Role of Transcriptional Regulation in Controlling Fluxes in Central Carbon Metabolism of *Saccharomyces cerevisiae*, a Chemostat Culture Study

**Running title**
Transcript levels and metabolic fluxes in *S. cerevisiae*

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Summary

In contrast to batch cultivation, chemostat cultivation allows the identification of carbon-source responses without interference by carbon-catabolite repression, accumulation of toxic products and differences in specific growth rate. This study focuses on the yeast *Saccharomyces cerevisiae*, grown in aerobic, carbon-limited chemostat cultures. Genome-wide transcript levels and *in vivo* fluxes were compared for growth on two sugars, glucose and maltose, and for two C2-compounds, ethanol and acetate. In contrast to previous reports on batch cultures, few genes (180 genes) responded to changes of the carbon source by a changed transcript level. Very few transcript levels were changed when glucose as the growth-limiting nutrient was compared to maltose (33 transcripts), or when acetate was compared to ethanol (16 transcripts). Although metabolic flux analysis using a stoichiometric model revealed major changes in the central carbon metabolism, only 117 genes exhibited a significantly different transcript level when sugars and C2-compounds were provided as the growth-limiting nutrient. Despite the extensive knowledge on carbon source regulation in yeast, many of the carbon-source responsive genes encoded proteins with unknown or incompletely characterized biological functions. *In silico* promoter analysis of carbon-source responsive genes confirmed the involvement of several known transcriptional regulators and suggested the involvement of additional regulators. Transcripts involved in the glyoxylate cycle and gluconeogenesis showed a good correlation with *in vivo* fluxes. This correlation was, however, not observed for other important pathways, including the pentose-phosphate pathway, TCA cycle and, in particular, glycolysis. These results indicate that *in vivo* fluxes in the central carbon metabolism of *S. cerevisiae* grown in steady-state, carbon-limited chemostat cultures are controlled to a large extent via post-transcriptional mechanisms.
Introduction

The yeast *Saccharomyces cerevisiae* is widely used as a model organism to study carbon-source-dependent metabolic regulation in eukaryotes. Wild-type *S. cerevisiae* strains have a narrow set of carbon sources that can support fast growth in synthetic media (1). The most widely studied of these are the hexoses glucose, fructose, galactose and mannose, the disaccharides maltose and sucrose and the C₂-compounds ethanol and acetate. The metabolic networks employed for the metabolism of the hexoses and disaccharides are very similar and only differ in the initial steps of metabolism (Fig. 1). For example, glucose and maltose metabolism only differ with respect to two reactions. The first reaction is the sugar transport through the plasma membrane: maltose uptake is catalyzed by an energy-dependent maltose-proton symport mechanism (Fig.1, step 30) (2), whereas glucose uptake is catalyzed exclusively by a facilitated diffusion mechanism (step 33) (3). The second reaction is the intracellular breakdown of maltose into glucose which involves a specific α-glucosidase (‘maltase’, step 29) (4). Similarly, the metabolism of the C₂-compounds ethanol and acetate only differ by the initial substrate-uptake step (steps 31 and 32) (5-7) and by two sequential NAD(P)+-dependent oxidation reactions that convert ethanol into acetate (steps 22 and 23).

Drastic changes are observed in central metabolism when the metabolism of sugars is compared with that of the C₂-compounds. During growth on sugars, all metabolic building blocks can be derived from glycolysis, TCA cycle and pentose phosphate pathway. During growth on C₂-compounds, gluconeogenesis and glyoxylate cycle are essential for the provision of some of these precursors. Furthermore, the higher ATP requirement for biosynthesis during growth on the C₂-compounds (and in particular acetate, (8)) implies that, at a fixed specific growth rate, dissimilatory fluxes have to be higher with the C₂-compounds than with a sugar as the sole carbon source.

So far, most studies on regulation of central carbon metabolism in *S. cerevisiae* have been performed in batch mode in shake-flask or reactors. This cultivation method, however, has several drawbacks for quantitative analysis. First of all, the concentrations of substrates and products change
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throughout cultivation, which makes it difficult to accurately measure fluxes through specific pathways or to assess the influence of carbon sources on cellular regulation. Furthermore, batch cultivation by definition requires the use of excess concentrations of the carbon source. When different carbon sources are compared, this will lead to different specific growth rates which, in itself, may already lead to changes in the make-up and activity of the metabolic network. Finally, the relatively high substrate concentrations in batch cultures lead to catabolite repression and inactivation phenomena (9;10).

Cultivation of microorganisms in chemostats offers numerous advantages for studying the structure and regulation of metabolic networks (11). In chemostat cultures, individual culture parameters can be changed, while keeping other relevant physical and chemical culture parameters (composition of synthetic medium, pH, temperature, aeration, etc.) constant. An especially important parameter in this respect is the specific growth rate which, in a chemostat, is equal to the dilution rate, which can be accurately controlled. This allows the experimenter to investigate the effects of environmental changes or genetic interventions at a fixed specific growth rate, even if these changes result in different specific growth rates in batch cultures. In a chemostat, growth can be limited by a single, selected nutrient. The very low residual concentrations of this growth-limiting nutrient in chemostat cultures alleviate effects of catabolite repression and inactivation. Furthermore, these low residual substrate concentrations prevent substrate toxicity which, for example, occurs when *S. cerevisiae* is grown on ethanol or acetate as the carbon source in batch cultures (12;13).

The central goal of the present study is to assess to what extent carbon-source dependent regulation of fluxes through central carbon metabolism in *S. cerevisiae* is regulated at the level of transcription. To this end, we compare the transcriptome of carbon-limited, aerobic chemostat cultures grown on four different carbon sources: glucose, maltose, ethanol and acetate. Data from the transcriptome analysis are compared with flux distribution profiles calculated with a stoichiometric metabolic network model. Questions that will be addressed are (i) does glucose-limited aerobic cultivation lead to a complete alleviation of glucose-catabolite repression, (ii) how (in)complete is our
understanding of the genes involved in the transcriptional response of *S. cerevisiae* to four of the most common carbon sources for this yeast, and (iii) to what extent do transcriptome analyses with microarrays provide a reliable indication of flux distribution in metabolic networks?

The complete dataset used in this study is available for download at http://www.bt.tudelft.nl/carbon-source.
Experimental Procedures

Strain and growth conditions

Wild-type *Saccharomyces cerevisiae* strain CEN.PK113-7D (MATa) (14) was grown at 30°C in 2-liter chemostats (Applikon), with a working volume of 1.0 liter as described in (15). Cultures were fed with a defined mineral medium that limited growth by glucose, ethanol, acetate or maltose with all other growth requirements in excess. 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 between 60% and 75% of air saturation. The off-gas was cooled by a condenser connected to a cryostat set at 2°C and analyzed as previously described (16). Steady-state samples were taken after approximately 10 to 14 volume changes to avoid strain adaptation due to long-term cultivation (17). Dry-weight, metabolite, dissolved oxygen and off-gas profiles had to be constant over at least 5 volume changes prior to sampling for RNA extraction.

Media

The defined mineral medium composition was based on that described by Verduyn *et al* (18). The carbon source was 256 ± 19 mmoles of carbon per liter.

Analytical methods

Culture supernatants were obtained after centrifugation of samples from the chemostats. For the purpose of glucose, ethanol, acetate and maltose determination and carbon recovery, culture supernatants and media were analyzed by HPLC, fitted with an AMINEX HPX-87H ion exchange column using 5 mM H₂SO₄ as the mobile phase. Culture dry weights were determined via filtration as described by Postma *et al*. (19).
Metabolic flux distribution

Intracellular metabolic fluxes were calculated through metabolic flux balancing using a compartmented stoichiometric model derived from the model developed by H.C. Lange (20).

Because the intracellular localization of certain enzymes as well as the trafficking of certain metabolites in *S. cerevisiae* are still a matter of debate, assumptions had to be made on these aspects. The main assumptions concern acetyl-Coenzyme A metabolism. It has been shown that transport of acetyl-CoA through the mitochondrial membrane cannot be performed in *S. cerevisiae* by the carnitine shuttle in the absence of exogenous carnitine (21;22). It is however possible to conceive growth on sugar and gluconeogenic carbon sources without Acetyl-CoA transport. During growth on sugar, mitochondrial acetyl-CoA is synthesized directly in this compartment via the pyruvate dehydrogenase complex, while the small amounts of necessary cytosolic acetyl-CoA are synthesized in the cytosol by the acetyl-CoA synthase (encoded by *ACS1* and *ACS2*, (15;23)). The gluconeogenic carbon sources acetate and ethanol can be converted by acetyl-CoA synthase into acetyl-CoA in the cytosol, acetyl-CoA being further converted in the cytosol to citrate by citrate synthase (*CIT2*, (23)). Citrate can then be transported through the mitochondrial membrane by the well-described citrate transporter (*CTP1*, (24)).

The set-up of the stoichiometric models for growth of *S. cerevisiae* on glucose, maltose, ethanol and acetate as well as the flux balancing was performed using dedicated software (SPAD it, Nijmegen, The Netherlands). The theory and practice of metabolic flux balancing has been described well in literature and will not be repeated here (25-29). For each carbon source the specific rates of growth, substrate consumption, carbon dioxide production and oxygen consumption during steady state chemostat cultivation were calculated from the measured concentrations and flow rates from three independent experiments.

The calculated specific conversion rates and their variances were used as input for the metabolic flux balancing procedure. In all cases the ATP-balance was omitted as constraint in the flux balancing. This is
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a prerequisite for proper balancing in case the ATP-stoichiometry of some reactions is insufficiently known (e.g. maintenance energy requirements, P/O-ratio etc.). However, also without the ATP-balance the number of measurements was sufficient to result in an over-determined system, thus making data reconciliation possible. In all cases the degree of redundancy was equal to 2.

The complete list of reactions and components used to build the model can be found at http://www.bt.tudelft.nl/carbon-source.

**Microarray analysis**

Sampling of cells from chemostats, probe preparation and hybridization to Affymetrix GeneChip® microarrays were performed as described previously (30). 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. 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.* (31).

For further statistical analyses Microsoft Excel running the Significance Analysis of Microarrays (SAM; v1.12) add-in was used (32) for all possible pair-wise comparisons of the four data sets. 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 conditions. Hierarchical clustering of the obtained set of significantly changed expression levels was subsequently performed by GeneSpring (Silicon Genetics).

Promoter analysis was performed using web-based softwares Regulatory Sequence Analysis Tools\(^1\) (RSA Tools, (33)) and AlignAce\(^2\) (34). The promoters (from -800 to -50) of each set of co-regulated genes were analyzed for over-represented motifs. When motifs shared largely overlapping sequences they were aligned to form longer conserved elements. All the individual promoter sequences contributing to these elements were then aligned and 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 using RSA Tools. The cluster coverage (Table 3) is then expressed as the number of genes in the cluster containing the motif at least once divided by the total number of genes in the cluster. Similarly, the genome coverage is expressed as the genome-wide number of genes containing the motif at least once divided by the total number of genes used by RSA Tools (6451 open reading frames).

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\(^1\) http://rsat.ulb.ac.be/rsat/
\(^2\) http://atlas.med.harvard.edu/
Results

Biomass yields and respiration rates in carbon-limited chemostat cultures.

Carbon-limited, aerobic chemostat cultures were grown on glucose, maltose, ethanol and acetate as single growth-limiting nutrients. At a dilution rate of 0.1 h\(^{-1}\) the concentration of all four carbon sources in the reservoir medium was ca. 250 mmoles of carbon per liter (Table 1), whereas their residual concentration in steady-state cultures were below their respective detection limits (i.e. less than 0.5 mM). For the glucose- and maltose-grown cultures, the respiratory quotient (ratio of the specific rate of carbon dioxide production and oxygen consumption) was close to 1.0, indicating a fully respiratory metabolism of these sugars. In all cultures over 95 % of the substrate carbon was recovered as either biomass or carbon dioxide (Table 1), and HPLC analysis of culture supernatants did not reveal the production of any low molecular weight metabolites.

Biomass yields, as well as the specific rates of oxygen consumption and carbon dioxide production, were very similar for cultures grown on maltose and glucose. This was expected, as maltose metabolism is initiated by the uptake and hydrolysis of the disaccharide to two glucose molecules (Fig. 1). In contrast to glucose uptake however, the uptake of maltose occurs via an energy-depending proton-symport mechanism (2), which is likely to be responsible for the slightly lower (8 %) biomass yield on maltose as compared to glucose (Table 1). Consistent with earlier studies, biomass yields on ethanol and, in particular, acetate were lower than that on glucose (8,35). These lower biomass yields and correspondingly higher respiration rates can be explained from the lower ATP yield from respiratory dissimilation of these substrates (which is largely due to the investment of 2 ATP equivalents in the acetyl-Coenzyme A synthetase reaction) and from the necessity to synthesize biosynthetic precursors via the glyoxylate cycle and gluconeogenesis. The difference in biomass yield between ethanol and acetate can be attributed to two factors: (i) energy-dependent uptake of acetate via a proton symport mechanism
Transcript levels and metabolic fluxes in *S. cerevisiae* (6;7) and (ii) the higher degree of reduction of ethanol, for which the ethanol and acetaldehyde dehydrogenases (Fig. 1, reactions 22 and 23, respectively) can yield NAD(P)H that can either be used for biosynthesis or yield ATP via oxidative phosphorylation.

**Flux distribution in central carbon metabolism.**

*In vivo* fluxes through central pathways in carbon metabolism were estimated by Metabolic Flux Analysis, using a stoichiometric model of the *S. cerevisiae* metabolic network. As *S. cerevisiae* is a eukaryote, this model took into account metabolic compartmentation by discriminating between reactions that occur in the yeast cytosol and in the mitochondrial matrix (36;37). To calculate intracellular fluxes, the model was fed with quantitative data on the biomass composition of *S. cerevisiae* (38) and with the substrate consumption and product formation rates observed in the carbon-limited chemostat cultures. The estimated fluxes of central carbon metabolism relevant for this study are summarized in Tables 2A and 2B.

With the exception of the sugar transport and maltose hydrolysis steps, there were only very few predicted changes in central carbon metabolism when either glucose or maltose was used as the carbon source (Tables 2A and 2B). The slight increase of catabolic fluxes in maltose-grown cultures was due to the ATP requirement for maltose uptake (2). Larger changes were predicted between the C2 substrates ethanol and acetate. When ethanol is the carbon source, NADP-dependent acetaldehyde dehydrogenase can make an important contribution to fulfill the cellular demand for NADPH (Table 2A). In contrast, in acetate-grown cultures, this important reduced cofactor has to be regenerated by NADP-dependent isocitrate dehydrogenase and possibly by the pentose-phosphate pathway (39). Furthermore, the lower degree of reduction of acetate and the associated lower yield of reducing equivalents during its dissimilation result in a lower ATP yield and necessitate higher fluxes through dissimilatory pathways. This was reflected by a substantially higher predicted *in vivo* activity of the TCA cycle in acetate-grown cells (Table 2B).
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Major metabolic rearrangements were predicted when ethanol or acetate replaced glucose or maltose as the sole carbon source for growth of \textit{S. cerevisiae}. Predicted fluxes through acetyl-Coenzyme A synthetase (Fig. 1, reaction 24) were low in glucose-grown cultures, where this enzyme is only required for the provision of relatively small amounts of cytosolic acetyl-Coenzyme A that are needed for lipid and lysine biosynthesis (40;41). In contrast, predicted fluxes through this enzyme were high in ethanol and acetate-grown cultures (Table 2A). Another important difference between growth on sugars and growth on C2-compounds is the involvement, for the latter substrates, of the glyoxylate cycle (Fig. 1, reactions 14, 15, 25, 26 and 21) and gluconeogenesis (steps 27 and 28). Furthermore, growth on C2-compounds led to a dramatic increase of predicted fluxes through the TCA cycle relative to sugar-grown cultures (Table 2B). Conversely, the flux through glycolysis was reversed (gluconeogenesis) and much lower in ethanol- and acetate-grown cells. Finally, the predicted flux through the oxidative pentose-phosphate pathway was reduced, as NADP-dependent acetaldehyde and/or isocitrate dehydrogenases provide alternative sources of NADPH during growth on C2-compounds.

Global transcriptome changes in chemostat cultures limited for different carbon sources. Independent triplicate chemostat cultures were run for each carbon-limitation, followed by genome-wide transcriptional analysis with oligonucleotide DNA microarrays. Consistent with the excellent reproducibility reported in earlier studies in which DNA microarray analysis was applied to chemostat cultures (30,32), the average coefficient of variation for the independent triplicate analyses did not exceed 0.18 (Table 3). Furthermore, the levels of \textit{ACT1} and \textit{PDA1} transcripts, which are commonly applied as loading standards for conventional Northern analysis, were not significantly different for cultures grown on different growth-limiting carbon sources (Table 3). The lowest measurable signal was 12 (arbitrary units) in all conditions (Table 3). 833 transcripts (13.7\% of the genome) remained below this detection limit for all four growth-limiting carbon sources tested.
As few as 180 genes (only 2.8% of the genome) were carbon-source responsive as defined by a significant change in the transcription profile (fold-change greater than 2 and false discovery rate of 1%, see Material and Methods section). This number seems low, especially when considering the major rearrangements in metabolism that have to occur when, for example, ethanol replaces glucose as the sole carbon source for growth (Table 1). As shown on Fig. 2, many of the carbon-source responsive genes (72 genes, 40%) have not yet been assigned a biological function as defined by MIPS³ (42). A significant number (50 genes, 28%) were found to be related to carbon metabolism encoding enzymes (35 genes), transporters (six genes) or proteins involved in regulation (nine genes). Finally, 11 genes (6%) were involved in nitrogen metabolism.

**Specific transcriptional responses to growth-limiting carbon sources.**

To analyze the specific transcriptional response of *S. cerevisiae* to the four growth-limiting carbon sources, the transcriptome data were subjected to hierarchical cluster analysis. This resulted in 6 distinct clusters (Fig. 3). Most of the C2-responsive genes found in the present study (117 out of 180) were present in clusters 2 and 5 (Fig. 3) which yielded different transcript levels for sugar-(glucose or maltose)-limited growth and for growth limited by C2-compounds (ethanol or acetate), respectively. The other clusters consisted of genes that specifically responded to a single growth-limiting carbon source: low expression under acetate limitation (cluster 1, 16 genes), high expression under glucose limitation (cluster 3, 13 genes), high expression under maltose limitation (cluster 4, 16 genes) and, finally, low expression under maltose limitation (cluster 6, 18 genes). No genes were found with a specific response to growth under ethanol limitation, or with a specifically increased transcript level in acetate- or glucose-limited cultures. The 6 clusters that were identified will be briefly discussed below.

³ Munich Information Center for Protein Sequences. http://mips.gsf.de/
Different transcript levels in sugar and C2-compound-limited cultures (Clusters 2 and 5)

Of the 117 genes that yielded different transcript levels in sugar-limited cultures and cultures limited by either ethanol or acetate, 79 were up-regulated (cluster 5) and 38 down-regulated (cluster 2) in cultures limited by the C2-compounds. Among the up-regulated transcripts, 21 encoded enzymes or regulatory proteins related to carbon metabolism and included the four structural genes for gluconeogenesis and glyoxylate cycle enzymes: \textit{PCK1} and \textit{FBP1} (encoding the gluconeogenic enzymes phosphoenol-pyruvate carboxykinase and fructose-1,6-bisphosphatase) and \textit{ICL1} and \textit{MLS1} (encoding the glyoxylate-cycle enzymes isocitrate lyase and malate synthase, respectively). A further seven of these genes encoded enzymes or subunits active in the TCA cycle. This included five cytosolic enzymes (\textit{IDH1}, \textit{IDH2}, \textit{FUM1}, \textit{SDH1}, \textit{SDH3}) and two mitochondrial enzymes (\textit{CIT2}, \textit{MDH2}) that were already known to be up-regulated during growth on non-fermentable carbon sources in batch cultures (43). Five additional genes encoded enzymes involved in acetyl-Coenzyme A metabolism and its trafficking across intracellular membranes (\textit{ACS1}, \textit{ACH1}, \textit{CAT1}, \textit{YAT1}, \textit{YAT2}), consistent with the key role of this intermediate in the metabolism of C2-compounds. Three transcripts that showed an increased level in cultures limited by C2-compounds are involved in transcriptional regulation of carbon metabolism: \textit{SIP4}, a transcriptional activator of gluconeogenic genes, \textit{SIP2} and \textit{REG2}. Finally, \textit{RKI1} (encoding ribose-phosphate isomerase, involved in the pentose-phosphate pathway), \textit{INO1} (encoding inositol-1-phosphate synthase) and \textit{ICL2} (encoding a 2-methylisocitrinate lyase involved in propionate metabolism) transcripts were also up-regulated in the presence of ethanol or acetate.

Eight of the genes that showed increased transcript levels in cultures limited by C2-compounds encoded proteins involved in transport across the cytosolic and the mitochondrial membranes. In addition to the \textit{CAT1}, \textit{YAT1} and \textit{YAT2} genes mentioned above, two further up-regulated genes were involved in acetyl-CoA trafficking via the L-carnitine shuttle. \textit{AGP2} encodes a plasma membrane carnitine transporter, while the gene product of \textit{CRC1} transports acetylcarnitine across the mitochondrial inner membrane. Consistent with published results on batch cultures, \textit{SFC1} which encodes the mitochondrial
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succinate-fumarate exchanger, was expressed at increased levels in ethanol- and acetate-limited chemostat cultures (44). Surprisingly also *STL1* exhibited elevated transcript levels in ethanol- and acetate-limited cultures (*ca.* 4-fold higher compared to sugars). Although Stl1p has been described has a member of the hexose transporters family (45) because of its homology to these transport proteins (26 to 28% identity), its physiological role in sugar transport has not been clearly established. Its transcriptional induction by gluconeogenic carbon sources may indicate its involvement in the transport of other compounds than hexoses. Three further transporter-encoding genes (*DUR3, MEP2* and *SAM3*) involved in nitrogen metabolism, as well as four genes (*DAL2, DAL5, GDH3* and *GCV2*) encoding enzymes involved in nitrogen metabolism showed higher transcript levels during carbon-limited growth on C2-compounds. Despite the high specific rates of respiration in the ethanol- and acetate-limited cultures as compared to the glucose- and maltose-limited cultures (Table 1), only a single gene (*NDE2*) involved in respiration showed a significantly higher transcript level in cultures limited for the C2-compounds. The 31 other transcripts responding to these carbon sources had unknown or poorly described functions.

From the 38 genes down-regulated in the presence of ethanol and acetate (cluster 2), more than half (20 genes) have not yet been assigned a clear biological role. Remarkably from the remaining 18, ten were linked to carbon metabolism: two glycolytic genes (*HXK1, TDH1*), four genes from the pentose-phosphate pathway (*TKL2, GND1, GND2* and *SOL3*), *VID24* encoding a protein involved in fructose-6-bisphosphate vacuolar transport and degradation, two members of the hexose transport family (*HXT2, HXT7*) and finally *MTH1*, involved in glucose signaling and repression (46). Surprisingly one gene (*CYC7*) encoding iso-2-cytochrome c and involved in respiration was repressed, while respiration rate was increased in cells grown with C2-compounds compared to sugars (Table 1). Among the remaining seven genes were *BAP2*, a branched-chain amino-acid permease, *SPS100* and *SWM1* both involved in sporulation, *FDH1* and *FDH2* encoding formate dehydrogenases of which precise role in yeast metabolism has not been clearly defined yet (47). Two other down-regulated genes respond to stress...
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conditions, *DOG2* responds to oxidative and osmotic stress and *PDR12* is involved in weak organic acid resistance.

*Up and down-regulation in response to maltose*

A set of 34 genes specifically responded to growth with maltose. Out of the 16 up-regulated transcripts, six had poorly described biological functions, four encoded ribosomal proteins (*RPS10A*, *RPS26B*, *RPL15B*, *RPL31B*), and six coded for proteins involved in maltose utilization. Genes necessary for maltose degradation (*i.e.* maltose permeases *MALx1*, maltases *MALx2* and maltose transcription activators *MALx3*) are clustered on *MAL* loci carried by subtelomeric regions (48). *S. cerevisiae* strains contain up to five highly homologous *MAL* loci and one locus is enough to sustain growth on maltose (4). Two *MAL* loci have been sequenced in *S. cerevisiae* S288C, the strain used for the genome sequencing program, although this yeast is not able to grow on maltose as the sole carbon source (49;50). Our laboratory strain, like all members from the CEN.PK family, can grow on maltose and contains four *MAL* loci. The Affymetrix microarrays contain four probe-sets that are specific to known *MAL* genes (*MAL11*, *MAL13*, *MAL33* and *MAL23*) and two that can hybridize to several homologues (*MALx2*, *MALx1*). As expected, genes encoding for maltose permeases and maltases were strongly induced in the presence of maltose (six to ten-fold), as did *FSP2*, *YJL216C* and *YGR287C*, genes sharing high homology with maltases. Despite their recent identification as *α*-glucoside transporters-encoding genes and their reported induction in the presence of maltose (51), two maltose permease homologues, *YDL247W* (*MPH2*) and *YJL160C* (*MPH3*), were not up-regulated in maltose-limited chemostats. Although their presence in CEN.PK background has been detected by genome-wide genotyping (52), their expression at a very low level in CEN.PK113-7D (average signal intensity below 50) may indicate a strain-specific regulation. Among the three genes coding for the maltose regulator, only *MAL13* and *MAL33* transcripts were detected, *MAL13* displaying a very low transcript level. Surprisingly, none of these transcripts were induced in the presence

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of maltose, pointing towards a probable control of their activation properties at the post-transcriptional level.

Of the 18 genes down-regulated in the presence of maltose, most have not been assigned a function yet. The remaining genes cover a broad range of functional categories (budding, protein processing, DNA repair, cell wall maintenance, etc.) and could not be directly linked to maltose utilization.

*Down-regulation in response to acetate*

As few as 16 transcriptional changes were observed between cells grown on ethanol or acetate, and all of them were down-regulations in the presence of acetate. Among these changes five were related to carbon metabolism: *ALD5, PFK26, GPH1, YNL134C* and *SOL4*. *ALD5*, encoding a mitochondrial minor isoform of acetaldehyde dehydrogenase, has previously been shown to be induced in the presence of ethanol (53). In apparent contrast, genome-wide transcription analysis indicated a mild repression of its expression after the diauxic shift (43). The specific physiological role of this acetaldehyde dehydrogenase isoenzyme has not been elucidated (54) and a proposed role in maintenance of the electron transport chain (53) does not shed light on its down-regulation in the presence of acetate. *PFK26* codes for a 6-phosphofructokinase catalyzing fructose-2,6-bisphosphate production. This metabolite has been shown to activate phosphofructokinase (Fig. 1, step 3) and to inactivate fructose-1,6-bisphosphatase (Fig. 1, step 28), although its role in the switch between glycolysis and gluconeogenesis remains unclear (55;56). This significant down-regulation of *PFK26* in the presence of acetate (2.3-fold) and minor down-regulation (1.6-fold) in the presence of ethanol are however indicating a transcriptional control of *PFK26* by carbon source in carbon-limited cultures. This may be relevant for controlling the *in vivo* fluxes through the antagonistic enzymes phosphofructokinase and fructose-1,6-bisphosphatase. *YNL134C* is a member of the zinc-binding dehydrogenases family which catalyzes the reversible oxidation of ethanol to acetaldehyde. This glucose-repressed gene (31;57) is the only alcohol dehydrogenase-encoding ORF displaying a
significant change in expression in response to carbon source identified in this study, however its physiological role has not been clearly identified so far. Finally \(GPH1\) encodes a glycogen phosphorylase involved in glycogen degradation (58), and \(SOL4\) expression product is a 6-phosphogluconolactonase involved in the pentose-phosphate pathway.

No transcripts related to acidic stress response were up-regulated, confirming that acetate-limitation efficiently resolved acid stress problems that are typically observed in batch cultures. Furthermore, three genes responding to acid and/or oxidative stress were down-regulated (\(MSC1\), \(AHP1\), \(GAD1\)).

**Up-regulation in response to glucose**

A puzzling cluster is the small subset of 13 transcripts that were specifically up-regulated when \(S.\ cerevisiae\) was grown with glucose as the sole carbon source. Six genes have unknown or poorly described functions. Four genes (\(SUC2\), \(SUC4\), \(HXT4\) and \(GIP2\)) are involved in carbon metabolism. Invertase, encoded by \(SUC\) homologues, is a well-described target of catabolite repression by glucose, both at the level of transcription and mRNA stability (59). However, it has been shown that low concentrations of glucose (0.1%) are necessary for a maximum expression of \(SUC\) genes (60). These findings are in good agreement with the significantly higher expression level of \(SUC2\) and \(SUC4\) (three to four-fold) measured when cells were grown in glucose compared to maltose, ethanol or acetate. \(HXT4\), encoding a moderate to low affinity hexose transporter, was also responding to low extracellular glucose concentration in our chemostat cultivations. The up-regulation of \(GIP2\) (protein phosphatase PP1 interacting protein), as well as \(GNP1\) (glutamine permease), \(SKN1\) (glucan synthase subunit) and \(MCH5\) (member of the major facilitator superfamily) in the presence of glucose does not have a clear physiological relevance.
**In silico promoter analysis of carbon source-regulated genes**

Co-regulation of global transcription is generally controlled by the specific binding of common activating or repressing proteins (transcription factors) to short sequences contained in promoter regions of the regulated genes. Searching the promoter regions of co-regulated genes for over-represented short sequences can identify these binding sites. We analyzed the upstream sequences of the genes from the six clusters defined above using web-based tools (for further information see Material & Methods Section). No significantly over-represented sequences were recovered from three clusters, namely low expression on acetate, high expression with glucose and low expression with maltose (Table 4). 11 putative promoter elements were over-represented in the three remaining clusters, of which only four could be associated to known transcription factors.

Among the promoter regions of the genes down-regulated in the presence of ethanol and acetate, three sequences were found to be over-represented compared to their genome coverage. One of them resembles the binding site targeted by the transcription factors Msn2p and Msn4p known as stress response element (STRE, Table 4). However, the down-regulation of Msn2p/Msn4p regulated genes seems unlikely as, in our experiments, cells were not exposed to any stress known to trigger the activity of these factors (reviewed by Estruch (61)). Furthermore, a comparison of the genome-wide and cluster 2-wide coverage of the core sequence of the STRE (AGGGG) did not show any significant over-representation of this element among the genes down-regulated with C2-carbon sources. This renders it unlikely that the recovered sequence is a ‘true’ STRE element.

The induction of genes necessary for maltose utilization (MALx1 and MALx2) requires the activation by the transcription factor Malx3p (48). It was therefore expected that one of the binding sites targeted by Malx3p would be over-represented in the promoter region of genes up-regulated when *S. cerevisiae* is grown in the presence of maltose. Indeed, a 10-nt sequence with significant homology to the Mal63p binding site (62) was retrieved from cluster 4 (Table 4). Surprisingly, three other sequences were
significantly over-represented (4 to 53 fold compared to genome coverage) in this relatively small cluster, but could not be associated to any known DNA-binding protein (Table 3).

Four rather well conserved sequences were recovered from the promoter regions of genes up-regulated in the presence of C2-carbon sources (cluster 5). Two of them could be identified as targets of known transcription factors: Mig1p and Cat8p/Sip4p (Table 4).

In glucose-grown batch cultures, many genes involved in the utilization of alternative carbon sources are repressed via a complex of signals known as carbon catabolite repression (10;63). Glucose repression of transcription is mainly exerted via the Mig1p binding protein. This is consistent with the finding that 24 genes (30%) up-regulated when S. cerevisiae is grown with acetate or ethanol contain a Mig1p binding site in their promoter region. From this set, most of the 18 genes with known function have previously been experimentally shown to contain a Mig1p binding site (REG2 (64), FBP1 (65), ICL1 (66)) or at least to be repressed by glucose (ACH1, MDH2, ICL1, NDE2, YAT1, CAT2, YAT2, CRC1, SFC1, SDH1 (31;67-69)). MIG1 transcription itself did not respond to carbon source, which is consistent with previous reports of Mig1p activity control by post-translational modification and nuclear export (70;71).

An important cis-acting element for the transcriptional response to gluconeogenic carbon sources is Cat8p (for review see (72)). Cat8p binds to an 11-bp upstream activation sequence element named CSRE (carbon-source responsive element) and most recently defined as CCrTysmCCG (r = A or G, y = C or T, S = C or G, n = A, T, G or C) (73). Most of the genes that have been shown to contain functional Cat8p binding sites were indeed up-regulated in response to growth with C2-carbon sources (SIP4, FBP1, ICL1, MLS1, PCK1, MDH2, SFC1, CAT2). Furthermore, screening the upstream untranslated regions of all genes up-regulated in the presence of C2-compounds allowed the identification of 20 additional genes containing one or more CSRE-related sequences (Table 5). Contrary to most of its targets, CAT8 itself was not affected by the carbon source (Fig. 3 and Fig. 4). This behavior is consistent with earlier reports of Cat8p activation at the post-translational level by phosphorylation (74).
No other binding sites corresponding to transcription factors known to be involved in carbon-source adaptation (Adr1p, Hap complex, Rtg regulators) could be recovered from this set of C2-compound co-regulated genes. *ACS1* and *ADH2* the main targets of Adr1p (75;76) were not significantly up-regulated in our culture conditions, and were therefore not included in the promoter analysis. Similarly, few of the potential targets of the Hap complex and the Rtg regulators (*SDH1, CIT2, IDH1, IDH2*) were up-regulated in this study and could therefore not result in a significant over-representation of their binding motifs in the cluster.
Discussion

Transcription analysis of chemostat cultures vs. batch cultures

In carbon-limited, aerobic chemostat cultures, remarkably few transcripts exhibited significant differences when the growth-limiting carbon source was changed. The change from a sugar (glucose or maltose) to a C2- compound (ethanol or acetate) resulted in only 117 genes with a significantly changed transcript level (Fig. 3). This robustness of the yeast transcriptome in response to changes of the carbon source is in contrast with previously reported data from batch cultures. In a transcriptome analysis of the diauxic shift in *S. cerevisiae*, which essentially represents the transition from growth on glucose to growth on ethanol, over 400 transcripts were found to change by more than two-fold (43). Similarly, in an independent study on glucose- and ethanol-grown batch cultures, over 600 transcripts were found to change (77). When comparing the carbon-source response in chemostat with these previous reports in batch cultivation, about half of the 117 genes that responded to C2-compounds in chemostat cultivations, were also transcriptionally regulated during these two transcriptome studies.

Several factors may have contributed to these different carbon-source-dependent transcriptional responses in batch and chemostat cultures. A large number of genes (225 based on work in our laboratory (31)) is transcriptionally regulated by glucose, for instance via glucose-catabolite repression. This phenomenon does not occur in glucose limited chemostat cultures, where the low residual glucose concentration (*ca.* 0.1 mM measured using a fast sampling technique (78)) prevents glucose catabolite repression. This absence of glucose repression is supported by our data: the transcript levels of several genes that are known to be regulated by glucose repression (*GAL* (79) and *MAL* (80) for instance) were clearly not further de-repressed when *S. cerevisiae* was grown with non-fermentable carbon sources. Another factor that may have influenced the transcriptional response in batch cultures is the toxicity of substrates and/or products. This is perhaps best illustrated by acetate. This weak acid, which is a normal metabolite of *S. cerevisiae* in glucose-grown batch cultures, uncouples the pH gradient across the yeast plasma membrane.
and is therefore likely to result in transcriptional stress responses (18). Production of ethanol and acetate
does not occur in aerobic, glucose-limited chemostat cultures and, when these compounds are used for
carbon-limited chemostat cultivation, their residual concentration is below 0.5 mM and therefore unlikely
to induce any stress responses. An additional factor that complicates interpretation of the data from the
classical study of DeRisi et al. on the diauxic shift (43) is the nature of the culture medium. This deRisi
study was performed with a complex medium, in which the yeast not only has to change carbon source at
the diauxic shift, but also has to sequentially utilize the many nitrogen sources that are present in the
medium. This complication does not influence the data from our chemostat study, in which ammonium
ions were the sole nitrogen source. Finally, whereas the specific growth rate drastically decreases after the
diauxic transition in batch cultures, our transcriptional analysis was performed at a fixed specific growth
rate. All these differences between chemostat and batch cultivation make the former a powerful tool to
study the influence of one parameter only, i.e., carbon source, without the inherent interferences that
occur in batch cultures. Unlike chemostat studies, carbon-source response studies performed in batch
cultivation can therefore not discriminate between carbon source de-repression and induction
mechanisms. In the present study, three different transcriptional responses to carbon source could be
identified. i) A strong C2-induction in addition to a de-repression, as observed for most of the Cat8-
dependent genes (FBP1, MLS1, ICL1, PCK1, SFC1, SIP4, Fig. 4A). ii) A strong induction by non-
fermentable carbon sources without glucose de-repression, as displayed by CIT2 and YAT1 (both involved
in acetyl-CoA metabolism, Fig. 4A). This glucose insensitivity of CIT2 is consistent with previous reports
(81), whereas contradictory reports of YAT1 repression by glucose may indicate a strain-specific response
of YAT1 to catabolite repression (68;81). iii) A more surprising set of genes, that had been so far
described as induced by C2-compounds (43;43;82-84), were strongly de-repressed under glucose-
limitation, but did not show any further significant induction in the presence of ethanol or acetate (ALD4,
JEN1, IDP2, ACS1, ADH2, Fig. 4B).
Carbon-source dependent transcripts and regulation mechanisms.

Carbon metabolism in *S. cerevisiae* is one of the most intensively studied metabolic systems. Indeed, in many cases, the carbon-source-dependent transcription of genes could be teleologically explained from the known catalytic or regulatory functions of their gene products. Some obvious examples include the high transcript levels of glyoxylate-cycle and gluconeogenic genes during growth on C2-compounds and the induction of MAL genes in maltose-limited chemostat cultures. However, in many other cases, the function of carbon-source-responsive genes is either entirely unknown (the frequency of genes with unknown function was 40%, which is significantly higher than that of the entire yeast genome (27% according to YPD™)) or difficult to interpret in terms of biochemical function of the gene product. Although there is no *a priori* proof that carbon-source-dependent transcriptional regulation correlates with physiological function, our data provide an interesting lead for future functional analysis research.

As expected, very few transcriptional differences were measured between cultures grown on glucose and maltose (34 changes). The MAL structural genes (maltose permeases and maltases), but not the MAL activators, were up-regulated in the presence of maltose. Analysis of the upstream region of these co-regulated genes resulted in the identification of the MAL regulator binding site (85) (Table 4). Cultivation on maltose however generated a puzzling set of 18 genes down-regulated compared to glucose cultivations. These changes suggest that maltose utilization, despite its closeness to glucose utilization, results in more metabolic perturbations than is generally recognized. Comparing the transcriptomes of ethanol or acetate-grown cells resulted in even less differences. As few as 16 genes yielded different transcript levels for these two gluconeogenic compounds, all being down-regulated with acetate. Acetate uptake is mediated by a protein which encoding gene has not been identified so far (6;7). Unfortunately no potential candidate for an acetate transporter could be identified from our dataset. Finally, comparing C2-compound-limited cultures with sugars-limited cultures resulted in the identification of 117 carbon-source responsive genes. Among these genes, a high proportion have unknown or poorly defined biological functions (48%), but as many as half of the remaining (34 genes, 29%) are involved in carbon
metabolism. The promoter analysis of co-regulated genes resulted in the definition of a new set of genes containing one or more sequences closely related to the carbon source responsive element (Table 5). Using the totally different approach of integrating transcriptome and proteome comparison of a wild-type and a cat8Δ S. cerevisiae strain, Haurie and coworkers (86) also proposed a list of Cat8p-dependent genes containing a CSRE-related binding site. Their gene list largely overlaps the list proposed in this work. From the set of C2-compounds up-regulated genes could also be identified a large number of genes containing a Mig1p binding site, which is consistent with the role of Mig1p in the repression of the genes involved in the utilization of alternative carbon sources. Two additional over-represented promoter elements were recovered from this set of genes that could not be related to any known transcription factor.

A recent study on FBP1 (87) nicely exemplified the potential complexity of carbon-source control of gene expression and strongly suggests, together with earlier studies (9;74), the involvement of additional, still unidentified transcription factors. The putative promoter elements identified in the present study could be the targets of these additional carbon-source regulators.

**Transcript levels versus metabolic fluxes**

From a combination of metabolic flux analysis and transcriptome analysis, we were able to compare metabolic fluxes in central carbon metabolism and transcript levels of key structural genes that encode enzymes of the central metabolic pathways (Tables 2A and 2B). Three distinct types of correlation between transcript levels and fluxes could be identified.

For the pathways that are specific for maltose metabolism (maltase) or for the metabolism of gluconeogenic carbon sources (gluconeogenesis and glyoxylate cycle), there was a strong qualitative correspondence between transcriptional regulation and estimated in vivo metabolic fluxes. The genes involved in these pathways are known to be strongly transcriptionally regulated, but several of the corresponding enzymes are also subject to post-translational regulation (inactivation by phosphorylation and/or ubiquitin-catalyzed degradation (88;89)). The time scale of these post-translational processes is
much shorter than that of transcriptional regulation. Although one would intuitively assume that post-translational processes are predominantly important under dynamic conditions, we cannot presently exclude the possibility that they also contribute to the regulation of in vivo enzyme activity during carbon-limited steady-state cultivation in chemostat cultures.

The changes in metabolic fluxes in the TCA cycle and in the pentose-phosphate pathway were only partially mirrored by changes in transcript levels. Little is known about the regulation of these two pathways. From our data it appears clearly that in order to meet the new flux requirements when C2 carbon sources are used, S. cerevisiae only enhanced or repressed the transcription of a few genes. One can assume that only the rate controlling steps need to have their protein concentration optimized in order to adjust the flux to the new requirement. In such a case, transcriptome analysis would help to identify the potential rate limiting steps i.e. succinate dehydrogenase and fumarase for the TCA cycle (CIT2 and MDH2 induction mainly reflecting the need of the corresponding proteins in a different compartment rather than a real rate-limiting step) and 6P-gluconolactonase, 6P-gluconate dehydrogenase, ribose-P isomerase and transketolase 2 for the pentose-phosphate pathway. However, the magnitude of change in transcript levels does not correlate well with the magnitude of change in metabolic fluxes, indicating that transcriptional control alone cannot explain the modifications in the flux distribution in the TCA cycle and in the pentose-phosphate pathway.

The model-predicted fluxes in the glycolytic pathway and in the enzymic reactions surrounding pyruvate were strongly dependent on the studied carbon sources. However, the different in vivo activities of the key enzymes of these pathways were not at all mirrored by their transcript levels. In glycolysis, only HXK1 (encoding hexokinase I) and TDH1 (encoding a minor isoform of glyceraldehyde dehydrogenase) displayed a reduced transcription level when the glycolytic flux was decreased at least three-fold. This is a clear indication that, during carbon-limited cultivation, fluxes through these central metabolic pathways in S. cerevisiae are not primarily controlled at the transcriptional level. Further research is required to assess the contribution of translational efficiency, post-translational modification
and regulation by intracellular concentrations of substrates, products and effectors to the regulation of in vivo activity of these pathways. Such discrepancies between fluxes and/or enzyme activities and transcript levels have already been reported by other integrative approaches with S. cerevisiae (90) and several bacteria (91-93).

Our study underlines that DNA microarrays, however useful for studying transcriptional regulation, comparative genotyping and purely correlation-based diagnostics, have limited value as indicators for in vivo activity of proteins. This limitation should be considered when applying DNA-microarrays as a tool for activities such as metabolic engineering or identification of potential drug targets.

Acknowledgements

The research group of Prof. J.T. Pronk is part of the Kluyver Centre for Genomics of Industrial Fermentation, which is supported by the Netherlands Genomics Initiative. This work was also supported by a STW grant and by a Marie-Curie fellowship to P. Daran-Lapujade. We thank H.C. Lange for providing the compartmented stoichiometric model. We also thank L.M. Serrano, M. de Herdt and J.M.A. Geertman for technical assistance and Prof. J.P. van Dijken for critically reading the manuscript.
Reference list


Transcript levels and metabolic fluxes in *S. cerevisiae*


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Table 1
Carbon source concentrations and physiological parameters of cultures used in this study

Data represent the average and S.D. of three separate chemostat cultivations grown to steady states at D = 0.1 h\(^{-1}\). Residual substrates in the culture medium were below detection limit.

<table>
<thead>
<tr>
<th>Carbon source in feed</th>
<th>Carbon source concentration</th>
<th>(Y_{sx})(^{a})</th>
<th>(^{b})q_{carbon source}</th>
<th>(^{b})q_{O2}</th>
<th>(^{b})q_{CO2}</th>
<th>RQ(^{b})</th>
<th>Carbon recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>41.4 ± 0.2</td>
<td>14.8 ± 0.0</td>
<td>1.15 ± 0.02</td>
<td>2.74 ± 0.03</td>
<td>2.85 ± 0.04</td>
<td>1.04 ± 0.02</td>
<td>97 ± 1</td>
</tr>
<tr>
<td>Maltose</td>
<td>19.6 ± 0.8</td>
<td>13.6 ± 0.3</td>
<td>0.61 ± 0.02</td>
<td>3.05 ± 0.18</td>
<td>3.05 ± 0.17</td>
<td>1.02 ± 0.01</td>
<td>97 ± 3</td>
</tr>
<tr>
<td>Ethanol</td>
<td>131.6 ± 3</td>
<td>13.4 ± 0.3</td>
<td>3.78 ± 0.06</td>
<td>6.87 ± 0.15</td>
<td>3.26 ± 0.04</td>
<td>0.47 ± 0.01</td>
<td>95 ± 2</td>
</tr>
<tr>
<td>Acetate</td>
<td>139.5 ± 4</td>
<td>8.4 ± 0.0</td>
<td>5.89 ± 0.09</td>
<td>7.4 ± 0.23</td>
<td>7.45 ± 0.18</td>
<td>1.01 ± 0.00</td>
<td>96 ± 2</td>
</tr>
</tbody>
</table>

\(^{a}\) Yield of biomass (g of dry biomass formed per mole of carbon source consumed)

\(^{b}\) Respiratory coefficient (\(^{b}\)q_{CO2}/\(^{b}\)q_{O2})
Table 2A: Flux distribution and transcript levels in *S. cerevisiae* grown in chemostat cultivation under maltose, glucose, ethanol and acetate limitation in glycolysis and metabolic steps around pyruvate. In gray background are indicated significantly up-regulated transcripts and increased fluxes. Significantly down-regulated transcripts and decreased fluxes are underlined.

### MALTOSE UTILIZATION

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Fluxes (mol/Cmol/h)</th>
<th>Gene</th>
<th>Transcripts (hybridisation intensities)</th>
<th>Loc^c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>Maltose</td>
<td>Ethanol</td>
<td>Acetate</td>
</tr>
<tr>
<td>29 Maltase</td>
<td>30.9 ± 0.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FSP2</td>
<td>74 ± 13</td>
<td>641 ± 87</td>
</tr>
<tr>
<td></td>
<td></td>
<td>YIL1216C</td>
<td>19 ± 7</td>
<td>165 ± 61</td>
</tr>
<tr>
<td></td>
<td></td>
<td>YGR287C</td>
<td>30 ± 1</td>
<td>66 ± 4</td>
</tr>
</tbody>
</table>

### GLYCOLYSIS

- **1 Hexokinase**: 29.6 ± 0.2
- **2 Glucose-6P isomerase**: 12.7 ± 0.1
- **3 Phosphofructokinase**: 18.8 ± 0.1
- **4 Fructose-1,6P aldolase**: 18.8 ± 0.1
- **5 Triose-P isomerase**: 18.5 ± 0.1
- **6 G3P dehydrogenase**: 40.1 ± 0.3
- **7 3P-glycerate kinase**: 40.1 ± 0.3
- **8 P-glycerate mutase**: 38.4 ± 0.3
- **9 Enolase**: 38.4 ± 0.3
- **10 Pyruvate kinase**: 37.2 ± 0.3

### PYRUVATE BRANCHPOINT

- **11 Pyruvate dehydrogenase**: 21.6 ± 0.2
- **12 Pyruvate carboxylase**: 5.3 ± 0.1
- **13 Pyruvate decarboxylase**: 5.9 ± 0.1
- **14 Alc. dehydrogenase, C**: 0
- **15 Alc. dehydrogenase, M**: 0
- **16 Acetaldehyde dehydrogenase, NAD**: 0
- **17 Acetyl-CoA synthase**: 6.1 ± 0.1

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*a* Sub-cellular localization of the protein is indicated by: C: cytosolic, M: mitochondrial, P: peroxisomal, NC: non-cytosolic, NM: non-mitochondrial.

*b* The number preceding the enzyme name corresponds to its metabolic step number on Fig. 1.

*c* Fluxes are expressed as biomass specific conversion rates, that is, as mol of reactant converted per Cmol of biomass (i.e. the amount of biomass containing one mol of carbon) per hour.
Table 2B: Flux distribution and transcript levels in *S. cerevisiae* grown in chemostat cultivation under maltose, glucose, ethanol and acetate limitation in the TCA cycle, the glyoxylate cycle, gluconeogenesis and the pentose-phosphate pathway. In gray background are indicated significantly up-regulated transcripts and increased fluxes. Significantly down-regulated transcripts and decreased fluxes are underlined.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Fluxes (mol/Cmolv)</th>
<th>Gene</th>
<th>Transcripts (hybridization intensities)</th>
<th>Loc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>Maltose</td>
<td>Ethanol</td>
<td>Acetate</td>
</tr>
<tr>
<td>TCA CYCLE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 Citrate synthase, M</td>
<td>21.6 ± 0.2</td>
<td>24.5 ± 0.7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Citrate synthase, C</td>
<td>0</td>
<td>0</td>
<td>59.3 ± 0.7</td>
<td>114.8 ± 1.4</td>
</tr>
<tr>
<td>15 Aconitase</td>
<td>21.6 ± 0.2</td>
<td>24.5 ± 0.7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>16 Isocitrate dehydrogenase NAD</td>
<td>19.2 ± 0.2</td>
<td>22.1 ± 0.7</td>
<td>30 ± 0.7</td>
<td>54.6 ± 1.7</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase NADP, C</td>
<td>0</td>
<td>0</td>
<td>31 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>17 α-ketoglutarate dehydrogenase</td>
<td>18.7 ± 0.2</td>
<td>21.7 ± 0.7</td>
<td>29.5 ± 0.7</td>
<td>85.1 ± 1.5</td>
</tr>
<tr>
<td>18 Succinyl-Co A ligase</td>
<td>18.7 ± 0.2</td>
<td>21.7 ± 0.7</td>
<td>29.5 ± 0.7</td>
<td>85.1 ± 1.5</td>
</tr>
<tr>
<td>19 Succinate dehydrogenase</td>
<td>18.7 ± 0.2</td>
<td>21.7 ± 0.7</td>
<td>56.2 ± 0.7</td>
<td>116.1 ± 1.4</td>
</tr>
<tr>
<td>20 Fumarase</td>
<td>1 ± 0</td>
<td>1 ± 0</td>
<td>27.7 ± 0.3</td>
<td>27.6 ± 0.4</td>
</tr>
<tr>
<td>21 Malate dehydrogenase, M</td>
<td>21.9 ± 0.2</td>
<td>24.8 ± 0.7</td>
<td>0.3 ± 0</td>
<td>0.3 ± 0</td>
</tr>
<tr>
<td>Malate dehydrogenase, NM</td>
<td>-2.1 ± 0</td>
<td>-2.1 ± 0</td>
<td>83.7 ± 0.7</td>
<td>138.9 ± 1.4</td>
</tr>
<tr>
<td>GLYOXYLATE CYCLE and GLUCONEOGENESIS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 Isocitrate lyase</td>
<td>0</td>
<td>0</td>
<td>26.7 ± 0.2</td>
<td>26.5 ± 0.4</td>
</tr>
<tr>
<td>26 Malate synthase</td>
<td>0</td>
<td>0</td>
<td>26.7 ± 0.2</td>
<td>26.5 ± 0.4</td>
</tr>
<tr>
<td>27 PEP carboxykinase</td>
<td>0</td>
<td>0</td>
<td>21.1 ± 0.2</td>
<td>20.5 ± 0.3</td>
</tr>
<tr>
<td>28 Fructose-bisphosphatase</td>
<td>0</td>
<td>0</td>
<td>6.2 ± 0.1</td>
<td>5.7 ± 0.1</td>
</tr>
<tr>
<td>PENTOSE-PHOSPHATE PATHWAY</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 Glucose-6P dehydrogenase</td>
<td>10.2 ± 0.1</td>
<td>10.1 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>31 6P-glucuronatase</td>
<td>10.2 ± 0.1</td>
<td>10.1 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>33 Transketolase 1</td>
<td>3.3 ± 0</td>
<td>3.3 ± 0</td>
<td>0.1 ± 0</td>
<td>0.1 ± 0</td>
</tr>
<tr>
<td>34 Transaldolase</td>
<td>3.3 ± 0</td>
<td>3.3 ± 0</td>
<td>0.1 ± 0</td>
<td>0.1 ± 0</td>
</tr>
<tr>
<td>35 Transketolase 2</td>
<td>2.7 ± 0</td>
<td>2.7 ± 0</td>
<td>-0.7 ± 0</td>
<td>-0.7 ± 0</td>
</tr>
</tbody>
</table>

a Sub-cellular localization of the protein is indicated by: C: cytosolic, M: mitochondrial, P: peroxisomal, NC: non-cytoplasmic, NM: non-mitochondrial.

b The number preceding the enzyme name corresponds to its metabolic step number on Fig. 1. (NS: not shown on Fig. 1)

c Fluxes are expressed as biomass specific conversion rates, that is, as mol of reactant converted per Cmol of biomass (i.e. the amount of biomass containing one mol of carbon) per hour.

d Although found both in the cytosol and in the mitochondria, fumarase is encoded by one gene and one transcript only (94). According to recent findings its localization depends on the folding of the protein (95).
Table 3

Summary of microarray experiment quality parameters for each carbon limitation

<table>
<thead>
<tr>
<th>Culture limiting nutrient</th>
<th>Average coefficient of variation(^a)</th>
<th>(ACT1^b)</th>
<th>(PDAI^c)</th>
<th>Lowest “measurable” signal(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.13</td>
<td>2556 ± 157</td>
<td>488 ± 67</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>Maltose</td>
<td>0.18</td>
<td>3119 ± 446</td>
<td>385 ± 8</td>
<td>13 ± 5</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.17</td>
<td>3028 ± 449</td>
<td>499 ± 12</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>Acetate</td>
<td>0.14</td>
<td>2615 ± 103</td>
<td>406 ± 30</td>
<td>12 ± 6</td>
</tr>
</tbody>
</table>

\(^a\) Represents the average of the coefficient of variation (standard deviation divided by the mean) for all genes except the genes with mean expression below 12.

\(^b\) Encoding actin.

\(^c\) Encoding pyruvate dehydrogenase complex E1-alpha subunit

\(^d\) Corresponds to the signal from the open reading frame with the lowest reliably detectable abundance.
<table>
<thead>
<tr>
<th>Regulatory cluster</th>
<th>Promoter element</th>
<th>Putative binding protein</th>
<th>Gene coverage (%)</th>
<th>Genome coverage (%)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cluster 1 – Low expression on acetate</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cluster 2 – Low expression on ethanol and acetate</td>
<td>GsGkrrGGGG</td>
<td>Msn2p/Msn4p</td>
<td>16</td>
<td>1</td>
<td>(61)</td>
</tr>
<tr>
<td></td>
<td>AnhArnAGTwCT</td>
<td>?</td>
<td>26</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>wwGkCnmmGmAA</td>
<td>?</td>
<td>31</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Cluster 3 – High expression with glucose</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cluster 4 – High expression with maltose</td>
<td>bbTTTCGCns</td>
<td>Mal63p</td>
<td>60</td>
<td>8</td>
<td>(62), (85)</td>
</tr>
<tr>
<td></td>
<td>SCCnCdATCC</td>
<td>?</td>
<td>53</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CkyTmCsGym</td>
<td>?</td>
<td>53</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CmnCGTkTbb</td>
<td>?</td>
<td>60</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Cluster 5 – High expression with ethanol and acetate</td>
<td>CCnynmnCCG</td>
<td>Cat8p/Sip4p</td>
<td>35</td>
<td>8</td>
<td>(96)</td>
</tr>
<tr>
<td></td>
<td>CCCsGms</td>
<td>Mig1p</td>
<td>30</td>
<td>15</td>
<td>(87;97)</td>
</tr>
<tr>
<td></td>
<td>TCnGCrGCnAww</td>
<td>?</td>
<td>10</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>kCsGsGCsr</td>
<td>?</td>
<td>14</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Cluster 6 – Low expression with maltose</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*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 NS, no significant patterns retrieved.
Table 5: CSRE-related sites in the upstream region of the genes up-regulated in the presence of gluconeogenic carbon source.

<table>
<thead>
<tr>
<th>Systematic name</th>
<th>Standard name</th>
<th>Molecular function</th>
<th>CSRE sequence</th>
<th>CSRE localization</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gene with previously characterised CSRE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YJL089W</td>
<td><strong>SIP4</strong></td>
<td>TF of gluconeogenic genes</td>
<td>CCGTTTCGACCG</td>
<td>-275 to -265</td>
</tr>
<tr>
<td>YLR377C</td>
<td><strong>FBP1</strong></td>
<td>Fructose-1,6-bisphosphatase</td>
<td>CCAATCCGTTCG</td>
<td>-504 to -494</td>
</tr>
<tr>
<td>YER065C</td>
<td><strong>ICL1</strong></td>
<td>Isocitrate lyase</td>
<td>CCAATTCAATCCG</td>
<td>-398 to -388</td>
</tr>
<tr>
<td>YNL117W</td>
<td><strong>MLS1</strong></td>
<td>Malate synthase</td>
<td>CCAATTGGGCGCG</td>
<td>-500 to -490</td>
</tr>
<tr>
<td>YKR097W</td>
<td><strong>PCK1</strong></td>
<td>PEP carboxylkinase</td>
<td>CTTTCTACTCCG</td>
<td>-518 to -508</td>
</tr>
<tr>
<td>YOL126C</td>
<td><strong>MDH2</strong></td>
<td>Cytosolic malate dehydrogenase</td>
<td>CTTTATACCG</td>
<td>-262 to -252</td>
</tr>
<tr>
<td>YJR095W</td>
<td><strong>SFC1</strong></td>
<td>Mito. succinate-fumarate transporter</td>
<td>CCAATTAAACCCG</td>
<td>-491 to -481</td>
</tr>
<tr>
<td>YML042W</td>
<td><strong>CAT2</strong></td>
<td>Carnitine O-acetyltransferase</td>
<td>CTTTCTGCGCG</td>
<td>-281 to -271</td>
</tr>
<tr>
<td><strong>Proposed new CSRE-genes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YBL015W</td>
<td><strong>ACH1</strong></td>
<td>Acetyl-CoA hydrolase</td>
<td>CGACGCGCCCG</td>
<td>-433 to -423</td>
</tr>
<tr>
<td>YLR308W</td>
<td><strong>CDA2</strong></td>
<td>Chitin Deacetylase</td>
<td>CGACGCGCCCG</td>
<td>-430 to -420</td>
</tr>
<tr>
<td>YOR316C</td>
<td><strong>COT1</strong></td>
<td>Involved in cobalt accumulation</td>
<td>CGCTCACCCG</td>
<td>-261 to -251</td>
</tr>
<tr>
<td>YOR100C</td>
<td><strong>CRC1</strong></td>
<td>Mito. carnitine carrier</td>
<td>CGATGATCCCG</td>
<td>-258 to -248</td>
</tr>
<tr>
<td>YKL096W</td>
<td><strong>CWP1</strong></td>
<td>Cell wall mannoprotein</td>
<td>CCTCGGCGCG</td>
<td>-345 to -335</td>
</tr>
<tr>
<td>YPL262W</td>
<td><strong>FUM1</strong></td>
<td>Mito. and cyto. fumarase</td>
<td>CCCCTGAGCCG</td>
<td>-334 to -324</td>
</tr>
<tr>
<td>YLR205C</td>
<td><strong>HMX1</strong></td>
<td>Unknown</td>
<td>CCATTGGACCG</td>
<td>-430 to -420</td>
</tr>
<tr>
<td>YDL085W</td>
<td><strong>NDE2</strong></td>
<td>Mito. NADH dehydrogenase</td>
<td>CGGCCGATCCG</td>
<td>-386 to -376</td>
</tr>
<tr>
<td>YPL156C</td>
<td><strong>PRM4</strong></td>
<td>Unknown</td>
<td>CGCTCTGCGCG</td>
<td>-383 to -373</td>
</tr>
<tr>
<td>YBR050C</td>
<td><strong>REG2</strong></td>
<td>Protein phosphatase type 1</td>
<td>CCATTGCGCG</td>
<td>-405 to -395</td>
</tr>
<tr>
<td>YOR095C</td>
<td><strong>RKI1</strong></td>
<td>Ribose-5-phosphate ketol-isomerase</td>
<td>CCATTGCCCG</td>
<td>-370 to -360</td>
</tr>
<tr>
<td>YGL056C</td>
<td><strong>SDS23</strong></td>
<td>Unknown</td>
<td>CGCTAACCG</td>
<td>-335 to -325</td>
</tr>
<tr>
<td>YGL208W</td>
<td><strong>SIP2</strong></td>
<td>Response to glucose starvation</td>
<td>CGTTGACCG</td>
<td>-148 to -138</td>
</tr>
<tr>
<td>YAR035W</td>
<td><strong>YAT1</strong></td>
<td>Outer carnitine acetyltransferase</td>
<td>CGCGGGTCCG</td>
<td>-167 to -157</td>
</tr>
<tr>
<td><strong>Proposed CSRE consensus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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**Proposed new CSRE-genes**

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<td><strong>Proposed CSRE consensus</strong></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>CCGTTTCGACCG</td>
<td>-275 to -265</td>
</tr>
</tbody>
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* With n=A, C, G or T, y = C or T, r = A or G
Figure legends


Figure 2: The 180 genes transcriptionally regulated by carbon source were sorted by their functional category as defined by MIPS. The two major categories were found to be genes with poorly described functions (40%) and genes related to carbon metabolism (28%).

Figure 3: Transcript profiles of carbon-source responsive genes. Each column represents the average expression intensity of three replicate genome-wide transcript profiles for carbon-limitation. Each row represents a gene. In green color are represented low expression levels, whereas red indicates high expression levels.

Figure 4: Expression profiles in chemostat cultivation of specific genes under glucose excess, glucose-, ethanol- or acetate-limitation. Glucose excess expression data represent the average hybridization intensity in aerobic chemostat cultivation under nitrogen-, phosphorus- and sulfur-limitation for the same *S. cerevisiae* strain and in perfectly identical culture conditions published by Boer *et al.* (31). A: C2-compound-responsive genes, B: C2-compound-nonresponsive genes.
### Cluster 1 – Low expression on acetate (16)
- **Carbohydrate-related**: ALD5, GPH1, SOL4, PKF26, YNL134C
- **Nitrogen metabolism**: CPA2, TRP4
- **Other**: RPL7B, YGP1, MEC1, AHPl, GAD1
- **Unknown, poorly defined**: YOR173W, YHR087W, YKL107W, YNL274C

### Cluster 2 – Low expression with BOTH ethanol and acetate (38)
- **Carbohydrate-related**: HXK1, TDH1, TKL2, GND2, GND1, SOL3, HXT2, MTH1, HXT7, VID24
- **Respiration**: CYC7
- **Nitrogen metabolism**: BAP2
- **Other**: PDR12, DOG2, SPS100, SWM1, FDH1, FDH2
- **Unknown, poorly defined**: ISF1, PIR3, SP118, GCY1, PHM8, YLR257W, YJR115W, YJL144W, YDL204W, YPL264C, YMR291W, YMR090W, YMR315W, YDR247W, YEL041W, YIR016W, YMR118C, YBR056W

### Cluster 3 – High expression with glucose (13)
- **Carbohydrate-related**: GIP2, SU2, SU4, HXT4
- **Nitrogen metabolism**: GNP1
- **Other**: SKN1, MCH5
- **Unknown, poorly defined**: YLR108C, YDR223W, YLR224W, YBR285W, YKR075C, YDR248C

### Cluster 4 – High expression with maltose (15)
- **Carbohydrate-related**: MAL32, MAL31, MAL11, FSP2, YJL216C, YGR287C
- **Other**: RPS10A, RPS268, RPL13B, RPL31B
- **Unknown, poorly defined**: SOL1, FIT3, YNL190W, YNL157W, YLR327C, YDR120W

### Cluster 5 – High expression BOTH with ethanol and acetate (79)
- **Carbohydrate-related**: ICL2, ACH1, FBPI, SIP4, REG2, MLS1, CIT2, ICL1, INO1, RKI1, PCK1, IDH2, IDH1, FUM1, MDH2, SDH1, SDH3, SIP2, CAT2, YAT1, YAT2
- **Respiration**: NDE2
- **Transport**: CRC1, STL1, SFC1, DUR3, MEP2, AGP2, SAM3, CTR3
- **Nitrogen metabolism**: DAL2, DAL5, GDH3, GCV2
- **Other**: MF42, SSP1, PEX21, COT1, CDA1, CDA2, ICR1, DON1, PTI1

### Cluster 6 – Low expression with maltose (18)
- **Other**: MSB2, BUD6, KCS1, RAD24, NOP2, MNN4, MAS2, DOT5, DSE2
- **Unknown, poorly defined**: VPS73, DAN4, DIA4, UBP5, YLR419W, YGR173W, YER113C, YDR286C, YBL107C