005. Continuous culture--making a comeback? Microbiology **151**:3153-3159.

139. **Hoskisson, P. A. and G. Hobbs**. 2006. One size doesn't fit all. Microbiology **152**:1891.
140. **Hughes, T. R., M. Mao, A. R. Jones, J. Burchard, M. J. Marton, K. W. Shannon, S. M. Lefkowitz, M. Ziman, J. M. Schelter, M. R. Meyer, S. Kobayashi, C. Davis, H. Dai, Y. D. He, S. B. Stephaniants, G. Cavet, W. L. Walker, A. West, E. Coffey, D. D. Shoemaker, R. Stoughton, A. P. Blanchard, S. H. Friend, and P. S. Linsley**. 2001. Expression profiling using microarrays fabricated by an ink-jet oligonucleotide synthesizer. Nat.Biotechnol. **19**:342-347.
141. **Huh, W. K., J. V. Falvo, L. C. Gerke, A. S. Carroll, R. W. Howson, J. S. Weissman, and E. K. O'Shea**. 2003. Global analysis of protein localization in budding yeast. Nature **425**:686-691.
142. **Hunter, K. and A. H. Rose**. 1972. Lipid composition of *Saccharomyces cerevisiae* as influenced by growth temperature. Biochim.Biophys.Acta **260**:639-653.
143. **Husnik, J. I., H. Volschenk, J. Bauer, D. Colavizza, Z. Luo, and H. J. van Vuuren**. 2006. Metabolic engineering of malolactic wine yeast. Metab Eng **8**:315-323.
144. **Iglewicz, B. and D. Hoaglin**. 1993. How to detect and handle outliers. ASQC Quality Press, Mikwaukee, Wisconsin.
145. **Ihssen, J. and T. Egli**. 2004. Specific growth rate and not cell density controls the general stress response in Escherichia coli. Microbiology **150**:1637-1648.
146. **Iraqui, I., S. Vissers, B. Andre, and A. Urrestarazu**. 1999. Transcriptional induction by aromatic amino acids in *Saccharomyces cerevisiae*. Mol.Cell Biol. **19**:3360-3371.
147. **James, T. C., S. Campbell, D. Donnelly, and U. Bond**. 2003. Transcription profile of brewery yeast under fermentation conditions. J.Appl.Microbiol. **94**:432-448.
148. **Jansen, M. L., P. Daran-Lapujade, J. H. de Winde, M. D. Piper, and J. T. Pronk**. 2004. Prolonged maltose-limited cultivation of *Saccharomyces cerevisiae* selects for cells with improved maltose affinity and hypersensitivity. Appl.Environ.Microbiol. **70**:1956-1963.
149. **Jansen, M. L., J. A. Diderich, M. Mashego, A. Hassane, J. H. de Winde, P. Daran-Lapujade, and J. T. Pronk**. 2005. Prolonged selection in aerobic, glucose-limited chemostat cultures of *Saccharomyces cerevisiae* causes a partial loss of glycolytic capacity. Microbiology **151**:1657-1669.
150. **Jensen, L. T. and V. C. Culotta**. 2002. Regulation of *Saccharomyces cerevisiae FET4* by oxygen and iron. J.Mol.Biol. **318**:251-260.
151. **Jia, Y., B. Rothermel, J. Thornton, and R. A. Butow**. 1997. A basic helix-loop-helix-leucine zipper transcription complex in yeast functions in a signaling pathway from mitochondria to the nucleus. Mol Cell Biol **17**:1110-1117.
152. **Jiang, B., J. L. Brown, J. Sheraton, N. Fortin, and H. Bussey**. 1994. A new family of yeast genes implicated in ergosterol synthesis is related to the human oxysterol binding protein. Yeast **10**:341-353.
153. **Jiang, Y., M. J. Vasconcelles, S. Wretzel, A. Light, C. E. Martin, and M. A. Goldberg**. 2001. *MGA2* is involved in the low-oxygen response element-dependent hypoxic induction of genes in *Saccharomyces cerevisiae*. Mol.Cell Biol. **21**:6161-6169.
154. **Jiranek, V., P. Langridge, and P. A. Henschke**. 1995. Regulation of hydrogen sulfide liberation in wine-producing *Saccharomyces cerevisiae* strains by assimilable nitrogen. Appl.Environ.Microbiol. **61**:461-467.
155. **Jones, P. G. and M. Inouye**. 1994. The cold-shock response--a hot topic. Mol.Microbiol. **11**:811-818.
156. **Jorgensen, P., I. Rupes, J. R. Sharom, L. Schneper, J. R. Broach, and M. Tyers**. 2004. A dynamic transcriptional network communicates growth potential to ribosome synthesis and critical cell size. Genes Dev. **18**:2491-2505.
157. **Kal, A. J., A. J. van Zonneveld, V. Benes, B. M. van den, M. G. Koerkamp, K. Albermann, N. Strack, J. M. Ruijter, A. Richter, B. Dujon, W. Ansorge, and H. F. Tabak**. 1999. Dynamics of gene expression revealed by comparison of serial analysis of gene expression transcript profiles from yeast grown on two different carbon sources. Mol.Biol.Cell **10**:1859-1872.
158. **Kandror, O., N. Bretschneider, E. Kreydin, D. Cavalieri, and A. L. Goldberg**. 2004. Yeast adapt to near-freezing temperatures by STRE/Msn2,4-dependent induction of trehalose synthesis and certain molecular chaperones. Mol.Cell **13**:771-781.
159. **Kane, M. D., T. A. Jatkoe, C. R. Stumpf, J. Lu, J. D. Thomas, and S. J. Madore**. 2000. Assessment of the sensitivity and specificity of oligonucleotide (50mer) microarrays. Nucleic Acids Res. **28**:4552-4557.
160. **Karpichev, I. V., L. Cornivelli, and G. M. Small**. 2002. Multiple regulatory roles of a novel *Saccharomyces cerevisiae* protein, encoded by YOL002c, in lipid and phosphate metabolism. J.Biol.Chem. **277**:19609-19617.
161. **Kellis, M., N. Patterson, M. Endrizzi, B. Birren, and E. S. Lander**. 2003. Sequencing and comparison of yeast species to identify genes and regulatory elements. Nature **423**:241-254.
162. **Khorlin, A. A., K. R. Khrapko, I. B. Ivanov, Y. Lysov, G. K. Yershov, S. K. Vasilenko, V. L. Florentiev, and A. D. Mirzabekov**. 1991. An oligonucleotide matrix hybridization approach to DNA sequencing. Nucleic Acids Symp.Ser. 191-192.

163. **Kimura, T., Y. Hosoda, Y. Sato, Y. Kitamura, T. Ikeda, T. Horibe, and M. Kikuchi**. 2005. Interactions among yeast protein-disulfide isomerase proteins and endoplasmic reticulum chaperone proteins influence their activities. J.Biol.Chem. **280**:31438-31441.
164. **Kirchberger, J., J. Bar, W. Schellenberger, H. Dihazi, and G. Kopperschlager**. 2002. 6-phosphofructokinase from Pichia pastoris: purification, kinetic and molecular characterization of the enzyme. Yeast. **19**:933-947.
165. **Koerkamp, M. G., M. Rep, H. J. Bussemaker, G. P. Hardy, A. Mul, K. Piekarska, C. A. Szigyarto, J. M. De Mattos, and H. F. Tabak**. 2002. Dissection of transient oxidative stress response in *Saccharomyces cerevisiae* by using DNA microarrays. Mol.Biol.Cell **13**:2783-2794.
166. **Kohlhaw, G. B.** 2003. Leucine biosynthesis in fungi: entering metabolism through the back door. Microbiol.Mol.Biol.Rev. **67**:1-15, table.
167. **Kolkman, A., P. Daran-Lapujade, A. Fullaondo, M. M. Olsthoorn, J. T. Pronk, M. Slijper, and A. J. Heck**. 2006. Proteome analysis of yeast response to various nutrient limitations. Mol.Syst.Biol. **2**:2006.
168. **Komeili, A., K. P. Wedaman, E. K. O'Shea, and T. Powers**. 2000. Mechanism of metabolic control. Target of rapamycin signaling links nitrogen quality to the activity of the Rtg1 and Rtg3 transcription factors. J.Cell Biol. **151**:863-878.
169. **Kondo, K. and M. Inouye**. 1991. TIP 1, a cold shock-inducible gene of *Saccharomyces cerevisiae*. J.Biol.Chem. **266**:17537-17544.
170. **Kondo, K., L. R. Kowalski, and M. Inouye**. 1992. Cold shock induction of yeast *NSR1* protein and its role in pre-rRNA processing. J.Biol.Chem. **267**:16259-16265.
171. **Kowalski, L. R., K. Kondo, and M. Inouye**. 1995. Cold-shock induction of a family of *TIP1*-related proteins associated with the membrane in *Saccharomyces cerevisiae*. Mol.Microbiol. **15**:341-353.
172. **Kresnowati, M. T., W. A. Van Winden, M. J. Almering, A. ten Pierick, C. Ras, T. A. Knijnenburg, P. Daran-Lapujade, J. T. Pronk, J. J. Heijnen, and J. M. Daran**. 2006. When transcriptome meets metabolome: fast cellular responses of yeast to sudden relief of glucose limitation. Mol.Syst.Biol. **2**:49.
173. **Krogan, N. J., G. Cagney, H. Yu, G. Zhong, X. Guo, A. Ignatchenko, J. Li, S. Pu, N. Datta, A. P. Tikuisis, T. Punna, J. M. Peregrin-Alvarez, M. Shales, X. Zhang, M. Davey, M. D. Robinson, A. Paccanaro, J. E. Bray, A. Sheung, B. Beattie, D. P. Richards, V. Canadien, A. Lalev, F. Mena, P. Wong, A. Starostine, M. M. Canete, J. Vlasblom, S. Wu, C. Orsi, S. R. Collins, S. Chandran, R. Haw, J. J. Rilstone, K. Gandi, N. J. Thompson, G. Musso, P. St Onge, S. Ghanny, M. H. Lam, G. Butland, A. M. Altaf-Ul, S. Kanaya, A. Shilatifard, E. O'Shea, J. S. Weissman, C. J. Ingles, T. R. Hughes, J. Parkinson, M. Gerstein, S. J. Wodak, A. Emili, and J. F. Greenblatt**. 2006. Global landscape of protein complexes in the yeast *Saccharomyces cerevisiae*. Nature **440**:637-643.
174. **Kulkarni, A. A., A. T. Abul-Hamd, R. Rai, H. El Berry, and T. G. Cooper**. 2001. Gln3p Nuclear Localization and Interaction with Ure2p in *Saccharomyces cerevisiae*. J.Biol.Chem. **276**:32136-32144.
175. **Kumar, A., P. M. Harrison, K. H. Cheung, N. Lan, N. Echols, P. Bertone, P. Miller, M. B. Gerstein, and M. Snyder**. 2002. An integrated approach for finding overlooked genes in yeast. Nat.Biotechnol. **20**:58-63.
176. **Kurganov, B. I., A. K. Dorozhko, Z. S. Kagan, and V. A. Yakovlev**. 1976. The theoretical analysis of kinetic behaviour of kinetic behaviour of "hysteretic" allosteric enzymes. III. Dissociating and associating enzyme systems in which the rate of installation of equilibrium between the oligomeric forms in comparable to that of enzymatic reaction. J.Theor.Biol. **60**:287-299.
177. **Kuyper, M., H. R. Harhangi, A. K. Stave, A. A. Winkler, M. S. Jetten, W. T. de Laat, J. J. den Ridder, H. J. Op den Camp, J. P. van Dijken, and J. T. Pronk**. 2003. High-level functional expression of a fungal xylose isomerase: the key to efficient ethanolic fermentation of xylose by *Saccharomyces cerevisiae*? FEMS Yeast Res. **4**:69-78.
178. **Kuyper, M., M. M. Hartog, M. J. Toirkens, M. J. Almering, A. A. Winkler, J. P. van Dijken, and J. T. Pronk**. 2005. Metabolic engineering of a xylose-isomerase-expressing *Saccharomyces cerevisiae* strain for rapid anaerobic xylose fermentation. FEMS Yeast Res. **5**:399-409.
179. **Kuyper, M., M. J. Toirkens, J. A. Diderich, A. A. Winkler, J. P. van Dijken, and J. T. Pronk**. 2005. Evolutionary engineering of mixed-sugar utilization by a xylose-fermenting *Saccharomyces cerevisiae* strain. FEMS Yeast Res. **5**:925-934.
180. **Kuyper, M., A. A. Winkler, J. P. van Dijken, and J. T. Pronk**. 2004. Minimal metabolic engineering of *Saccharomyces cerevisiae* for efficient anaerobic xylose fermentation: a proof of principle. FEMS Yeast Res. **4**:655-664.
181. **Kwast, K. E., P. V. Burke, B. T. Staahl, and R. O. Poyton**. 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.

182. **Kwast, K. E., L. C. Lai, N. Menda, D. T. James, III, S. Aref, and P. V. Burke**. 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.
183. **Lancashire, B. and C. Gopal**. 1998. The selection of yeast strains for brewing. Brewer Guardian Lecture 26-31.
184. **Lange, H.** 2002. TU Delft. Quantitative physiology of *Saccharomyces cerevisiae* using metabolic network analysis.
185. **Large, P. J.** 1986. Degradation of organic nitrogen compounds by yeasts. Yeast **2**:1-34.
186. **Larsson, C., A. Nilsson, A. Blomberg, and L. Gustafsson**. 1997. Glycolytic flux is conditionally correlated with ATP concentration in *Saccharomyces cerevisiae*: a chemostat study under carbon- or nitrogen-limiting conditions. J.Bacteriol. **179**:7243-7250.
187. **Larsson, C., U. von Stockar, I. Marison, and L. Gustafsson**. 1993. Growth and metabolism of *Saccharomyces cerevisiae* in chemostat cultures under carbon-, nitrogen-, or carbon- and nitrogen-limiting conditions. J.Bacteriol. **175**:4809-4816.
188. **Lau, W.-T. W., R. W. Howson, P. Malkus, R. Schekman, and E. K. O'Shea**. 2000. Pho86p, an endoplasmic reticulum (ER) resident protein in *Saccharomyces cerevisiae*, is required for ER exit of the high-affinity phosphate transporter Pho84p. PNAS **97**:1107-1112.
189. **Lee, K. S., J. L. Patton, M. Fido, L. K. Hines, S. D. Kohlwein, F. Paltauf, S. A. Henry, and D. E. Levin**. 1994. The *Saccharomyces cerevisiae PLB1* gene encodes a protein required for lysophospholipase and Phospholipase-B Activity. J.Biol.Chem. **269**:19725-19730.
190. **Lee, T. I., N. J. Rinaldi, F. Robert, D. T. Odom, Z. Bar-Joseph, G. K. Gerber, N. M. Hannett, C. T. Harbison, C. M. Thompson, I. Simon, J. Zeitlinger, E. G. Jennings, H. L. Murray, D. B. Gordon, B. Ren, J. J. Wyrick, J. B. Tagne, T. L. Volkert, E. Fraenkel, D. K. Gifford, and R. A. Young**. 2002. Transcriptional regulatory networks in *Saccharomyces cerevisiae*. Science **298**:799-804.
191. **Lee, W. C., Z. X. Xue, and T. Melese**. 1991. The NSR1 gene encodes a protein that specifically binds nuclear localization sequences and has two RNA recognition motifs. J.Cell Biol. **113**:1-12.
192. **Lee, W. C., D. Zabetakis, and T. Melese**. 1992. NSR1 is required for pre-rRNA processing and for the proper maintenance of steady-state levels of ribosomal subunits. Mol.Cell Biol. **12**:3865-3871.
193. **Lelandais, G., S. Le Crom, F. Devaux, S. Vialette, G. M. Church, C. Jacq, and P. Marc**. 2004. yMGV: a cross-species expression data mining tool. Nucl.Acids.Res. **32**:D323-D325.
194. **Lewis, J. G., R. P. Learmonth, P. V. Attfield, and K. Watson**. 1997. Stress co-tolerance and trehalose content in baking strains of *Saccharomyces cerevisiae*. J.Ind.Microbiol.Biotechnol. **18**:30-36.
195. **Lewis, J. G., R. P. Learmonth, and K. Watson**. 1993. Role of growth phase and ethanol in freeze-thaw stress resistance of *Saccharomyces cerevisiae*. Appl.Environ.Microbiol. **59**:1065-1071.
196. **Li, K. C.** 2002. Genome-wide coexpression dynamics: theory and application. Proc.Natl.Acad.Sci.U.S.A **99**:16875-16880.
197. **Liao, X. and R. A. Butow**. 1993. *RTG1* and *RTG2*: two yeast genes required for a novel path of communication from mitochondria to the nucleus. Cell **72**:61-71.
198. **Linn, S. C., R. M. van de, and G. Giaccone**. 2003. [Novel approaches; improved diagnostics and therapeutics with DNA microarrays. II. Applications]. Ned.Tijdschr.Geneeskd. **147**:800-804.
199. **Lipshutz, R. J., D. Morris, M. Chee, E. Hubbell, M. J. Kozal, N. Shah, N. Shen, R. Yang, and S. P. Fodor**. 1995. Using oligonucleotide probe arrays to access genetic diversity. Biotechniques **19**:442-447.
200. **Liu, Y. and S. L. Sturley**. 2004. Nutritional genomics in yeast models. Nutrition **20**:166-172.
201. **Liu, Z. and R. A. Butow**. 1999. A transcriptional switch in the expression of yeast tricarboxylic acid cycle genes in response to a reduction or loss of respiratory function. Mol Cell Biol **19**:6720-6728.
202. **Liu, Z., T. Sekito, M. Spirek, J. Thornton, and R. A. Butow**. 2003. Retrograde signaling is regulated by the dynamic interaction between Rtg2p and Mks1p. Mol.Cell **12**:401-411.
203. **Lodi, T., A. Alberti, B. Guiard, and I. Ferrero**. 1999. Regulation of the *Saccharomyces cerevisiae DLD1* gene encoding the mitochondrial protein D-lactate ferricytochrome c oxidoreductase by *HAP1* and *HAP2/3/4/5*. Mol.Gen.Genet. **262**:623-632.
204. **Lowry, C. V., M. E. Cerdan, and R. S. Zitomer**. 1990. A hypoxic consensus operator and a constitutive activation region regulate the ANB1 gene of *Saccharomyces cerevisiae*. Mol.Cell Biol. **10**:5921-5926.
205. **Luttik, M. A., K. M. Overkamp, P. Kotter, S. de Vries, J. P. van Dijken, and J. T. Pronk**. 1998. The *Saccharomyces cerevisiae NDE1* and *NDE2* genes encode separate mitochondrial NADH dehydrogenases catalyzing the oxidation of cytosolic NADH. J.Biol.Chem. **273**:24529-24534.
206. **Lyons, T. J., N. Y. Villa, L. M. Regalla, B. R. Kupchak, A. Vagstad, and D. J. Eide**. 2004. Metalloregulation of yeast membrane steroid receptor homologs. Proc.Natl.Acad.Sci.U.S.A **101**:5506-5511.

207. **MacDonald, J. A. and K. B. Storey**. 1999. Regulation of ground squirrel Na+K+-ATPase activity by reversible phosphorylation during hibernation. Biochem.Biophys.Res.Commun. **254**:424-429.
208. **MacDonald, J. A. and K. B. Storey**. 2002. Purification and characterization of fructose bisphosphate aldolase from the ground squirrel, *Spermophilus lateralis*: enzyme role in mammalian hibernation. Arch.Biochem.Biophys. **408**:279-285.
209. **MacDonald, J. A. and K. B. Storey**. 2005. Temperature and phosphate effects on allosteric phenomena of phosphofructokinase from a hibernating ground squirrel (*Spermophilus lateralis*). FEBS J. **272**:120-128.
210. **Mackenzie, D. E. and A. G. Christy**. 2005. The role of soil chemistry in wine grape quality and sustainable soil management in vineyards. Water Sci.Technol. **51**:27-37.
211. **Magasanik, B. and C. A. Kaiser**. 2002. Nitrogen regulation in *Saccharomyces cerevisiae*. Gene **290**:1-18.
212. **Maier, A., B. Volker, E. Boles, and G. F. Fuhrmann**. 2002. Characterisation of glucose transport in *Saccharomyces cerevisiae* with plasma membrane vesicles (countertransport) and intact cells (initial uptake) with single *Hxt1, Hxt2, Hxt3, Hxt4, Hxt6, Hxt7* or *Gal2* transporters. FEMS Yeast Res. **2**:539-550.
213. **Mao, C., J. D. Saba, and L. M. Obeid**. 1999. The dihydrosphingosine-1-phosphate phosphatases of *Saccharomyces cerevisiae* are important regulators of cell proliferation and heat stress responses. Biochem.J. **342 Pt 3:667-75.**:667-675.
214. **Marguet, D., X. J. Guo, and G. J. Lauquin**. 1988. Yeast gene *SRP1* (serine-rich protein). Intragenic repeat structure and identification of a family of *SRP1*-related DNA sequences. J.Mol.Biol. **202**:455-470.
215. **Marini, A. M., S. Soussi-Boudekou, S. Vissers, and B. Andre**. 1997. A family of ammonium transporters in *Saccharomyces cerevisiae*. Mol.Cell Biol. **17**:4282-4293.
216. **Marks, V. D., G. K. van der Merwe, and H. J. van Vuuren**. 2003. Transcriptional profiling of wine yeast in fermenting grape juice: regulatory effect of diammonium phosphate. FEMS Yeast Res. **3**:269-287.
217. **Martin, D. E., P. Demougin, M. N. Hall, and M. Bellis**. 2004. Rank Difference Analysis of Microarrays (RDAM), a novel approach to statistical analysis of microarray expression profiling data. BMC.Bioinformatics. **5**:148.
218. **Mashego, M. R., W. M. Van Gulik, J. L. Vinke, and J. J. Heijnen**. 2003. Critical evaluation of sampling techniques for residual glucose determination in carbon-limited chemostat culture of *Saccharomyces cerevisiae*. Biotechnol.Bioeng. **83**:395-399.
219. **Mashego, M. R., L. Wu, J. C. Van Dam, C. Ras, J. L. Vinke, W. A. Van Winden, W. M. Van Gulik, and J. J. Heijnen**. 2004. MIRACLE: mass isotopomer ratio analysis of U-13C-labeled extracts. A new method for accurate quantification of changes in concentrations of intracellular metabolites. Biotechnol.Bioeng. **85**:620-628.
220. **Mattivi, F., C. Zulian, G. Nicolini, and L. Valenti**. 2002. Wine, biodiversity, technology, and antioxidants. Ann.N.Y.Acad.Sci. **957**:37-56.
221. **Mauricio, J. C., E. Valero, C. Millan, and J. M. Ortega**. 2001. Changes in nitrogen compounds in must and wine during fermentation and biological aging by flor yeasts. J.Agric.Food Chem. **49**:3310-3315.
222. **McAleer, W. J., E. B. Buynak, R. Z. Maigetter, D. E. Wampler, W. J. Miller, and M. R. Hilleman**. 1984. Human hepatitis B vaccine from recombinant yeast. Nature **307**:178-180.
223. **Mellor, J., M. J. Dobson, N. A. Roberts, M. F. Tuite, J. S. Emtage, S. White, P. A. Lowe, T. Patel, A. J. Kingsman, and S. M. Kingsman**. 1983. Efficient synthesis of enzymatically active calf chymosin in *Saccharomyces cerevisiae*. Gene **24**:1-14.
224. **Merkel, O., M. Fido, J. A. Mayr, H. Pruger, F. Raab, G. Zandonella, S. D. Kohlwein, and F. Paltauf**. 1999. Characterization and function in vivo of two novel phospholipases B/lysophospholipases from *Saccharomyces cerevisiae*. J.Biol.Chem. **274**:28121-28127.
225. **Merkel, O., O. V. Oskolkova, F. Raab, R. El Toukhy, and F. Paltauf**. 2005. Regulation of activity in vitro and in vivo of three phospholipases B from *Saccharomyces cerevisiae*. Biochem.J. **387**:489-496.
226. **Messenguy, F. and T. G. Cooper**. 1977. Evidence that specific and "general" control of ornithine carbamoyltransferase production occurs at the level of transcription in *Saccharomyces cerevisiae*. J.Bacteriol. **130**:1253-1261.
227. **Messenguy, F. and E. Dubois**. 1993. Genetic evidence for a role for *MCM1* in the regulation of arginine metabolism in *Saccharomyces cerevisiae*. Mol.Cell Biol. **13**:2586-2592.
228. **Messenguy, F., E. Dubois, and C. Boonchird**. 1991. Determination of the DNA-binding sequences of ARGR proteins to arginine anabolic and catabolic promoters. Mol.Cell Biol. **11**:2852-2863.
229. **Mewes, H. W., C. Amid, R. Arnold, D. Frishman, U. Guldener, G. Mannhaupt, M. Munsterkotter, P. Pagel, N. Strack, V. Stumpflen, J. Warfsmann, and A. Ruepp**. 2004. MIPS: analysis and annotation of proteins from whole genomes. Nucl.Acids.Res. **32**:D41-D44.

230. **Meyhack, B., W. Bajwa, H. Rudolph, and A. Hinnen**. 1982. Two yeast acid phosphatase structural genes are the result of a tandem duplication and show different degrees of homology in their promoter and coding sequences. EMBO J. **1**:675-680.
231. **Miller, S. M. and B. Magasanik**. 1991. Role of the complex upstream region of the *GDH2* gene in nitrogen regulation of the NAD-linked glutamate dehydrogenase in *Saccharomyces cerevisiae*. Mol.Cell Biol. **11**:6229-6247.
232. **Monod, J.** 1942. Recherche sur la croissance des cultures bacteriennes. Hermann et Cie, Paris .
233. **Moskvina, E., C. Schuller, C. T. Maurer, W. H. Mager, and H. Ruis**. 1998. A search in the genome of *Saccharomyces cerevisiae* for genes regulated via stress response elements. Yeast **14**:1041-1050.
234. **Murata, Y., T. Homma, E. Kitagawa, Y. Momose, M. S. Sato, M. Odani, H. Shimizu, M. Hasegawa-Mizusawa, R. Matsumoto, S. Mizukami, K. Fujita, M. Parveen, Y. Komatsu, and H. Iwahashi**. 2006. Genome-wide expression analysis of yeast response during exposure to 4 degrees C. Extremophiles. **10**:117-128.
235. **Murray, M. and M. L. Greenberg**. 2000. Expression of yeast *INM1* encoding inositol monophosphatase is regulated by inositol, carbon source and growth stage and is decreased by lithium and valproate. Mol.Microbiol. **36**:651-661.
236. **Naftalin, R. J.** 1997. Evidence from studies of temperature-dependent changes of D-glucose, D-mannose and L-sorbose permeability that different states of activation of the human erythrocyte hexose transporter exist for good and bad substrates. Biochim.Biophys.Acta **1328**:13-29.
237. **Nakagawa, Y., N. Sakumoto, Y. Kaneko, and S. Harashima**. 2002. Mga2p is a putative sensor for low temperature and oxygen to induce *OLE1* transcription in *Saccharomyces cerevisiae*. Biochem.Biophys.Res.Commun. **291**:707-713.
238. **Nakao, Y., Y. Kodama, T. Fujimura, W. Miki, T. Ito, K. Shirahige, and T. Ashikari**. 2005. Construction of Lager brewing yeast DNA microarray and genome wide gene expression analysis during the lager brewing process. Yeast **22**:S43.
239. **Nelissen, B., R. De Wachter, and A. Goffeau**. 1997. Classification of all putative permeases and other membrane plurispanners of the major facilitator superfamily encoded by the complete genome of *Saccharomyces cerevisiae*. FEMS Microbiol.Rev. **21**:113-134.
240. **Ng, R. and J. Abelson**. 1980. Isolation and sequence of the gene for actin in *Saccharomyces cerevisiae*. Proc.Natl.Acad.Sci.U.S.A **77**:3912-3916.
241. **Niederberger, P., R. Prasad, G. Miozzari, and H. Kacser**. 1992. A strategy for increasing an in vivo flux by genetic manipulations. The tryptophan system of yeast. Biochem.J. **287 (Pt 2)**:473-479.
242. **Nielsen, J.** 2000. Metabolic engineering. Appl.Microbiol.Biotechnol. **55**:263-283.
243. **Nomura, M. and H. Takagi**. 2004. Role of the yeast acetyltransferase *Mpr1* in oxidative stress: regulation of oxygen reactive species caused by a toxic proline catabolism intermediate. Proc.Natl.Acad.Sci.U.S.A **101**:12616-12621.
244. **Noots, I., J. A. Delcour, and C. W. Michiels**. 1999. From field barley to malt: detection and specification of microbial activity for quality aspects. Crit Rev.Microbiol. **25**:121-153.
245. **Norbeck, J., A. K. Pahlman, N. Akhtar, A. Blomberg, and L. Adler**. 1996. Purification and characterization of two isoenzymes of DL-glycerol-3-phosphatase from *Saccharomyces cerevisiae*. Identification of the corresponding *GPP1* and *GPP2* genes and evidence for osmotic regulation of Gpp2p expression by the osmosensing mitogen-activated protein kinase signal transduction pathway. J.Biol.Chem. **271**:13875-13881.
246. **Norgaard, P., V. Westphal, C. Tachibana, L. Alsoe, B. Holst, and J. R. Winther**. 2001. Functional differences in yeast protein disulfide isomerases. J.Cell Biol. **152**:553-562.
247. **NOVICK, A. and L. SZILARD**. 1950. Experiments with the Chemostat on spontaneous mutations of bacteria. Proc.Natl.Acad.Sci.U.S.A **36**:708-719.
248. **Novo, M. T., G. Beltran, M. J. Torija, M. Poblet, N. Rozes, J. M. Guillamon, and A. Mas**. 2003. Changes in wine yeast storage carbohydrate levels during preadaptation, rehydration and low temperature fermentations. Int.J.Food Microbiol. **86**:153-161.
249. **Ogawa, N., J. DeRisi, and P. O. Brown**. 2000. New components of a system for phosphate accumulation and polyphosphate metabolism in *Saccharomyces cerevisiae* revealed by genomic expression analysis. Mol.Biol.Cell **11**:4309-4321.
250. **Ohlmeier, S., A. J. Kastaniotis, J. K. Hiltunen, and U. Bergmann**. 2004. The yeast mitochondrial proteome, a study of fermentative and respiratory growth. J.Biol.Chem. **279**:3956-3979.
251. **Olesen, K., T. Felding, C. Gjermansen, and J. Hansen**. 2002. The dynamics of the *Saccharomyces carlsbergensis* brewing yeast transcriptome during a production-scale lager beer fermentation. FEM.Yeast Res. **2**:563-573.
252. **Oliver, S.** 2002. 'To-day, we have naming of parts...'. Nat.Biotechnol. **20**:27-28.
253. **Overkamp, K. M., B. M. Bakker, P. Kotter, M. A. Luttik, J. P. van Dijken, and J. T. Pronk**. 2002. Metabolic engineering of glycerol production in *Saccharomyces cerevisiae*. Appl.Environ.Microbiol. **68**:2814-2821.

254. **Owttrim, G. W.** 2006. RNA helicases and abiotic stress. Nucleic Acids Res. **34**:3220-3230.
255. **Panadero, J., C. Pallotti, S. Rodriguez-Vargas, F. Randez-Gil, and J. A. Prieto**. 2006. A downshift in temperature activates the high osmolarity glycerol (HOG) pathway, which determines freeze tolerance in *Saccharomyces cerevisiae*. J.Biol.Chem. **281**:4638-4645.
256. **Parrou, J. L., B. Enjalbert, L. Plourde, A. Bauche, B. Gonzalez, and J. Francois**. 1999. Dynamic responses of reserve carbohydrate metabolism under carbon and nitrogen limitations in *Saccharomyces cerevisiae*. Yeast **15**:191-203.
257. **Parrou, J. L. and J. Francois**. 1997. A simplified procedure for a rapid and reliable assay of both glycogen and trehalose in whole yeast cells. Anal.Biochem. **248**:186-188.
258. **Parrou, J. L., M. A. Teste, and J. Francois**. 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.
259. **Persson, B. L., A. Berhe, U. Fristedt, P. Martinez, J. Pattison, J. Petersson, and R. Weinander**. 1998. Phosphate permeases of *Saccharomyces cerevisiae*. Biochim.Biophys.Acta **1365**:23-30.
260. **Petersen, D., G. V. Chandramouli, J. Geoghegan, J. Hilburn, J. Paarlberg, C. H. Kim, D. Munroe, L. Gangi, J. Han, R. Puri, L. Staudt, J. Weinstein, J. C. Barrett, J. Green, and E. S. Kawasaki**. 2005. Three microarray platforms: an analysis of their concordance in profiling gene expression. BMC.Genomics **6**:63.
261. **Petit, T., J. A. Diderich, A. L. Kruckeberg, C. Gancedo, and K. van Dam**. 2000. Hexokinase regulates kinetics of glucose transport and expression of genes encoding hexose transporters in *Saccharomyces cerevisiae*. J.Bacteriol. **182**:6815-6818.
262. **Piper, M. D., P. Daran-Lapujade, C. Bro, B. Regenberg, S. Knudsen, J. Nielsen, and J. T. Pronk**. 2002. Reproducibility of oligonucleotide microarray transcriptome analyses. An interlaboratory comparison using chemostat cultures of *Saccharomyces cerevisiae*. J.Biol.Chem. **277**:37001-37008.
263. **Pir, P., B. Kirdar, A. Hayes, Z. Y. Onsan, K. O. Ulgen, and S. G. Oliver**. 2006. Integrative investigation of metabolic and transcriptomic data. BMC.Bioinformatics. **7**:203.
264. **Postma, E., A. Kuiper, W. F. Tomasouw, W. A. Scheffers, and J. P. van Dijken**. 1989. Competition for glucose between the yeasts *Saccharomyces cerevisiae* and Candida utilis. Appl.Environ.Microbiol. **55**:3214-3220.
265. **Postma, E., C. Verduyn, W. A. Scheffers, and J. P. van Dijken**. 1989. Enzymic analysis of the crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. Appl Environ Microbiol **55**:468-477.
266. **Powers, T. and P. Walter**. 1999. Regulation of ribosome biogenesis by the rapamycin-sensitive TOR-signaling pathway in *Saccharomyces cerevisiae*. Mol.Biol.Cell **10**:987-1000.
267. **Pronk, J. T.** 2002. Auxotrophic yeast strains in fundamental and applied research. Appl.Environ.Microbiol. **68**:2095-2100.
268. **Quackenbush, J.** 2001. Computational analysis of microarray data. Nat.Rev.Genet. **2**:418-427.
269. **Quackenbush, J.** 2003. Genomics. Microarrays--guilt by association. Science **302**:240-241.
270. **Rainieri, S., Y. Kodama, Y. Kaneko, K. Mikata, Y. Nakao, and T. Ashikari**. 2006. Pure and mixed genetic lines of Saccharomyces bayanus and Saccharomyces pastorianus and their contribution to the lager brewing strain genome. Appl.Environ.Microbiol. **72**:3968-3974.
271. **Regenberg, B., L. During-Olsen, M. C. Kielland-Brandt, and S. Holmberg**. 1999. Substrate specificity and gene expression of the amino-acid permeases in *Saccharomyces cerevisiae*. Curr.Genet. **36**:317-328.
272. **Regnacq, M., P. Alimardani, B. El Moudni, and T. Berges**. 2001. SUT1p interaction with Cyc8p(Ssn6p) relieves hypoxic genes from Cyc8p-Tup1p repression in *Saccharomyces cerevisiae*. Mol.Microbiol. **40**:1085-1096.
273. **Reifenberger, E., E. Boles, and M. Ciriacy**. 1997. Kinetic characterization of individual hexose transporters of *Saccharomyces cerevisiae* and their relation to the triggering mechanisms of glucose repression. Eur.J.Biochem. **245**:324-333.
274. **Robyr, D., Y. Suka, I. Xenarios, S. K. Kurdistani, A. Wang, N. Suka, and M. Grunstein**. 2002. Microarray deacetylation maps determine genome-wide functions for yeast histone deacetylases. Cell **109**:437-446.
275. **Rodriguez-Vargas, S., F. Estruch, and F. Randez-Gil**. 2002. Gene expression analysis of cold and freeze stress in Baker's yeast. Appl.Environ.Microbiol. **68**:3024-3030.
276. **Rosenfeld, E. and B. Beauvoit**. 2003. Role of the non-respiratory pathways in the utilization of molecular oxygen by *Saccharomyces cerevisiae*. Yeast **20**:1115-1144.
277. **Rossignol, T., L. Dulau, A. Julien, and B. Blondin**. 2003. Genome-wide monitoring of wine yeast gene expression during alcoholic fermentation. Yeast **20**:1369-1385.
278. **Rudra, D., Y. Zhao, and J. R. Warner**. 2005. Central role of Ifh1p-Fhl1p interaction in the synthesis of yeast ribosomal proteins. EMBO J. **24**:533-542.
279. **Ruis, H. and C. Schuller**. 1995. Stress Signaling in Yeast. Bioessays **17**:959-965.

280. **Sahara, T., T. Goda, and S. Ohgiya**. 2002. Comprehensive expression analysis of time-dependent genetic responses in yeast cells to low temperature. J.Biol.Chem. **277**:50015-50021.
281. **Saiardi, A., H. Erdjument-Bromage, A. M. Snowman, P. Tempst, and S. H. Snyder**. 1999. Synthesis of diphosphoinositol pentakisphosphate by a newly identified family of higher inositol polyphosphate kinases. Curr.Biol. **9**:1323-1326.
282. **Saldanha, A. J., M. J. Brauer, and D. Botstein**. 2004. Nutritional homeostasis in batch and steady-state culture of yeast. Mol.Biol.Cell **15**:4089-4104.
283. **Sandager, L., A. Dahlqvist, A. Banas, U. Stahl, M. Lenman, M. Gustavsson, and S. Stymne**. 2000. An acyl-CoA:cholesterol acyltransferase (ACAT)-related gene is involved in the accumulation of triacylglycerols in *Saccharomyces cerevisiae*. Biochem.Soc.Trans. **28**:700-702.
284. **Sauer, U.** 2001. Evolutionary engineering of industrially important microbial phenotypes. Adv.Biochem.Eng Biotechnol. **73**:129-169.
285. **Schaaff, I., J. Heinisch, and F. K. Zimmermann**. 1989. Overproduction of glycolytic enzymes in yeast. Yeast **5**:285-290.
286. **Schade, B., G. Jansen, M. Whiteway, K. D. Entian, and D. Y. Thomas**. 2004. Cold adaptation in budding yeast. Mol.Biol.Cell **15**:5492-5502.
287. **Schena, M., R. A. Heller, T. P. Theriault, K. Konrad, E. Lachenmeier, and R. W. Davis**. 1998. Microarrays: biotechnology's discovery platform for functional genomics. Trends Biotechnol. **16**:301-306.
288. **Schena, M., D. Shalon, R. W. Davis, and P. O. Brown**. 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science **270**:467-470.
289. **Schirmer, T. and P. R. Evans**. 1990. Structural basis of the allosteric behaviour of phosphofructokinase. Nature. **343**:140-145.
290. **Schmelzle, T. and M. N. Hall**. 2000. TOR, a central controller of cell growth. Cell **103**:253-262.
291. **Schmidt, U. and C. G. Begley**. 2003. Cancer diagnosis and microarrays. Int.J.Biochem.Cell Biol. **35**:119-124.
292. **Schuller, H. J.** 2003. Transcriptional control of nonfermentative metabolism in the yeast *Saccharomyces cerevisiae*. Curr.Genet. **43**:139-160.
293. **Seki, M., M. Narusaka, J. Ishida, T. Nanjo, M. Fujita, Y. Oono, A. Kamiya, M. Nakajima, A. Enju, T. Sakurai, M. Satou, K. Akiyama, T. Taji, K. Yamaguchi-Shinozaki, P. Carninci, J. Kawai, Y. Hayashizaki, and K. Shinozaki**. 2002. Monitoring the expression profiles of 7000 Arabidopsis genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. Plant J. **31**:279-292.
294. **Seong, K. T., Y. Katakura, K. Ninomiya, Y. Bito, S. Katahira, A. Kondo, M. Ueda, and S. Shioya**. 2006. Effect of flocculation on performance of arming yeast in direct ethanol fermentation. Appl.Microbiol.Biotechnol.
295. **Seytter, T., F. Lottspeich, W. Neupert, and E. Schwarz**. 1998. Mam33p, an oligomeric, acidic protein in the mitochondrial matrix of *Saccharomyces cerevisiae* is related to the human complement receptor gC1q-R. Yeast **14**:303-310.
296. **Shamji, A. F., F. G. Kuruvilla, and S. L. Schreiber**. 2000. Partitioning the transcriptional program induced by rapamycin among the effectors of the Tor proteins. Curr.Biol. **10**:1574-1581.
297. **Sinclair, K., J. P. Warner, and D. T. Bonthron**. 1994. The *ASP1* gene of *Saccharomyces cerevisiae*, encoding the intracellular isozyme of L-asparaginase. Gene **144**:37-43.
298. **Skacel, M., B. Skilton, J. D. Pettay, and R. R. Tubbs**. 2002. Tissue microarrays: a powerful tool for high-throughput analysis of clinical specimens: a review of the method with validation data. Appl.Immunohistochem.Mol.Morphol. **10**:1-6.
299. **Snoek, I. S. and H. Y. Steensma**. 2006. Why does *Kluyveromyces lactis* not grow under anaerobic conditions? Comparison of essential anaerobic genes of *Saccharomyces cerevisiae* with the *Kluyveromyces* lactis genome. FEMS Yeast Res. **6**:393-403.
300. **Somer, L., O. Shmulman, T. Dror, S. Hashmueli, and Y. Kashi**. 2002. The eukaryote chaperonin *CCT* is a cold shock protein in *Saccharomyces cerevisiae*. Cell Stress.Chaperones. **7**:47-54.
301. **Somero, G. N.** 2004. Adaptation of enzymes to temperature: searching for basic "strategies". Comp Biochem.Physiol B Biochem.Mol.Biol. **139**:321-333.
302. **Sonderegger, M., M. Jeppsson, B. Hahn-Hagerdal, and U. Sauer**. 2004. Molecular basis for anaerobic growth of *Saccharomyces cerevisiae* on xylose, investigated by global gene expression and metabolic flux analysis. Appl.Environ.Microbiol. **70**:2307-2317.
303. **Stanbrough, M. and B. Magasanik**. 1995. Transcriptional and posttranslational regulation of the general amino acid permease of *Saccharomyces cerevisiae*. J.Bacteriol. **177**:94-102.
304. **Stanbrough, M., D. W. Rowen, and B. Magasanik**. 1995. Role of the GATA factors Gln3p and Nil1p of *Saccharomyces cerevisiae* in the expression of nitrogen-regulated genes. Proc.Natl.Acad.Sci.U.S.A **92**:9450-9454.

305. **Stefano, G. B., J. D. Burrill, S. Labur, J. Blake, and P. Cadet**. 2005. Regulation of various genes in human leukocytes acutely exposed to morphine: expression microarray analysis. Med.Sci.Monit. **11**:MS35-MS42.
306. **Steinmetz, L. M. and R. W. Davis**. 2004. Maximizing the potential of functional genomics. Nat.Rev.Genet. **5**:190-201.
307. **Stewart, G. G. and I. Russell**. 1986. One Hundred Years of Yeast Research and Development in the Brewing Industry. J.Inst.Brew. **92**:537-558.
308. **Stückrath, I., H. C. Lange, P. Kötter, W. M. Van Gulik, K. D. Entian, and J. J. Heijnen**. 2002. Characterization of null mutants of the glyoxylate cycle and gluconeogenic enzymes in *S. cerevisiae* through metabolic network modeling verified by chemostat cultivation. Biotechnol.Bioeng. **77**:61-72.
309. **Stukey, J. E., V. M. McDonough, and C. E. Martin**. 1990. 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. J.Biol.Chem. **265**:20144-20149.
310. **Sudarsanam, P., Y. Pilpel, and G. M. Church**. 2002. Genome-wide co-occurrence of promoter elements reveals a cis-regulatory cassette of rRNA transcription motifs in *Saccharomyces cerevisiae*. Genome Res. **12**:1723-1731.
311. **Suter, B., D. Auerbach, and I. Stagljar**. 2006. Yeast-based functional genomics and proteomics technologies: the first 15 years and beyond. Biotechniques **40**:625-644.
312. **Tachibana, C. and T. H. Stevens**. 1992. The yeast *EUG1* gene encodes an endoplasmic reticulum protein that is functionally related to protein disulfide isomerase. Mol.Cell Biol. **12**:4601-4611.
313. **Tai, S. L., V. M. Boer, P. Daran-Lapujade, M. C. Walsh, J. H. de Winde, J. M. Daran, and J. T. Pronk**. 2005. Two-dimensional Transcriptome Analysis in Chemostat Cultures: COMBINATORIAL EFFECTS OF OXYGEN AVAILABILITY AND MACRONUTRIENT LIMITATION IN *SACCHAROMYCES CEREVISIAE*. J.Biol.Chem. **280**:437-447.
314. **Tate, J. J. and T. G. Cooper**. 2003. Tor1/2 regulation of retrograde gene expression in *Saccharomyces cerevisiae* derives indirectly as a consequence of alterations in ammonia metabolism. J.Biol.Chem. **19;278**:36924-36933.
315. **Tate, J. J., K. H. Cox, R. Rai, and T. G. Cooper**. 2002. Mks1p is required for negative regulation of retrograde gene expression in *Saccharomyces cerevisiae* but does not affect nitrogen catabolite repression-sensitive gene expression. J.Biol.Chem. **277**:20477-20482.
316. **Ter Linde, J. J., H. Liang, R. W. Davis, H. Y. Steensma, J. P. van Dijken, and J. T. Pronk**. 1999. Genome-wide transcriptional analysis of aerobic and anaerobic chemostat cultures of *Saccharomyces cerevisiae*. J.Bacteriol. **181**:7409-7413.
317. **Ter Linde, J. J. and H. Y. Steensma**. 2002. A microarray-assisted screen for potential Hap1 and Rox1 target genes in *Saccharomyces cerevisiae*. Yeast **19**:825-840.
318. **ter Schure, E. G., H. H. Sillje, A. J. Verkleij, J. Boonstra, and C. T. Verrips**. 1995. The concentration of ammonia regulates nitrogen metabolism in *Saccharomyces cerevisiae*. J.Bacteriol. **177**:6672-6675.
319. **ter Schure, E. G., H. H. Sillje, E. E. Vermeulen, J. W. Kalhorn, A. J. Verkleij, J. Boonstra, and C. T. Verrips**. 1998. Repression of nitrogen catabolic genes by ammonia and glutamine in nitrogen-limited continuous cultures of *Saccharomyces cerevisiae*. Microbiology **144 (Pt 5)**:1451-1462.
320. **ter Schure, E. G., N. A. van Riel, and C. T. Verrips**. 1999. The role of ammonia metabolism in nitrogen catabolite repression in *Saccharomyces cerevisiae*. FEMS Microbiol.Rev. **24**:67-83.
321. **Teusink, B., M. C. Walsh, K. van Dam, and H. V. Westerhoff**. 1998. The danger of metabolic pathways with turbo design. Trends Biochem.Sci. **23**:162-169.
322. **Thevelein, J. M. and S. Hohmann**. 1995. Trehalose synthase: guard to the gate of glycolysis in yeast? Trends Biochem.Sci. **20**:3-10.
323. **Thieringer, H. A., P. G. Jones, and M. Inouye**. 1998. Cold shock and adaptation. Bioessays **20**:49-57.
324. **Thomas, D. and Y. Surdin-Kerjan**. 1997. Metabolism of sulfur amino acids in *Saccharomyces cerevisiae*. Microbiol.Mol.Biol.Rev. **61**:503-532.
325. **Tong, A. H., M. Evangelista, A. B. Parsons, H. Xu, G. D. Bader, N. Page, M. Robinson, S. Raghibizadeh, C. W. Hogue, H. Bussey, B. Andrews, M. Tyers, and C. Boone**. 2001. Systematic genetic analysis with ordered arrays of yeast deletion mutants. Science **294**:2364-2368.
326. **Tong, A. H., G. Lesage, G. D. Bader, H. Ding, H. Xu, X. Xin, J. Young, G. F. Berriz, R. L. Brost, M. Chang, Y. Chen, X. Cheng, G. Chua, H. Friesen, D. S. Goldberg, J. Haynes, C. Humphries, G. He, S. Hussein, L. Ke, N. Krogan, Z. Li, J. N. Levinson, H. Lu, P. Menard, C. Munyana, A. B. Parsons, O. Ryan, R. Tonikian, T. Roberts, A. M. Sdicu, J. Shapiro, B. Sheikh, B. Suter, S. L. Wong, L. V. Zhang, H. Zhu, C. G. Burd, S. Munro, C. Sander, J. Rine, J. Greenblatt, M. Peter, A. Bretscher, G. Bell, F. P. Roth, G. W. Brown, B. Andrews, H. Bussey, and C. Boone**. 2004. Global mapping of the yeast genetic interaction network. Science **303**:808-813.

327. **Torija, M. J., G. Beltran, M. Novo, M. Poblet, J. M. Guillamon, A. Mas, and N. Rozes**. 2003. Effects of fermentation temperature and Saccharomyces species on the cell fatty acid composition and presence of volatile compounds in wine. Int.J.Food Microbiol. **85**:127-136.
328. **Torija, M. J., N. Rozes, M. Poblet, J. M. Guillamon, and A. Mas**. 2003. Effects of fermentation temperature on the strain population of *Saccharomyces cerevisiae*. Int.J.Food Microbiol. **80**:47-53.
329. **Trobner, W. and R. Piechocki**. 1985. Competition between the dam mutator and the isogenic wild-type of Escherichia coli. Mutat.Res. **144**:145-149.
330. **Tusher, V. G., R. Tibshirani, and G. Chu**. 2001. Significance analysis of microarrays applied to the ionizing radiation response. Proc.Natl.Acad.Sci.U.S.A **98**:5116-5121.
331. **Upadhya, R., J. Lee, and I. M. Willis**. 2002. Maf1 is an essential mediator of diverse signals that repress RNA polymerase III transcription. Mol.Cell **10**:1489-1494.
332. **Van Dam, J. C., M. R. Eman, J. Frank, H. C. Lange, G. W. K. van Dedem, and S. J. Heijnen**. 2002. Analysis of glycolytic intermediates in *Saccharomyces cerevisiae* using anion exchange chromatography and electrospray ionization with tandem mass spectrometric detection. Analytica Chimica Acta **460**:209-218.
333. **van den Berg, M. A., P. Jong-Gubbels, C. J. Kortland, J. P. van Dijken, J. T. Pronk, and H. Y. Steensma**. 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.
334. **Van Dijck, P., D. Colavizza, P. Smet, and J. M. Thevelein**. 1995. Differential importance of trehalose in stress resistance in fermenting and nonfermenting *Saccharomyces cerevisiae* cells. Appl.Environ.Microbiol. **61**:109-115.
335. **van Dijken, J. P., J. Bauer, L. Brambilla, P. Duboc, J. M. Francois, C. Gancedo, M. L. Giuseppin, J. J. Heijnen, M. Hoare, H. C. Lange, E. A. Madden, P. Niederberger, J. Nielsen, J. L. Parrou, T. Petit, D. Porro, M. Reuss, N. van Riel, M. Rizzi, H. Y. Steensma, C. T. Verrips, J. Vindelov, and J. T. Pronk**. 2000. An interlaboratory comparison of physiological and genetic properties of four *Saccharomyces cerevisiae* strains. Enzyme Microb.Technol. **26**:706-714.
336. **van Helden, J., B. Andre, and J. Collado-Vides**. 2000. A web site for the computational analysis of yeast regulatory sequences. Yeast **16**:177-187.
337. **van Hoek, P.** 2000. Fermentative Capacity in Aerobic Cultures of Bakers' Yeast. IOS Press, Amsterdam.
338. **van Hoek, P., M. T. Flikweert, Q. J. van der Aart, H. Y. Steensma, J. P. van Dijken, and J. T. Pronk**. 1998. Effects of pyruvate decarboxylase overproduction on flux distribution at the pyruvate branch point in *Saccharomyces cerevisiae*. Appl.Environ.Microbiol. **64**:2133-2140.
339. **van Hoek, P., J. P. van Dijken, and J. T. Pronk**. 2000. Regulation of fermentative capacity and levels of glycolytic enzymes in chemostat cultures of *Saccharomyces cerevisiae*. Enzyme Microb.Technol. **26**:724-736.
340. **Van Maris, A. J., B. M. Bakker, M. Brandt, A. Boorsma, M. J. Teixeira De Mattos, L. A. Grivell, J. T. Pronk, and J. Blom**. 2001. Modulating the distribution of fluxes among respiration and fermentation by overexpression of *HAP4* in *Saccharomyces cerevisiae*. FEMS Yeast Res. **1**:139-149.
341. **van Maris, A. J., A. A. Winkler, D. Porro, J. P. van Dijken, and J. T. Pronk**. 2004. Homofermentative lactate production cannot sustain anaerobic growth of engineered *Saccharomyces cerevisiae*: possible consequence of energy-dependent lactate export. Appl.Environ.Microbiol. **70**:2898-2905.
342. **Venter, U. and W. Horz**. 1989. The acid phosphatase genes *PHO10* and *PHO11* in S. cerevisiae are located at the telomeres of chromosomes VIII and I. Nucleic Acids Res. **17**:1353-1369.
343. **Verduyn, C., E. Postma, W. A. Scheffers, and J. P. van Dijken**. 1990. Energetics of *Saccharomyces cerevisiae* in anaerobic glucose-limited chemostat cultures. J.Gen.Microbiol. **136**:405-412.
344. **Verduyn, C., E. Postma, W. A. Scheffers, and J. P. van Dijken**. 1990. Physiology of *Saccharomyces cerevisiae* in anaerobic glucose-limited chemostat cultures. J.Gen.Microbiol. **136 (Pt 3)**:395-403.
345. **Verduyn, C., E. Postma, W. A. Scheffers, and J. P. van Dijken**. 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.
346. **Verduyn, C., J. P. van Dijken, and W. A. Scheffers**. 1984. Colorimetric alcohol assays with alcohol oxidase. J.Microbiol.Methods **2**:15-25.
347. **Verstrepen, K. J. and F. M. Klis**. 2006. Flocculation, adhesion and biofilm formation in yeasts. Mol.Microbiol. **60**:5-15.
348. **Visser, W., W. A. Scheffers, Batenburg-van der Vegte WH, and J. P. van Dijken**. 1990. Oxygen requirements of yeasts. Appl.Environ.Microbiol. **56**:3785-3792.

349. **Viswanathan, M., G. Muthukumar, Y. S. Cong, and J. Lenard**. 1994. Seripauperins of *Saccharomyces cerevisiae*: a new multigene family encoding serine-poor relatives of serine-rich proteins. Gene **148**:149-153.
350. **Vuralhan, Z., M. A. Luttik, S. L. Tai, V. M. Boer, M. A. Morais, D. Schipper, M. J. Almering, P. Kotter, J. R. Dickinson, J. M. Daran, and J. T. Pronk**. 2005. Physiological characterization of the *ARO10*-dependent, broad-substrate-specificity 2-oxo acid decarboxylase activity of *Saccharomyces cerevisiae*. Appl.Environ.Microbiol. **71**:3276-3284.
351. **Vuralhan, Z., M. A. Morais, S. L. Tai, M. D. Piper, and J. T. Pronk**. 2003. Identification and characterization of phenylpyruvate decarboxylase genes in *Saccharomyces cerevisiae*. Appl.Environ.Microbiol. **69**:4534-4541.
352. **Walker, G.** 2004. The 4th Brewing Yeast Fermentation Performance Congress, Oxford, England, 9-12 September, 2003. FEMS Yeast Res. **4**:567-570.
353. **Walker, S. J., Y. Wang, K. A. Grant, F. Chan, and G. M. Hellmann**. 2006. Long versus short oligonucleotide microarrays for the study of gene expression in nonhuman primates. J.Neurosci.Methods **152**:179-189.
354. **Walker-Simmons, M. and D. E. Atkinson**. 1977. Functional capacities and the adenylate energy charge in *Escherichia coli* under conditions of nutritional stress. J.Bacteriol. **130**:676-683.
355. **Walsh, M. C., H. P. Smits, M. Scholte, and K. van Dam**. 1994. Affinity of glucose transport in *Saccharomyces cerevisiae* is modulated during growth on glucose. J.Bacteriol. **176**:953-958.
356. **Warner, J. R.** 1999. The economics of ribosome biosynthesis in yeast. Trends Biochem.Sci. **24**:437-440.
357. **Watson, T. G.** 1976. Amino-acid pool composition of *Saccharomyces cerevisiae* as a function of growth rate and amino-acid nitrogen source. J.Gen.Microbiol. **96**:263-268.
358. **Wei, C. L., M. Kainuma, and J. W. Hershey**. 1995. Characterization of yeast translation initiation factor 1A and cloning of its essential gene. J.Biol.Chem. **270**:22788-22794.
359. **Weikert, C., U. Sauer, and J. E. Bailey**. 1997. Use of a glycerol-limited, long-term chemostat for isolation of *Escherichia coli* mutants with improved physiological properties. Microbiology **143 (Pt 5)**:1567-1574.
360. **Wenzel, T. J., A. W. Teunissen, and H. Y. Steensma**. 1995. *PDA1* mRNA: a standard for quantitation of mRNA in *Saccharomyces cerevisiae* superior to *ACT1* mRNA. Nucleic Acids Res. **23**:883-884.
361. **Werner-Washburne, M., E. Braun, G. C. Johnston, and R. A. Singer**. 1993. Stationary phase in the yeast *Saccharomyces cerevisiae*. Microbiol.Rev. **57**:383-401.
362. **Whitesell, R. R., D. M. Regen, A. H. Beth, D. K. Pelletier, and N. A. Abumrad**. 1989. Activation energy of the slowest step in the glucose carrier cycle: break at 23 degrees C and correlation with membrane lipid fluidity. Biochemistry **28**:5618-5625.
363. **Wiederrecht, G., D. Seto, and C. S. Parker**. 1988. Isolation of the gene encoding the *S. cerevisiae* heat shock transcription factor. Cell **54**:841-853.
364. **Wilcox, L. J., D. A. Balderes, B. Wharton, A. H. Tinkelenberg, G. Rao, and S. L. Sturley**. 2002. Transcriptional profiling identifies two members of the ATP-binding cassette transporter superfamily required for sterol uptake in yeast. J.Biol.Chem. **277**:32466-32472.
365. **Winzeler, E. A., C. I. Castillo-Davis, G. Oshiro, D. Liang, D. R. Richards, Y. Zhou, and D. L. Hartl**. 2003. Genetic diversity in yeast assessed with whole-genome oligonucleotide arrays. Genetics **163**:79-89.
366. **Winzeler, E. A., D. D. Shoemaker, A. Astromoff, H. Liang, K. Anderson, B. Andre, R. Bangham, R. Benito, J. D. Boeke, H. Bussey, A. M. Chu, C. Connelly, K. Davis, F. Dietrich, S. W. Dow, M. El Bakkoury, F. Foury, S. H. Friend, E. Gentalen, G. Giaever, J. H. Hegemann, T. Jones, M. Laub, H. Liao, R. W. Davis, and .** 1999. Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. Science **285**:901-906.
367. **Wolfe, D. M. and D. A. Pearce**. 2006. Channeling studies in yeast: yeast as a model for channelopathies? Neuromolecular.Med. **8**:279-306.
368. **Wu, J., N. Zhang, A. Hayes, K. Panoutsopoulou, and S. G. Oliver**. 2004. Global analysis of nutrient control of gene expression in *Saccharomyces cerevisiae* during growth and starvation. Proc.Natl.Acad.Sci.U.S.A .
369. **Wu, L., M. R. Mashego, J. C. Van Dam, A. M. Proell, J. L. Vinke, C. Ras, W. A. Van Winden, W. M. Van Gulik, and J. J. Heijnen**. 2005. Quantitative analysis of the microbial metabolome by isotope dilution mass spectrometry using uniformly 13C-labeled cell extracts as internal standards. Anal.Biochem. **336**:164-171.
370. **Wu, L., J. van Dam, D. Schipper, M. T. Kresnowati, A. M. Proell, C. Ras, W. A. Van Winden, W. M. Van Gulik, and J. J. Heijnen**. 2006. Short-term metabolome dynamics and carbon, electron, and ATP balances in chemostat-grown *Saccharomyces cerevisiae* CEN.PK 113-7D following a glucose pulse. Appl.Environ.Microbiol. **72**:3566-3577.
371. **Wu, L. F., T. R. Hughes, A. P. Davierwala, M. D. Robinson, R. Stoughton, and S. J. Altschuler**. 2002. Large-scale prediction of *Saccharomyces cerevisiae* gene function using overlapping transcriptional clusters. Nat.Genet. **31**:255-265.

372. **Yan, C. and T. Melese**. 1993. Multiple regions of *NSR1* are sufficient for accumulation of a fusion protein within the nucleolus. J.Cell Biol. **123**:1081-1091.
373. **Zahringer, H., J. M. Thevelein, and S. Nwaka**. 2000. Induction of neutral trehalase *NTH1* by heat and osmotic stress is controlled by STRE elements and Msn2/Msn4 transcription factors: variations of PKA effect during stress and growth. Mol.Microbiol. **35**:397-406.
374. **Zhao, H. and D. J. Eide**. 1997. Zap1p, a metalloregulatory protein involved in zinc-responsive transcriptional regulation in *Saccharomyces cerevisiae*. Mol.Cell Biol. **17**:5044-5052.
375. **Zhao, S. H., J. Recknor, J. K. Lunney, D. Nettleton, D. Kuhar, S. Orley, and C. K. Tuggle**. 2005. Validation of a first-generation long-oligonucleotide microarray for transcriptional profiling in the pig. Genomics **86**:618-625.

Summary

The genome-wide transcriptome analysis of the eukaryotic cell *Saccharomyces cerevisiae* is no longer a tool that is within the reach of just the very few. The rapid expansion of microarray technology has paved a highway in molecular biology to obtain and analyze high-density information on the DNA level on what is actually happening in a cell's response to perturbations. This thesis deals primarily with this technology to further understand the intrinsic control of gene expression regulation. This knowledge is invaluable especially for industrial fermentation processes as gene expression data in principle allows for the use of the microorganisms themselves as the ultimate biosensor. DNA microarray identifies transcripts that may serve for diagnostic purposes or for rational design and optimization of such processes. However, a potential pitfall in transcriptome responses is that many assumptions have been made to underlay an explanation towards the changes seen on the gene expression. When a gene changes its expression levels in reaction to a perturbation, it is generally accepted that the response is a direct reply to the perturbation to counteract the disturbances for cell survival. This statement is of course debatable.

S. cerevisiae can strive for growth under anaerobic conditions with relative ease compared to other microorganisms. There is not yet a clear understanding of the main features of *S. cerevisiae* that underlie this phenotype. The genome-wide transcriptome response to anaerobiosis was therefore analyzed in Chapter 2 under four different limitations of carbon, nitrogen, phosphorus and sulfur to further dive into this enigma. It was noted that the two-dimensional analysis yielded 155 genes that were specific to anaerobiosis regardless of the limitation it was grown on. In previous studies of oxygen availability related transcriptome response in carbon-limited cultures, the changes were close to 900 genes. This mark filtering of responsive genes to anaerobiosis has allowed us to decipher more clearly gene regulation to oxygen availability and also to set a mold for indicator 'signature' genes that could be used for diagnostic purposes in custom array designs. Accompanying these signature genes however were also genes with context-dependency to the growth limitations. This is indicative that the transcriptome response forms a multi-dimensional space in which a unique response to a perturbation can only be found by comparing gene responses to a plethora of backgrounds. If the 155 genes were further analyzed to for example a gradient of pH or growth rates, we would further breakdown these set of genes to a handful that can truly be identified as anaerobic responsive genes.

Of the 155 transcripts that had changed expression under anaerobiosis, 65 were up-regulated. In these 65 transcripts, were genes whose functions could be clearly related to a benefit for anaerobic growth, for instance those involved in sterol uptake and unsaturated fatty acid metabolism. However, many of these genes were also still unknown in function or have previously not been identified to be biologically meaningful to anaerobiosis. A very important question regarding the assumptions on transcriptome response is, would an up-regulation of a gene infer a beneficial function for fitness of a cell? We set out in chapter 3 by analyzing 27 single null mutant strains selected from the 65 genes found up-regulated under anaerobiosis in an anaerobic competitive chemostat fermentation. Only 5 mutant strains showed a

significant reduction in fitness when compared to a reference 'wild-type' strain. When grown in an aerobic competitive chemostat, none of the mutant strains showed significant reduction in fitness. The low hit rate found from this experiment could be explained by the genetic robustness of *S. cerevisiae*. Backup genes and homolog genes that are constitutively expressed can probably compensate for the lost of function when these genes are deleted individually. Alternatively, an up-regulation of a gene under anaerobiosis may not necessarily have a beneficial impact on the fitness of the cell. This up-regulation may be a coordinated response of family genes for instance in the up-regulation of genes related to alternative nitrogen sources even when they are not present in the medium. So why does *S. cerevisiae* then invest such amount of energy to up-regulate these genes? Most likely, many of these genes do play an integral role under anaerobiosis but a single deletion would probably not have affected the fitness of the cells enough to show an apparent disadvantage. Clearly, multiple gene deletion is vital for further answers and experiments like synthetic lethality screening could lead to identify 'partner' genes that coordinate in cell survival.

Where Chapter 2 deals with the transcriptome response to anaerobiosis and macronutrient limitations, Chapter 4 investigates gene expression on the consumption of different amino acids as the sole nitrogen source. In aerobic carbon-limited cultures, we grew yeast on a sole nitrogen source of ammonium or one of five amino acids, namely asparagine, proline, methionine, leucine and phenylalanine. Synonymous to Chapter 2, signature transcripts were sorted according to each nitrogenous source present in the medium. Here we found sets of genes that were uniquely responsive however care should be taken on the uniqueness of these gene responses. Since only 5 of the 20 amino acids have been tested in this study, an extension on this work would definitely further disintegrate the distinctive response. Since amino acids share many properties (e.g. polarity, structure, acidity, pathway), instead of relying on simple 'indicator' genes, complex sets of genes are more likely meaningful analysis for industrial fermentations. This was readily seen within this dataset when we compared similar transcriptome response patterns across these 5 amino acids. We identified a large simultaneous change in gene expression in particular to methionine, leucine and phenylalanine. From this response we were able to shade more light on the catabolism of these compounds via the Ehrlich pathway in nitrogen utilization and fusel alcohol/acid formation. The result was that two genes, *ARO10* and *PDR12* have been extensively categorized and implicated with this catabolic pathway. These findings have been published elsewhere (see List of publications).

In the beverage industry, fermentations are normally carried out at temperatures that are less than favorable for *S. cerevisiae* growth. At these lower temperatures (10-18 ^{o}C), favorable flavor and aroma compounds are prized but the adverse effects accompanying the reduction in temperature like reduced growth rates and enzyme kinetics, slow protein folding, increased stability of RNA and increased cellular viscosity cannot be circumvented. Chapter 5 was set out to identify the effects of low temperature on gene expression. In carbon- and nitrogen-limited anaerobic fermentations at 12 ^{o}C, we found that the background of the growth limiting substrate had significant effects on the transcriptome and in particular related

to the corresponding transport and metabolism routes. What was particularly surprising to notice was the large discrepancy seen with already published low temperature transcriptome analyses that were mainly analyzed in dynamic batch conditions. The characteristic accumulation of trehalose and up-regulation of the environmental stress response (ESR) did not occur. Instead we saw the total opposite effects of low temperature cultivation at 12 ^{o}C in chemostats. One likely explanation for this would be the response of reduced growth rates that are normally accompanied with the reduction of temperature. ESR and the stress response element (STRE) have also been implicated with changes in growth rates and since growth rates are kept constant in chemostat cultures these effects were decoupled. Based on the transcriptome response alone, we found that growth at 12 ^{o}C is not as detrimental as previously thought.

The interesting results obtained in Chapter 5 however did not answer many questions on the physiology and homeostasis of cell adaptation to low temperature. From the fluxes obtained from Chapter 5, we observed that even with the reduction in temperature from 30 ^{o}C to 12 ^{o}C, the glycolytic flux was maintained at similar levels in both cultivating temperatures. Hence, in Chapter 6, we aimed to understand the behaviour of this flux via a systems biology approach on the glycolytic pathway in carbon-limited cultures. The results demonstrated that there were two main mechanisms for control of *in vivo* glycolytic fluxes. The glucose uptake assays suggested that there was large reprogramming of the glucose-transport capacities with temperature change. The changes in gene expression of the hexose transporters had allowed for a higher glucose-transport capacity and this is in agreement with the higher measured fermentative capacity for low temperatures. The other mechanism that influenced the glycolytic flux was on the metabolite level, where intracellular concentrations of ATP and glycolytic intermediates were unanimously increased while ADP, AMP and trehalose intermediate T6P were decreased in concentrations at 12 ^{o}C. Although these metabolites have been implicated as activators and inhibitors to the glycolytic enzymes, the pattern of change was puzzling and was difficult to show a consistent correlation to those already found in literature. However, all these changes that were seen altered the homeostasis of *S. cerevisiae* to allow growth at low temperature at the same growth rate with little changes to the glycolytic fluxes, biomass yield and biomass composition. In evolutionary terms, these changes may be advantageous to maintain an adequate pool of metabolites and proteins for adaptation to diurnal temperature cycles. In conclusion we see that transcriptome studies alone cannot provide all the answers for the regulation of *in vivo* glycolytic fluxes. In fact the regulation as previously seen in other systems biology approaches on the glycolytic pathway seems to be mainly demonstrated on the metabolite level. Other than the vertical control of the glucose transport, metabolic control plays a dominant role in maintaining a sustainable glycolytic flux.

This thesis has covered a plethora of conditions that are interesting for industrial fermentations. We discovered that transcriptomics is an indispensable tool for a genome-wide analysis as it is the most widely available tool to date. However, the limitations of microarrays have also been addressed, but new technologies are constantly updating the many problems especially in terms of sensitivity and

accuracy. Transcriptomics can nonetheless cover only a small dissection of what is a vast complex network of communication and regulation in the cell. New insights in proteomics and metabolomics are paving a new direction for yeast molecular biology with integration of different levels of information that make up the whole metabolic circuit control. But in order to fully understand changes that are implicated to the molecular basis of the cell, fundamental research as demonstrated in this thesis is needed to cement a strong foundation for other exciting work to go on.

Samenvatting

De techniek om de transcriptie van een heel genoom te analyseren, zoals voor het eukaryote micro-organisme *Saccharomyces cerevisiae*, is inmiddels wijdverspreid. De snelle introductie van microarray technologie heeft het voor moleculair biologen mogelijk gemaakt om informatiestromen op het niveau van het DNA te meten en te analyseren. Zo kan achterhaald worden wat er in de cel gebeurd wanneer deze zich aanpast aan veranderingen in zijn milieu. In dit proefschrift ligt de nadruk op het gebruik van deze techniek om meer begrip te krijgen van de intrinsieke controle op de regulatie van genexpressie. Deze kennis kan van onschatbare waarde zijn. Bij industriële fermentatie processen zou het micro-organisme zelf gebruikt kunnen worden als ultieme biosensor door het meten van de expressie van zijn genen. Met DNA microarrays kunnen transcripten worden geselecteerd die geschikt zijn voor de diagnostische doeleinden of voor het rationeel ontwerpen en optimaliseren van zulke fermentatie processen. Voorzichtigheid is echter geboden bij het zoeken naar verklaringen voor gemeten veranderingen in genexpressie wanneer er grote aannames worden gemaakt. Veranderingen in genexpressie na een verstoring in het milieu van een organisme worden in het algemeen opgevat als een direct antwoord van de cel om de effecten die de verstoring teweeg brengt te compenseren. De juistheid van deze aanname is natuurlijk te betwisten.

S. cerevisiae heeft relatief weinig moeite om zich te vermenigvuldigen in de afwezigheid van zuurstof. Het is nog niet duidelijk waarin *S. cerevisiae* afwijkt ten opzichte van andere micro-organismes dat dit verschil in fenotype verklaart. Om hiervan meer begrip te ontwikkelen is het transcriptoom van *S. cerevisiae* onder zowel aerobe en anaerobe condities geanalyseerd onder vier verschillende limitaties: koolstof, stikstof, fosfor en zwavel (Hoofdstuk 2). Een twee-dimensionale analyse leverde 155 genen op met een verschil in expressie tussen anaerobe en aerobe condities onder alle vier de limitaties. In eerdere studies naar de respons van het transcriptoom op zuurstofbeschikbaarheid in koolstofgelimiteerde cultures werden bijna 900 genen geïdentificeerd. Het gebruik van meerdere limitaties zorgt voor een meer beperkte selectie van genen waarvan met een hogere betrouwbaarheid gesteld kan worden dat hun expressie specifiek reageert op verandering in zuurstofbeschikbaarheid. De genen binnen deze selectie kunnen gebruikt worden om maatwerk arrays te maken voor het testen op de aanwezigheid van zuurstof. De expressie van een andere groep genen reageert contextgevoelig op de verschillende groeilimitaties. Het is belangrijk om te beseffen dat de keuze voor de verschillende contexten bepalend is voor de groep genen die geïdentificeerd worden als genen waarvan de expressie specifiek veranderd door de aan- of afwezigheid van zuurstof.

Het gebruik van andere, extra contexten, zoals het groeien van *S. cerevisiae* bij verschillende pH-waardes of groeisnelheden, zal leiden tot een verdere selectie binnen deze groep genen.

Binnen de groep van 155 transcripten waarvan de expressie verschilde onder anaerobe condities waren er 65 opgereguleerd. Sommige van deze genen hadden functies die gerelateerd kunnen worden aan een voordeel bij anaerobe groei, zoals sterol-opname en vetzuurmetabolisme. Daarnaast waren er veel genen met een nog onbekende functie. Ten slotte zijn er genen, waaraan al wel functies zijn toegekend, maar waarvan opregulatie geen duidelijk voordeel biedt onder anaerobe condities. Hieruit vloeit de vraag voort of een toename in genexpressie betekent dat de functie van dat gen een positief effect heeft op de fitheid van de cel. In Hoofdstuk 3 is de analyse beschreven van een selectie van 27 stammen waarin steeds één van de 65 genen was uitgeschakeld. Deze stammen zijn getest onder anaerobe condities in een competitieve chemostaat fermentatie. Slechts 5 mutant-stammen vertoonden een verminderde fitheid ten opzichte van de referentie 'wild-type' stam. In een aerobe competieve chemostaat fermentatie vertoonden geen van de mutanten een significante afname in fitheid. Deze uitkomsten kunnen verklaard worden vanuit de genetische robustheid van *S. cerevisiae*. De aanwezigheid van 'backup genes' en van genen met homologe functies die constitutief tot expressie komen, zorgt waarschijnlijk voor compensatie in het geval van verlies van de functionaliteit van één van deze genen. Daarnaast is het mogelijk dat opregulatie van een gen onder anaerobe condities geen positief effect heeft op de fitheid van de cel. Hele families van genen kunnen worden opgereguleerd, zoals in het geval van genen met een functie in de opname van alternatieve stikstofbronnen, terwijl deze bronnen niet aanwezig hoeven te zijn in het medium. Maar waarom zou S. cerevisiae dan grote hoeveelheden energie investeren om deze genen tot overexpressie te brengen? Waarschijnlijk spelen veel van deze genen een integrale rol onder anaerobe condities waarbij een enkele deletie geen duidelijk meetbare invloed heeft op de fitheid van de cel. Deze hypothese kan getest worden door het tegelijk uitschakelen van meerdere genen. Daarnaast kunnen door het uitvoeren van experimenten zoals 'synthetic lethality screenings' genen worden geïdentificeerd die samen gecoördineerd invloed hebben op het overleven van de cel.

Waar Hoofdstuk 2 de respons van het transcriptoom op anaerobe condities en groeilimitaties behandeld, richt Hoofdstuk 4 zich op de verschillen in genexpressie bij de consumptie van een aantal aminozuren die als enige stikstofbron zijn toegevoegd. In aerobe koolstofgelimiteerde cultures hebben we gist laten groeien. Als stikstofbron werd ammonium toegevoegd, of een aminozuur (asparagine, proline, methionine, leucine of fenylalanine). Net als in Hoofdstuk 2 zijn transcripten geordend op basis van hun expressie bij de verschillende stikstofbronnen. Een set met genen was gevonden die bij slechts één specifieke stikstofbron van expressie veranderden. Hierbij moet worden benadrukt dat veel van deze genen waarschijnlijk niet aan deze eis voldoen wanneer het experiment met alle 20 aminozuren wordt herhaald. Aangezien aminozuren in veel eigenschappen overeenkomsten vertonen (b.v. in polariteit, structuur, zuursterkte, metabole routes), is het waarschijnlijk dat meerdere genen gebruikt moeten worden als 'indicator' om voldoende onderscheid te kunnen maken bij analyses van industriële fermentaties. Wij kwamen snel tot deze

conclusie bij het vergelijken van de respons in het transcriptoom op de aanwezigheid van de 5 aminozuren. Met name bij methionine, leucine en phenylalaninen werden grotendeels vergelijkbare veranderingen in genexpressie waargenomen. Deze uitkomst scheen meer licht op het catabolisme van deze stoffen via de Ehrlich route voor stikstof gebruik en de productie van fusel-alcoholen en -zuren. Twee genen, *ARO10* en *PDR12* zijn op basis van deze resultaten uitgebreid onderzocht en geïmpliceerd in deze catabole route. Deze vindingen zijn elders gepubliceerd (zie List of publications).

In de drankenindustrie worden fermentaties meestal uitgevoerd bij te lage temperaturen (10-18 ^{o}C) voor goede groei van S. cerevisiae. Deze temperaturen bevorderen de productie van smaakstoffen en aroma's, maar zorgen onvermijdelijk voor lage groeisnelheden, langzamere enzymkinetiek en eiwitvouwing en een verhoging van de stabiliteit van RNA en van viscositeit in de cel. In Hoofdstuk 5 zijn de effecten een lagere temperatuur op de genexpressie onderzocht. In koolstof- en stikstofgelimiteerde anaerobe fermentaties bij 12 ^{o}C kwam naar voren dat de keuze van het groeilimiterende substraat grote invloed had op het transcriptoom, met name dat deel dat correspondeert met transportroutes en routes binnen het metabolisme. Het meest verrassend waren de grote verschillen met eerder bij lage temperatuur uitgevoerde transcriptoom analyses, waarbij de experimenten hoofdzakelijk in dynamische batch condities waren verricht. De karakteristieke ophoping van trehalose en de opregulatie van de 'environmental stress response' (ESR) werden niet waargenomen. In plaats hiervan zagen we totaal tegenovergestelde effecten bij het cultiveren van S. cerevisiae bij lage temparaturen (12 ^{o}C) in chemostaten. Een mogelijke uitleg hiervoor is dat lage temperaturen normaliter zorgen voor een afname in de groeisnelheid. Er zijn aanwijzingen dat veranderingen in groeisnelheden invloed hebben op de ESR en het 'stress reponse element' (STRE). Het constant houden van de groeisnelheid in een chemostat bij een afname in temperatuur leidt tot een ontkoppeling van deze effecten. Op basis van alleen de transcriptoom respons concludeerden we dat groei bij 12 ^{o}C niet zo nadelig is als eerder gedacht.

De interessante resultaten die in Hoofdstuk 5 zijn verkregen gaven echter geen antwoord op vragen over veranderingen in fysiology en homeostase van de cel bij het aanpassen aan lage temperaturen. Gemeten fluxes (Hoofdstuk 5) lieten zien dat bij een temperatuurafname van 30 ^{o}C naar 12 ^{o}C de flux door de glycolyse op een vergelijkbaar niveau bleef. Om het bedrag van deze flux beter te begrijpen is een systeembiologie-aanpak toegepast op de glycolytische route in koolstof-gelimiteerde cultures (Hoofdstuk 6). De resultaten lieten zien dat er twee belangrijke mechanismes waren voor de controle van de *in vivo* glycolytische fluxen. De metingen aan glucoseopname suggereerden dat er veel herprogrammering plaatsvond van de capaciteiten van glucosetransport bij een verandering in de temperatuur. De veranderingen in genexpressie van de hexose transporters maakten een verhoging van glucosetransportcapaciteit mogelijk en dit is in overeenstemming met de toename in de gemeten fermentatieve capaciteit bij lage temperaturen. Het andere mechanisme met een invloed op de glycolytische flux lag op het metabolietniveau, waar de intracellulaire concentraties van ATP en alle glycolytische tussenproducten waren toegenomen, terwijl die van ADP, AMP en het tussenproduct naar trehalose, T6P, waren afgenomen bij 12 ^{o}C. Hoewel aan deze metabolieten

rollen zijn toegewezen als activatoren en inhibitoren van glycolytische enzymen, stelde het patroon van concentratieveranderingen ons voor raadsels en dit patroon kwam niet eenduidig overeen met eerder in literatuur beschreven resultaten. Echter, al deze waargenomen veranderingen veranderden de homeostase van *S. cerevisiae* zodanig om groei bij lage temperatuur mogelijk te maken met weinig veranderingen in glycolytische fluxes en de opbrengst en samenstelling van biomassa. Geschreven in evolutionaire termen lijken deze veranderingen voordelig in het handhaven van voldoende voorraden van metabolieten en eiwitten in de cel zodat deze zich kan aanpassen aan de temperatuurverschillen die optreden tijdens de dag en nacht. Concluderend zien we dat onderzoek naar alleen het transcriptoom niet alle antwoorden kan geven op hoe in vivo glycolytische fluxen worden gereguleerd. In studies met een andere systeembiologie-aanpak naar de regulatie van de glycolytisch route lijkt deze regulatie zich voornamelijk te bevinden op het metabolietniveau. Op de verticale controle van glucose transport na, speelt metabole controle een dominante rol in het onderhouden van een constante glycolytische flux.

Dit proefschrift heeft een groot aantal condities beschreven die interessant zijn voor industriële fermentaties. We hebben gezien dat transcriptomics een onmisbaar gereedschap is voor genoomonderzoek vanwege zijn brede beschikbaarheid. Daarnaast zijn ook de beperkingen van microarrays besproken, alhoewel deze nieuwe techniek constant vernieuwd wordt om met name de problemen met gevoeligheid en nauwkeurigheid te verminderen. Toch beslaat transcriptomics slechts een klein deel van het complexe netwerk van communicatie en regulatie binnen de cel. Nieuwe inzichten in proteomics en metabolomics leggen het grondwerk voor een nieuwe tak binnen het onderzoek naar de moleculaire biology van gist waarbij verschillende niveaus van informatie worden geintegreerd om een completer model te maken van de controle van metabole routes. Maar om volledig inzicht te krijgen in cellulaire veranderingen en hun relatie met de moleculaire opbouw van de cel is meer fundamenteel onderzoek, zoals beschreven in dit proefschrift, noodzakelijk. Alleen zo kan een basis gelegd worden voor ander boeiend werk.

A thank you note

Without a doubt, if I had to thank everyone, I could have another thesis ready for print just on all the help I have received, not only academically but also for my time here in the Netherlands. Therefore, I first apologize if I have missed anyone out, it was done without intentions.

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The cover of this thesis was designed with an inspiration from an agarose gel picture. The vertical lines entwined with changes and crossovers signify the use of bioinformatics in representing biological data. Biology can no longer be separated from information technology. In fact, it is dependent on it.

Curriculum vitae

Siew Leng Tai was born on 7 October 1977 in Kuala Lumpur, Malaysia. Having completed his pre-university with Cambridge 'A'-Levels at Taylor's College in Malaysia, he ventured in 1996 to Manchester, England to study Chemical Engineering. He spent 3 years at the University of Manchester Institute of Science and Technology (UMIST) and graduated with a Bachelor's degree in engineering (BEng). Having completed his university degree he returned to Malaysia where he worked for about a year as a sales engineer in the field of gas analyzers and detection systems. In August 2000, he left Malaysia again to continue his studies at TU Delft, The Netherlands. August 2002 saw him graduate with a M.Sc. in Chemical Engineering with specialization in biotechnology. For this final M.Sc. thesis, he studied the possibilities of over producing phenylalanine in *S. cerevisiae* by means of metabolic engineering on the biosynthesis pathway. He was supervised by Prof. Jack Pronk during this six month period. Continuing his passion for science, he continued working under the supervisor of Prof. Jack Pronk and Dr. Jean-Marc Daran for his PhD studies. During this period, he worked mainly with the utilization of transcriptomics and chemostat cultivations in understanding how yeast gene expression is influenced by growth conditions. The results of his work have been published in this thesis. As of October 2006, Siew Leng is in South Africa working again with yeast in the group of Prof. Florian Bauer of the Wine Biotechnology Institute in Stellenbosch.

List of Publications

Vuralhan Z, Morais MA, Tai SL, Piper MD, Pronk JT. Identification and characterization of phenylpyruvate decarboxylase genes in *Saccharomyces cerevisiae*. Appl Environ Microbiol. 2003 Aug;69(8):4534-41

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 Jan 7;280(1):437-47.

Vuralhan Z, Luttik MA, Tai SL, Boer VM, Morais MA, Schipper D, Almering MJ, Kotter P, Dickinson JR, Daran JM, Pronk JT. Physiological characterization of the ARO10-dependent, broad-substrate-specificity 2-oxo acid decarboxylase activity of *Saccharomyces cerevisiae*. Appl Environ Microbiol. 2005 Jun;71(6):3276-84.

Hazelwood LA, Tai SL, Boer VM, de Winde JH, Pronk JT, Daran JM. A new physiological role for Pdr12p in *Saccharomyces cerevisiae*: export of aromatic and branched-chain organic acids produced in amino acid catabolism. FEMS Yeast Res. 2006 Sep;6(6):937-45.

Tai SL, Snoek I, Luttik MA, Almering MJ, Walsh MC, Pronk JT, Daran JM. Competitive chemostat cultivation of *Saccharomyces cerevisiae* mutants indicates a weak correlation between oxygen-dependent transcriptional regulation and fitness of deletion strains under anaerobic conditions. Accepted for publication in Microbiology UK 2006.

Boer VM, Tai SL, Vuralhan Z, Arifin Y, Walsh MC, Piper MD, de Winde JH, Pronk JT, Daran JM. Transcriptional responses of *Saccharomyces cerevisiae* to preferred and non-preferred nitrogen sources in glucose-limited chemostat cultures. Accepted for publication in FEMS Yeast Research 2006.

Tai SL, Daran-Lapujade P, Luttik MA, Walsh MC, Diderich JA, Krijger GC, van Gulik WM, Pronk JT, Daran JM. Control of Glycolytic Flux in *Saccharomyces cerevisiae* Grown at Low Temperature: A Multi-level Analysis in Anaerobic Chemostat Cultures. Accepted for publication in J Biol Chem 2006.