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The Genome-wide Transcriptional Responses of *Saccharomyces cerevisiae* Grown on Glucose in Aerobic Chemostat Cultures Limited for Carbon, Nitrogen, Phosphorus, or Sulfur*

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Profiles of genome-wide transcriptional events for a given environmental condition can be of importance in the diagnosis of poorly defined environments. To identify clusters of genes constituting such diagnostic profiles, we characterized the specific transcriptional responses of *Saccharomyces cerevisiae* to growth limitation by carbon, nitrogen, phosphorus, or sulfur. Microarray experiments were performed using cells growing in steady-state conditions in chemostat cultures at the same dilution rate. This enabled us to study the effects of one particular limitation while other growth parameters (pH, temperature, dissolved oxygen tension) remained constant. Furthermore, the composition of the media fed to the cultures was altered so that the concentrations of excess nutrients were comparable between experimental conditions. In total, 1881 transcripts (31% of the annotated genome) were significantly changed between at least two growth conditions. Of those, 484 were significantly higher or lower in one limitation only. The functional annotations of these genes indicated cellular metabolism was altered to meet the growth requirements for nutrient-limited growth. Furthermore, we identified responses for several active transcription factors with a role in nutrient assimilation. Finally, 51 genes were identified that showed 10-fold higher or lower expression in a single condition only. The transcription of these genes can be used as indicators for the characterization of nutrient-limited growth conditions and provide information for metabolic engineering strategies.

Growth of microorganisms in their natural environment and in many industrial applications is often limited by nutrient availability (1, 2). In these situations the specific growth rate of the organism is determined by the low (non-saturating) concentration of a single nutrient. For example, in the industrial production of bakers' yeast sugar-limited, aerobic cultivation at relatively low specific growth rates is essential to achieve high biomass yields. On the other hand processes such as beer fermentation occur at high concentrations of fermentable sugars and are limited by other nutrients (*e.g.* oxygen, nitrogen). As a result the yeast's metabolic activities are altered. This situation is different from nutrient starvation in which the absence of a nutritional component is often the cause of stress responses that result in growth arrest or cell death (3, 4).

In the laboratory, cultivation of microorganisms is predominantly performed in shake-flasks, in which all relevant nutrients are at least initially present in excess. During the course of batch cultivation the physical and chemical environment constantly changes, which directly affects the specific growth rate and the regulation of many metabolic processes (5). With the use of chemostat cultures, it is possible to study steady-state physiological adaptations to nutrient-limited growth. The medium that is continuously fed into the culture can be designed such that growth is limited by a single, defined nutrient, whereas all other nutrients remain present in excess. In conjunction with this continuous feed of fresh media into the vessel, waste media and cells are removed at the same rate. This results in a constant dilution rate (h^{-1}) which in steady-state cultures is equal to the specific growth rate μ (6). This offers the unique possibility to study metabolism and its regulation at a fixed and constant specific growth rate under tightly defined nutritional conditions.

Microorganisms have evolved a multitude of strategies to cope with nutrient limitations. Low, growth-limiting amounts of important nutrients often lead to the induction of high affinity transport systems and/or metabolic systems that allow more efficient incorporation of the nutrients into biomass constituents. A classical example is the high affinity glutamine synthetase/glutamine oxoglutatrate aminotransferase system from *Klebsiella pneumoniae* (formerly *Aerobacter aerogenes*) that can replace glutamate dehydrogenase as the primary ammonia-assimilating enzyme system during ammonia-limited growth (7). In other cases, the final biomass composition itself has a reduced content of the growth-limiting nutrient. For example, in *Saccharomyces cerevisiae* and *Escherichia coli*, it has been observed that the amino acid composition of the subset of structural enzymes used in the assimilation of sulfur, carbon, or nitrogen have a reduced content of the respective element compared with their average content in the predicted proteome (8).

The physiological responses of microorganisms to different nutrient limitation regimes can be regulated at various levels. At the level of transcription, DNA microarrays allow the accurate, genome-wide mapping of regulatory responses. With few exceptions (9–12) DNA microarray analyses have been performed using cells grown in shake flasks. With the use of chemostats, detailed analyses of the transcriptional responses of *S. cerevisiae* to nutrient limitations may aid in the development of new, DNA array-based approaches for diagnosis of industrial fermentation processes. Furthermore, such studies provide valuable information for the functional analysis of genes whose encoded protein has no known or only poorly

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defined function. However, as yet there have been no systematic investigations into the impact on genome-wide transcriptional regulation of different nutrient limitation regimes in chemostat cultures.

The main chemical elements that are assimilated into yeast biomass are carbon, hydrogen, oxygen, nitrogen, sulfur, and phosphorus (13). Previous work from our laboratory already addressed the impact of oxygen supply on the transcriptome of *S. cerevisiae* (9, 12). The aim of the present study was to determine which of the currently recognized genes of *S. cerevisiae* have uniquely higher or lower expression when growth is limited for each of the macro nutrients, carbon, nitrogen, sulfur, or phosphorus. To this end, a wild type strain of *S. cerevisiae* was grown on glucose under strictly defined conditions in aerobic, nutrient-limited chemostat cultures at a constant growth rate. These experiments revealed the transcriptional differences that contribute to altered yeast metabolism and so can serve as molecular identifiers to diagnose the status of nutrient-limited fermentations or refine metabolic engineering strategies. The complete data set is available for download at www.nutrient-limited.bt.tudelft.nl.

EXPERIMENTAL PROCEDURES

Strain and Growth Conditions—Wild type *S. cerevisiae* strain CEN.PK113–7D (*MAT***a**) (14) was grown at 30 °C in 2-liter chemostats (Applikon) with a working volume of 1.0 liter as described in van den Berg *et al.* (15). Cultures were fed with a defined mineral medium that limited growth by either carbon, nitrogen, phosphorus, or sulfur with all other growth requirements in excess and at a constant residual concentration. 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 bio controller. Stirrer speed was 800 rpm, and the airflow was 0.5 liters·min^{-1}. Dissolved oxygen tension was measured on line with an Ingold model 34-100-3002 probe and was above 50% of air saturation. The off-gas was cooled by a condenser connected to a cryostat set at 2 °C, and oxygen and carbon dioxide were measured off line with an ADC 7000 gas analyzer. Steady-state samples were taken after ~10–14 volume changes to avoid strain adaptation due to long term cultivation (16). Dry weight, metabolite, dissolved oxygen and gas profiles had to be constant over at least 3 volume changes before sampling for RNA extraction.

Media—The defined mineral medium composition was based on that described by Verduyn *et al.* (17). In all limitations except for carbon, the residual glucose concentration was targeted to 17 g·$liter^{-1}$ to sustain glucose repression at the same level. For each limitation, the medium contained the following components (per liter). For carbon-limited, the composition was 5.0 g of $(NH_4)_2SO_4$, 3.0 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, and 7.5 g of glucose. For nitrogen-limited, the composition was 1.0 g of $(NH_4)_2SO_4$, 5.3 g of K_2SO_4, 3.0 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, and 59 g of glucose. For phosphorus-limited, the composition was 5.0 g of $(NH_4)_2SO_4$, 1.9 g of K_2SO_4, 0.12 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, and 59 g of glucose . For sulfur-limited, the composition was 4.0 g of NH_4Cl, 0.05 g of $MgSO_4 \cdot 7H_2O$, 3.0 g of KH_2PO_4, 0.4g of $MgCl_2$, and 42 g of glucose.

Analytical Methods—Culture supernatants were obtained after centrifugation of samples from the chemostats or by a rapid sampling technique using steel balls precooled to −20 °C.[1] 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 glucose in the glucose-limited chemostats was determined enzymatically using a commercial glucose determination kit from Roche Molecular Biochemicals. Ammonium concentrations were determined by a modified method of the Boehringer ureum test. Phosphate and sulfate 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.* (19).

Microarray Analysis—Sampling of cells from chemostats, probe preparation, and hybridization to Affymetrix GeneChip® microarrays was performed as described previously (12). 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. For further statistical analyses, Microsoft Excel Significance Analysis of Microarrays (SAM; v1.12) add-in was used (20). The data representation used in Figs. 2 and 3 were generated using the mean and variance normalize function of the software J-Express v2.1.

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 9335 transcript features on the YG-S98 arrays a filter was applied to extract 6383 yeast open reading frames of which there were 6084 different genes. This discrepancy was due to several genes being represented more than once when suboptimal probe sets were used in the array design.

To represent the variation in triplicate measurements, the coefficient of variation (standard deviation divided by the mean) was first calculated for each transcript. When the genes were ordered by increasing average signal, the average coefficient of variation displayed a sharp increase for the 900 genes with the lowest abundance. The average coefficient of variation for the remaining 5483 signals was used to represent the average error for each condition (for further explanation and use of these values, see Piper *et al.* 12). Because the lowest 900 transcripts were unable to be reliably measured, their level was set to a value of 12 (see "Lowest measurable level" in Table II) for the comparison analyses.

Clusters of expression profiles were identified from all possible pairwise comparisons of the four data sets. A transcript fell into one of the eight expression clusters (significantly higher or lower in only one condition) if it was called significantly changed using Significance Analysis of Microarrays (expected median false positive rate of 1%) by at least 2-fold from each other condition. In our experience, these criteria establish a data set able to be reproduced by an independent laboratory (12).

Promoter analyses were performed using the web-based software Regulatory Sequence Analysis Tools (bioinformatics.bmc.uu.se/~jvanheld/rsa-tools) (21). The promoters (from −800 to −50) 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 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.

RESULTS

Physiology and Global Transcriptional Responses of S. cerevisiae during Nutrient-limited Growth—Metabolic changes, mediated partly by transcriptional regulation, are required for successful adaptation to environmental changes. Here we have measured the genome-wide transcriptional responses of *S. cerevisiae* to four different macronutrient limitations during steady-state growth in chemostats. To verify that each of the nutrient limitations was appropriately achieved, we measured the concentrations of nutrients in the culture supernatants (Table I). This showed that when a nutrient was growth-limiting, its residual concentration was below the detection limit, whereas each other nutrient was in excess. Furthermore, the concentrations of each excess nutrient were comparable between cultures. This ability to control the concentrations of excess nutrients is a unique feature of chemostat cultivation and one that is especially important for ammonia and glucose in light of their impact on transcriptional regulation via sensors of extracellular nutrients (22). Indeed, from work in our laboratory beyond the scope of this paper, we have noted systematic alterations in global transcription as a result of differences in excess extracellular glucose concentrations (data not shown).

For each culture, the rates of glucose and oxygen consumption as well as carbon dioxide and ethanol evolution were determined (Table I). In the glucose-limited culture, no ethanol was produced, and cells grew with a biomass yield on glucose of 0.5 g·g^{-1}, reflecting complete respiratory catabolism. This is typical of steady-state growth of *S. cerevisiae* strain

[1] M. R. Mashego, W. M. van Gulik, J. L. Vinke, and J. J. Heijnen (2002) *Biotechnol. Bioeng.*, in press.

TABLE I

Nutrient concentrations and physiological parameters of cultures used in this study

Except where indicated, data represent the average and S.D. of three separate chemostat steady states.

Growth-limiting nutrient	Residual nutrient measurements				Physiological parameters						
	Glucose	NH_4^+	PO_4^{2-}	SO_4^{2-}	Y_{SX} (g/g)[a]	$q_{glucose}$[b]	$q_{ethanol}$[c]	q_{O2}[d]	q_{CO2}[e]	RQ[f]	Carbon recovery
	g/liter	*mM*	*mM*	*mM*							%
Carbon	0.014 ± 0.003	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[h]	18.6 ± 1.0[i]	40.7 ± 1.0[i]	0.09 ± 0.00	5.8 ± 0.1	8.0 ± 0.1	2.7 ± 0.1	12.1 ± 0.2	4.5 ± 0.2	96 ± 1
Phosphorus	18.1 ± 1.0	54.3 ± 0.0	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	3.4 ± 0.0	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	2.7 ± 0.2	96 ± 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 limit of assay.
[i] Average of two measurements.

CEN.PK113–7D under glucose limitation at dilution rates below 0.3 h^{-1} (23). The three nutrient-limited cultures containing residual glucose exhibited mixed respiratory (indicated by high q_{O_2}) and fermentative glucose catabolism, indicating that excess glucose in the medium does not fully repress respiration. Similarly high rates of oxygen consumption in the presence of excess glucose have been noted previously (24).

For the transcriptome analyses, the variation for each condition was measured from the three independent array replicates performed (Table II). The average coefficient of variation was no more than 0.21 and for sulfur limitation was as low as 0.13, reflecting the high reproducibility between replicate arrays. Furthermore, the level of the *ACT1* transcript and the signal from the gene with lowest measurable expression were both unchanged between culture conditions. This indicated that the transcriptomes from each condition were similar in their overall magnitude of expression, thus supporting the use of global scaling for these comparisons.

In total 1881 genes (31% of the genome) had altered expression levels in at least one condition, whereas 3558 (58%) were unchanged, and 645 (11%) remained below reliable detection in all four conditions (Fig. 1). It is not surprising that such a large proportion of the genome was altered across our experiments since the four limiting nutrients are major constituents of biomass. For each nutrient it is believed that metabolic changes occur in the cell that result in both sparing of accessible nutrients and initiation of methods to make alternative forms of that nutrient available. In both cases, this requires the differential regulation of many genes involved in transport and metabolism of the required compounds.

Among the changes observed, a division was made to separate the genes that had a significantly higher or lower abundance in only one condition when compared with each other limitations and those with more complex regulatory patterns (Fig. 1). Within the former class, there were four regulatory patterns that showed higher expression under only one limitation and four patterns that showed lower expression in only one limitation (Fig. 2). We rationalize that these eight patterns of expression should be the most informative for promoter and functional analysis studies as well as forming the basis for the list of transcripts that could be used as specific molecular identifiers to characterize specific growth limitations. Because our aim was to define transcripts that could be used for this purpose, we chose to only concentrate on the 484 open reading frames that fell into these eight classes.

Identification of Putative Regulatory Elements Responsible for Transcriptional Regulation during Nutrient-limited Growth—Coordinated regulation of global transcription is driven by the action of transcription factors that generally act once bound to short elements in gene promoters. Searching the promoters of co-regulated genes for short sequences that are over-represented can identify these elements. We analyzed the genes from the eight regulatory classes defined above using the web-based tool RSAT (21). Because our classifications selected for genes that were differentially regulated under only one of

TABLE II

Summary of microarray experiment quality parameters for each growth limitation

Culture limiting nutrient	Average coefficient of variation[a]	*ACT1*[b]	Lowest measurable signal[c]
Carbon	0.18	2489 ± 81	12 ± 6
Nitrogen	0.14	2265 ± 106	12 ± 3
Phosphorus	0.21	2314 ± 266	13 ± 3
Sulfur	0.13	2172 ± 249	12 ± 3

[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] Corresponds to the signal from the open reading frame with the lowest reliably detectable abundance.

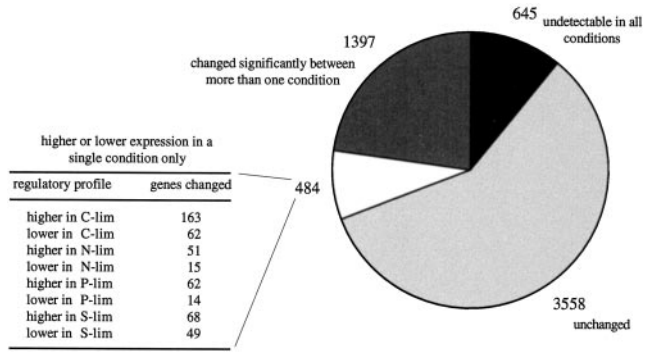

FIG. 1. **Summary of the global transcriptional responses to growth in nutrient-limited chemostats.** The global transcript profiles from yeast grown under limitation for either carbon, nitrogen, phosphorus, or sulfur were compared, and the classes of expression profiles were scored. More than two-thirds of the predicted genome (69%) were either not measurable or unchanged across all four conditions. The remaining (significantly changed) genes were either specifically higher or lower in a single condition (484 transcripts) or subject to more complex regulation (1397 transcripts). Those genes subject to unique regulation are further subdivided into the eight possible expression profiles.

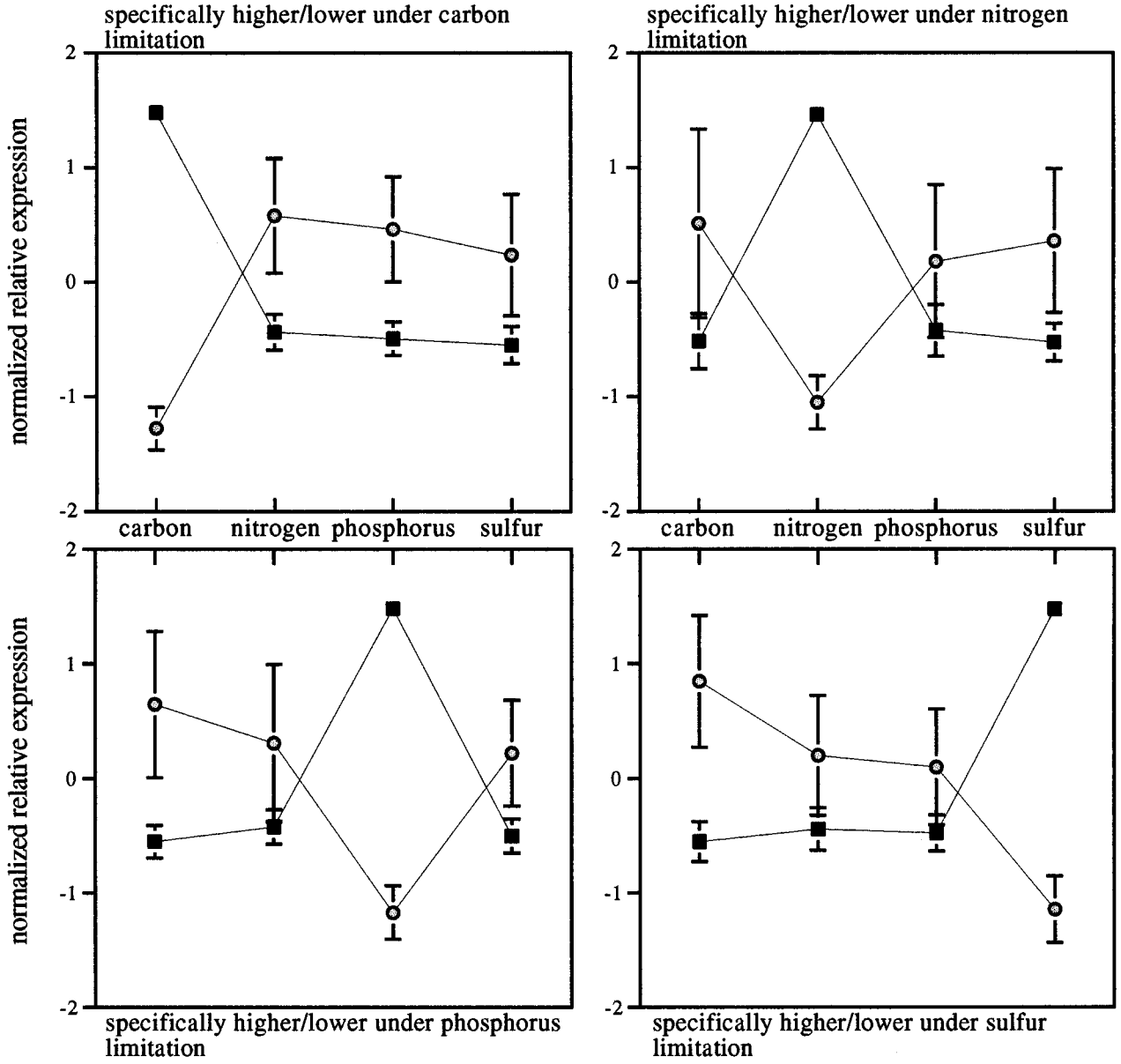

FIG. 2. **Representative regulatory profiles of genes with specifically higher (*black squares*) or lower (*gray circles*) transcript abundance in a single growth limitation.** The transcript level across the four conditions for each gene was mean and variance normalized to give an average of 0 and variance of 1. The data plotted here represent the average abundance of all transcripts in a class for each growth limitation. *Error bars* represent the S.D.

the nutrient limitations tested, each group should have been enriched for the regulatory elements that are specifically required for an appropriate response.

Several significantly over-represented elements were recovered from each group of genes except for those that had specifically lower expression under nitrogen or phosphorus limitation (Table III). Because it is known that many transcriptionally active elements have an enhanced effect when present in more than one copy (for example, see Ref. 25), the proportion of gene promoters in each subgroup with at least two elements was compared with the proportion found in all promoters of the genome (Table III). For each regulatory profile in which a known transcriptional regulator is thought to act, its corresponding binding sequence was found among the multiple elements recovered. In addition, several unknown elements were found that could not be associated with the binding of known transcription factors. To define unique transcriptional responses to distinct nutrient limitations, only the known elements are discussed further below.

The Regulatory Response of S. cerevisiae Specific to Carbon-limited Growth—S. cerevisiae has the ability to use several different carbohydrate molecules as its sole source of carbon and energy. The carbon source that is most directly incorporated into central metabolism is glucose, which is often referred to as a preferred carbon source. At the molecular level, the availability of glucose to cells signals repression of many genes involved in the utilization of alternative carbon sources via a complex of signals collectively known as carbon catabolite repression (for reviews, see Refs. 26 and 27). We found 163 transcripts with significantly higher levels and 62 transcripts with significantly lower levels in a manner specific to glucose-limited growth. This set of genes is the largest of each of the four groups represented here (Fig. 1), reflecting the central importance of adaptation to changes in carbon source availability.

Among the genes that had higher transcript levels, there were 47 known to be involved in uptake and phosphorylation of glucose (4 genes), uptake and metabolism of fatty acids and storage carbohydrates (17 genes), glyoxylate cycle and glucone-

TABLE III
Gene coverage of over-represented sequences retrieved from the promoters of co-regulated genes
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]	Ref.
	Forward	Reverse				
				%	%	
Specifically higher in C limitation	dCCCCdh	dhGGGGh	Miglp	57	28	(28)
	drCGGCT	AGCCGyh	?	15	4	
	wCTCCA	TGGAGs	Adrlp	22	16	(29)
	mwCCGG	GGCCsk	?	25	15	
	dnnCCGCCG	CGGCGGnnh	Ume6p	2	1	(30)
Specifically lower in C limitation	rGAAAAA	TTTTTCy	Hxk2p/Med8p	61	38	(32)
	GAAGAA	TTCTTC	?	42	34	
	dnyTCGAGAndd	hhnTCTCGArnh	?	8	1	
Specifically higher in N limitation	wGATAAs	WTTATCs	Gln3p/Gat1p/Dal80p/Gzf3p	53	9	(33)
	dnCAGCAA	TTGCTGng	?	22	7	
Specifically lower in N limitation	NS[c]					
Specifically higher in P limitation	mACGTGb	vCTCGTK	Pho4p	39	5	(38)
	GCAGCAnndd	hhnnTGCTGC	?	11	3	
	sdTGGAdnh	dnhTCCAhw	?	46	38	
Specifically lower in P limitation	NS					
Specifically higher in S limitation	hGCCACA	TGTGGCd	Cbflp/Met4p/Met28p	25	3	(41)
	dCACGTGAh	dTCACGTGh	Met31p/Met32p	13	2	(42)
	hACAGwk	bsCTGTd	?	49	33	
Specifically lower in S limitation	AGGGG	CCCCT	Msn2p/Msn4p	56	14	(43)
	CCGCGC	GCGCGG	?	8	1	
	GGmACm	kGTkCC	?	52	25	

[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] NS, no significant patterns retrieved by the algorithm employed by RSA Tools.

ogenesis (5 genes), uptake and utilization of alternative carbon sources (7 genes), energy generation (6 genes), and other areas of carbohydrate metabolism (8 genes). When also including the associated regulators *ADR1*, *CAT8*, *SIP2*, *SIP4*, and *GAL4*, the alterations to cellular metabolism that allow carbon and energy scavenging become apparent. Interestingly, there are also 4 genes (*CTA1*, *GPX1*, *GTT1*, and *TSA2*) that were specifically higher with a role in protecting cells against oxidative stress that is known to arise at least partially from respiratory metabolism and the oxidation of fatty acids. For the remaining 104 transcripts, there were 25 of known function that could not be directly related to carbohydrate metabolism and 82 of only poorly defined or no known function (Fig. 3).

Using the promoters of these co-regulated genes to look for over-represented sequences, several potential regulatory elements were found (Table III). Because this comparison identified genes that were specifically higher when glucose was limiting when compared with conditions in which glucose was in excess, it was no surprise to find the sequences for the glucose-sensitive repressor Mig1p (28) and the glucose-repressible activator Adr1p (29). Although the element for the meiosis-specific transcriptional repressor/activator Ume6p was also found (30), its relatively low coverage of the regulated genes indicates it is not critical for their coordinated regulation. A notable omission from this list is the Cat8p/Sip4p binding site or carbon source-responsive element (5′-CCrTyCrTCCG-3′, r is A or G, and y is C or T (31)). Because only a relatively small number of genes have been shown to be regulated through this element (26), its retrieval from among these 162 gene promoters is unlikely. There is a weak possibility that the element 5′-drCGGCT-3′ (d is A, G, or T, and y is C or T), which was retrieved represents this site.

The largest functional category of the 62 genes that were specifically lower during glucose-limited growth was those concerned with transport (10 genes). This included three hexose transporters of low or moderate affinity (*HXT1*, *HXT3*, and *HXT4*) that would not be required when external glucose concentrations are low. There were also three genes required for the regulation of glucose repression (*MIG2*, *STD1*, and *TUP1*) as well as two isogenes of components of central carbon metabolism (*PFK27* and *TDH3*). Finally, there were 18 transcripts of various (known) functions that were specifically down-regulated during glucose-limited growth. It is of note that this group contained several genes involved in cell proliferation and differentiation (*TPO1*, *TPO2*, *TPO3*, *PHD1*, *BUD9*, *GIC2*, *SST2*, *STE2*, *STE6*, and *SUN4*). The remaining 31 transcripts had no known or only poorly defined function.

The promoter analysis of these genes revealed the binding site for the regulatory proteins Med8p and Hxk2p (Table III) that play an important role in the repression and activation of genes during glucose (de)repressing conditions (32). Although an abundant sequence in the genome, its presence is consistent with the observation that the glucose-limited chemostats were fully respiratory and that Hxk2p plays a critical role in the control of respiration (23).

The Regulatory Response of S. cerevisiae Specific to Nitrogen-limited Growth—From our experiments, there were 51 transcripts whose level was significantly higher in a manner that was specific for growth limitation by the nitrogen source. Of these, 21 had no known functions or only poorly defined functions, whereas the remainder were principally involved in the metabolism of nitrogen-containing compounds (Fig. 3). Not surprisingly, the gene encoding the high affinity ammonia permease (and signal transducer), *MEP2*, showed a strong increase in expression. It was also notable that the multicopy suppressor of the differentiation defect in a *mep2* null strain, *HMS1*, was increased as well as four other genes involved in cellular differentiation. Of the structural genes for catabolism of nitrogenous compounds, the complete set of genes encoding the uptake and utilization of allantoin (*DAL* genes), proline (*PUT* genes), and urea (*DUR* genes) were represented as well as two genes encoding peptidases that are involved in recycling proteins targeted to the vacuole. Other genes whose enzyme activities yield ammonia (from serine, cystathionine, or amide bonds) or glutamate (from glutathione) were also induced as well as *GAP1* for the uptake of all amino acids. The functional

specifically higher expression under carbon limitation
transport
HXT2, HXT5, PXA1, BIO5, CRC1, CTR3, FRE4, PCA1, PMA2, ODC1, SFC1, JEN1, MAL11, STL1, ALP1, BAP2
carbohydrate-related metabolism
HXK1, FOX2, ECI1, ECI2, FAA2, INO1, PEX1, PLB2, POT1, POX1, TES1, MRF1, IDP3, GUT1, ATH1, GLG2, GCY1, FBP1, IDP2, MDH2, ICL1, MAL32, SUC2, SUC4, ACS1, CYB2, FDH1, FDH2, NDE2, SPS19, GUT2, ADH2, ALD4, CAT2, YAT2, CIT3, PFK26, GND2, TKL2
other metabolism
CPA2, ARO10, ICL2, THI11, BIO3
regulators of carbohydrate metabolism
ADR1, CAT8, GAL4, SIP2, SIP4
cell defense
CTA1, GPX1, GTT1, TSA2, SSA4, HSP26
other (splicing, cell fate & organization, protein synthesis)
SMX3, CDA1, IME1, SPO1, ECM4, SED1, MIP6, MRPL31, RPS14B
poorly defined/unknown
SNZ1, BNS1, SIP18, MBR1, SNO1, SRD1, SOL1, ISF1, FUN34, KNH1, MOM45, MRK1, PAI3, SRL3, USV1, ZTA1, YGR067C, YLR327C, YCR010C, YDR384C, YFL030W, YGR043C, YJL045W, YJL216C, YJR096W, YMR118C, YGR201C, YPL113C, YAR068W, YBR116C, YBR150C, YBR157C, YBR230C, YDL009C, YDL204W, YDL214C, YDL223C, YDR018C, YDR070C, YEL041W, YEL057C, YER067W, YER121W, YFR017C, YGL080W, YGL117W, YGR236C, YGR243W, YGR250C, YHR209W, YIL057C, YIL117C, YIR016W, YJL037W, YJL144W, YJL161W, YJL185C, YJL217W, YKL107W, YKL171W, YKL187C, YKR049C, YKR067W, YKR075C, YLR194C, YML128C, YMR107W, YMR191W, YMR206W, YNL194C, YNL335W, YOL083W, YOL101C, YPL109C, YPL156C, YPL186C, YPL201C, YPR002W, YPR030W

specifically lower expression under carbon limitation
transport
HXT1, HXT3, HXT4, AGP1, MEP3, OPT1, PTR2, TPO1, TPO2, TPO3, BST1, COX5B
metabolism
PFK27, TDH3, OYE2, LCB4
regulation
STD1, MIG2, TUP1, MSN4, PHD1
other (cell cycle, DNA processing, cell fate, cell organization, protein fate)
PIF1, BUD9, GIC2, SST2, STE2, STE6, SUN4, WSC4, ALG2, MNN1
poorly defined/unknown
YER053C, YHR210C, YER028C, YBR161W, ISR1, YBL029W, YBL095W, YCR043C, YCR102C, YDR352W, YDR533C, YEL016C, YER124C, YER188W, YGL157W, YGR125W, YHR048W, YHR049W, YJL213W, YJR030C, YLR108C, YLR179C, YLR460C, YMR057C, YMR269W, YMR285C, YNL134C, YNR032C, YPL095C, YPL245W, YPR013C

specifically higher expression under nitrogen limitation
transport
DUR3, DAL4, DAL5, GAP1, PUT4, MEP2, FET4
metabolism
DAL1, DAL2, DAL3, AMD2, DUR1, 2, PUT1, PUT2, YFR055W, SDL1, ECM38, MLS1, DAL7, PDC5, ALD2
regulation
DAL80, HMS1
other (cell fate, protein degradation)
MFα, SPS4, BAR1, FUS1, CPS1, LAP4
poorly defined/unknown
YBR139W, YMR088C, YPR194C, YLR004C, DCG1, THI3, YGR190C, YIL059C, YLR053C, YNL129W, YHR029C, YIL037C, YGR035C, YOL128C, YDL239C, YDR090C, YHR180W, YMR090W, YIL089W, YNR068C, YPL150W, YCL061C

specifically lower expression under nitrogen limitation
transport
GNP1, BAP2, HIP1, HXT2, AQY2, YLL053C, FET3
other (cell fate, subcellular localization, cell defence)
NCA3, NSR1, GRE1, HSP30, HSP26
poorly defined/unknown
YOL155C, YGR146C, YPL250C

specifically higher expression under phosphorus limitation
transport
PHO84, PHO89, PHO86, ALR1, TRK2
phosphate assimilation
PHO11, PHO3, PHO81
other metabolism
GIT1, KCS1, PLB3, INM1, HOR2, PYK2, PRS4, NTH2, PPR1
polyphosphate accumulation
VTC3, VTC4, VTC1
other (protein fate, regulation, stress, cellular morphology)
ERC1, MAF1, AST1, UFO1, ZAP1, DDP1, APG2
poorly defined/unknown
AUT4, BUD23, FLO9, MRS7, PHM6, PHM8, YGL224C, YPL110C, PMU1, SHE9, SPL2, YDL109C, VIP1, YHR210C, YIL120W, YMR279C, YML056C, YBL070C, YNL217W, YAR069C, YCL036W, YMR195W, YOR343C, YJL119C, YGR079W, YER186C, YNR075W, YJL083W, YMR211W, YLR346C, YMR194C, YNL224C, YNR014W, YNL046W, YOL154W

specifically lower expression under phosphorus limitation
transport & metabolism
YIP3, DIP5, SIT1, SUL1, IPT1
protein synthesis
RPS22B, RPL7B
poorly defined/unknown
YDR111C, YLR327C, YBR099C, YBL049W, YLR264C, YIL037C, YKL051W

specifically higher expression under sulfur limitation
transport
SUL2, MMP1, MUP1, MUP3, SAM3, GNP1, HGT1, ATM1, COT1
sulfate assimilation
AGP3, SER33, MET1, MET8, MET2, MET3, MET10, MET16, MET22, MHT1, CYS3, STR3
other metabolism
ARN1, PDC6, DLD3
regulation
MET28, MET32, YOR302W
detoxification/stress response
GTT2, YHR176W, OYE3, CTT1, RAD59, FLR1. YLL057C
other (cell cycle and structure)
CSM2, KIC1, ASE1, CWP1
poorly defined/unclassified
TIS11, CBP1, SOH1, CHL4, YOL163W, MCH5, YBR293W, YOR378W, YIL166C, YLL055W, BNA3, YJL060W, YFL057C, YLL058W, ICY2, PCL5, YLR364W, YBR281C, YBR292C, YEL072W, YFL067W, YGR154C, YIR042C, YKL071W, YLL056C, YML018C, YNL095C, YNL191W, YOL162W, YOL164W, YPL052W

specifically lower expression under sulfur limitation
transport
PUT4, HXT6, HXT7, JEN1, CTR1, CTR3, SSU1, PDR12
metabolism
BNA1, GAD1, GPH1, GSY1, GSY2, GLC3, TSL1, GND2, HXK1, TDH1, GPM2, PGM2, MDH2, SCS3, SUR2
regulation
TYE7, SOL1, SOL4
other (cell cycle, cell differentiation & structure, stress response)
TFS1, SPS100, HSP12, SPI1
poorly defined/unknown
USV1, SDS24, YRO2, PAU2, DIA3, YOL153C, YBR056W, YDR070C, YER035W, YGL121C, YJL016W, YJR115W, YLR053C, YMR291W, YMR322C, YNL194C, YOL053C, YOR173W, UIP4

FIG. 3. **The transcript profiles and identities of the genes that were specifically up- or down-regulated in each of the four limitations.** The average of three replicate genome-wide transcript profiles were averaged for each condition 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 full data set containing all transcript abundance measurements as well as those for the eight categories of changes can be found at www.nutrient-limited.bt.tudelft.nl.

relevance of the regulation of the remaining three genes (*FET4*, *PDC5*, and *ALD2*) that were specifically higher is somewhat more difficult to explain. For *PDC5* and *ALD2* it is possible they have a role in catabolism of the branched chain and aromatic amino acids via the Ehrlich pathway. Evidence for this role of *PDC5* has been found in our laboratory when cells were grown with phenylalanine as the sole nitrogen source.[2]

In the first 800 nucleotides of the promoters of these co-regulated genes, we found the core sequence for binding the transcriptional activators Gln3p and Gat1p and repressors Dal80p and Gzf3p (Table III (33)). These regulators are central to the control of genes under nitrogen catabolite repression (34, 35), and therefore, it is understandable that this regulatory system has an important role when cellular growth is limited by the nitrogen source. Furthermore, it is interesting to note that the six known genes (*MLS1*, *PDC5*, *MFα*, *FUS1*, *HMS1*, and *FET4*) whose promoters do not contain this element are less directly related by function to metabolism involved in scavenging nitrogen. These data indicate that GATA-mediated regulation is the principle requirement for the observed regulatory profile.

During nitrogen-limited growth there were also 15 genes whose expression was significantly lower compared with the other three conditions. On close inspection of expression of these genes across conditions, it was apparent that as well as being significantly lower during nitrogen-limited growth, all but three (*NSR1*, *FET3*, and *HIP1*) varied between two other conditions as well (see Fig. 3, *top right panel*). This together with the fact that the group was small and that the promoter analysis failed to detect an over-represented sequence indicated that they were subject to more complex regulation than simply repression under nitrogen limitation. If this were true, their down-regulation was probably not unique to nitrogen-limited growth but rather the downstream consequences of the physiological changes that occur when nitrogen was limiting.

The Regulatory Response of S. cerevisiae Specific to Phosphorus-limited Growth—The range of substrates *S. cerevisiae* can use as a source of phosphate is relatively limited when compared with carbon or nitrogen. The two principle sources are inorganic phosphate that can be scavenged and taken up from the growth medium and glycerophosphoinositol, which is imported then degraded (36). In support of this, it has been shown previously that when cells were grown in low phosphate-containing media, many genes required for the uptake and assimilation of these molecules changed (37).

When we grew cells under phosphate-limiting conditions, we found there were 62 genes significantly higher and 14 genes significantly lower than the other three limitations (Fig. 3). Of the genes that were increased, there were 35 that had no known or only poorly defined functions, including several genes implicated in phosphate metabolism by sequence similarity (*SPL2* and *YPL110c*) or expression pattern (*PHM* genes). Of the remaining 27, there were 6 *PHO* genes that function in the regulation and utilization of inorganic phosphate from the medium as well as 3 genes (*GIT1*, *KCS1*, and *INM1*) involved in the uptake and metabolism of inositol phosphates. Three additional open reading frames known to be involved in the accumulation of polyphosphates in the vacuole (*VTC* genes) were present as well as *PLB3*, *HOR2*, *PYK2*, *PRS4*, and *DDP1*, which are directly involved in the metabolism of phosphorylated metabolites. Finally there were 10 genes for which there is no simple explanation for their specifically higher level under hosphate-limiting conditions.

From the promoter analyses, there was a clear over-representation of a redundant sequence that is inclusive of the binding site for the transcriptional activator Pho4p (38), which is nuclear-localized in response to phosphate starvation (Table III; for review, see Ref. 39). This sequence was also reported in the study of Ogawa *et al.* (37), who reported a good correlation between the presence of the element and induction during growth in low phosphate-containing media. It is also apparent here that the Pho4p regulator plays an important role for optimal growth under phosphate limitation.

Phosphate-limited growth also resulted in significantly lower levels in the expression of 14 genes. Like those that were low under nitrogen limitation, the small size of this group and the failure of the promoter analysis to detect over-represented sequences argues for more complex mechanisms of control than a single regulator. However, unlike those that were lower during nitrogen-limited growth, the nature of the repression here was more specific since there were six transcripts (*RPS22B*, *YBR099c*, *YBL049w*, *YIP3*, *YLR264c*, and *IPT1*) whose levels were not significantly changed between any other two conditions. However, a promoter analysis of the genes in this subgroup revealed no significant elements, consistent with the earlier conclusion for nitrogen limitation that the group is not under the specific control of a single transcriptional regulator.

The Regulatory Response of S. cerevisiae Specific to Sulfur-limited Growth—Sulfur is principally used by *S. cerevisiae* for the synthesis of the amino acids cysteine and methionine and the closely related metabolite *S*-adenosylmethionine. From these molecules, the important redox properties of sulfur are exploited by the cell in diverse roles such as cellular defense and detoxification as well as being a structural component of prosthetic groups, cofactors, and proteins. Several of these roles are represented among the 68 yeast genes that were specifically higher during sulfate limitation.

Transcripts encoding proteins for the uptake of sulfate (*SUL2*) and sulfur-containing molecules (*MMP1*, *MUP1*, *MUP3*, *SAM3*, *GNP1*, and *HGT1*) were specifically higher. Furthermore, genes whose products are involved in acquiring and generating intermediates and cofactors for sulfate assimilation (serine: *AGP3*, *SER33*; siroheme: *MET1*, *MET8*) were found along with structural components of the assimilation pathway (*MET3*, *MET16*, *MET22*, *MET10*, *MET2*, *STR3*, *CYS3*, and *MHT1*) and their transcriptional regulators (*MET28* and *MET32*). In terms of the wider role of sulfur in the cell, transcripts related to glutathione metabolism (*GTT2*) and its redox cycling (*YHR176w* and *OYE3*) were found to be higher in this condition as well as genes required for iron-sulfur cluster generation (*ATM1* and *ARN1*). We also found the pyruvate decarboxylase isozyme *PDC6* dramatically induced in agreement with a recent hypothesis that yeast can remodel its biomass composition to spare sulfur (40). Finally there were seven known genes whose increase could not be easily rationalized during sulfur-limited growth as well as 30 genes of unknown or only poorly defined function.

Two promoter sequences have been identified as important in the regulation of genes for sulfate assimilation in *S. cerevisiae*. These are the binding sites for the Cbf1p-Met4p-Met28p transcriptional activation complex (41) and that for the Met31p and Met32p transcription factors (42). Together, these drive the coordinated expression of the genes encoding components of the sulfur amino acid biosynthetic pathway. Redundant forms of both of these elements were found specifically over-represented in the promoters of the genes expressed at a higher level during sulfur limitation (Table III).

In contrast with the lower abundance transcripts for nitrogen and phosphate limitations, the genes specifically lower

[2] Z. Vuralhan, M. A. Morais, S. L. Tai, M. D. W. Piper, and J. T. Pronk, unpublished data.

under sulfur limitation formed a relatively large group. Of 49 genes, 19 were unknown whereas the next largest group (4 genes) was that involved in glycogen metabolism. One open reading frame (*SSU1*) is required for the export of (and resistance to) sulfite, an intermediate in the reduction of sulfate to sulfide for homocysteine synthesis. This may result in conservation of intracellular sulfite that results in sulfur sparing. Two other interesting genes (*CTR1* and *CTR3*) encode copper transporters whose down-regulation could be important to reduce the import of metal ions that are potentially harmful to thiol-compromised cells. Although the remainder of the genes cover a broad range of functional categories, they appear to be transcriptionally unified through the presence of stress-responsive elements in their promoters for the binding of transcription factors Msn2p and Msn4p (43).

These two transcription factors are responsible for transcriptional induction of many genes when cells are exposed to a variety of stresses, including nutritional (glucose starvation) stress (reviewed by Estruch (44)). The specific reduction in expression of stress-responsive element-driven transcripts seen here is difficult to rationalize since none of the cultures in our comparisons was challenged with stresses that are known to modulate the activity of these factors. One explanation is that the promoter context is especially important here. This is tentatively supported by the presence of unknown elements also found over-represented in the promoters (Table III).

DISCUSSION

During macronutrient-limited growth of steady-state cultures of *S. cerevisiae* we measured the differential expression of 1881 different genes (31% of the predicted genome). From this group of transcripts, we studied 484 that were found to have significantly higher or significantly lower abundance in one condition when compared with the other three conditions tested. The information distilled in the functions and promoters of these open reading frames offers us insights into the metabolic responses of the cell that result in more efficient utilization of the growth limiting nutrient. Furthermore, because these regulatory events were coupled to highly specific environmental changes using the controlled environment of the chemostat, these genes were used to generate a set of molecular "indicators." It is proposed that this list can be used for the diagnosis of media deficiencies from fermentations using undefined media or regulatory anomalies arising during rounds of strain improvement by metabolic engineering.

One consequence of our specific goal to study these limited regulatory events is that many interesting phenomena were excluded from the present work (such as the previously described connection between phosphorus and sulfur metabolism (45) or carbon and nitrogen metabolism (46)). These analyses are, however, beyond the scope of this paper and will, therefore, be investigated in future studies.

Global Transcriptional Regulation to Optimize Assimilation of Growth-limiting Nutrients—Almost all nutrients enter the cell via transport proteins whose capacity and affinity properties vary. When the growth of a microorganism is limited by the low abundance of a nutrient in the medium, an increase in high affinity uptake systems is essential to cell survival. This situation is exemplified in *S. cerevisiae* by the hexose transport family of proteins (encoded by the *HXT* genes) that contains as many as 20 members (47). In our study, when growth was limited by the carbon source (glucose) we found a gene for high affinity glucose uptake (*HXT2*) and a reserve transporter (*HXT5*) elevated, whereas genes encoding low affinity uptake (*HXT1*, *HXT3*, and *HXT4*) were reduced relative to conditions of glucose excess. This was in agreement with previous chemostat studies by Diderich *et al.* (48, 49). Furthermore, the genes encoding high affinity transport proteins of the other nutrients (*MEP2*, *PHO84*/*PHO89*, and *SUL2* for ammonium, phosphorus, and sulfate, respectively) were all relatively increased in a manner specific to the appropriate nutrient-limited culture. In the case of phosphorus limitation, the genes for vacuolar polyphosphate synthesis were also transcribed at a higher level. This agreed with the hypothesis that polyphosphate synthesis is a mechanism to maintain low intracellular free phosphate levels to enhance high affinity uptake (37).

The second strategy that was identified to enhance survival during nutrient-limited growth is to exploit other possible sources of that nutrient. Amino acids can serve to supply the cell with carbon, nitrogen, and sulfur, which was reflected by elevated levels of several amino acid transporter genes in these three limitations. Furthermore, strategies to scavenge carbon (in the form of maltose, sucrose, fatty acids, and carboxylic acids), energy (from lactate and formate), nitrogen (from allantoin, proline, and urea), phosphorus (from glycerophosphoinositol), and sulfur (from *S*-adenosylmethionine, isethionate, taurine, and glutathione) were reflected in the specific transcript changes for each limitation.

The third regulatory outcome was the elevation of genes whose products are involved in the mobilization and utilization of storage compounds. This was principally seen during nitrogen-limited and sulfate-limited cultivation. Both protein degradation (51) and allantoin utilization (52) are recognized routes for the mobilization of stored nitrogen, whereas glutathione catabolism can be used as a source of sulfur for *S. cerevisiae* (53). Several genes involved in these pathways were elevated in a manner specific to their respective limitations.

Finally, biomass remodeling for nutrient sparing has also been proposed as a mechanism for successful adaptation to nutrient-limiting conditions. Previous reports of the preferential expression of low sulfur-containing proteins during sulfur-limited growth in other microbes (54, 55) have recently been complemented by a yeast study that analyzed the proteome (8). This work proposed a model for the evolutionary adaptation of proteins to low nutrient levels through their reduced carbon, nitrogen, or sulfur content following a model recognized by Pardee (50) for a sulfur-binding protein from *Salmonella typhimurium*. More recently still, sulfur conservation in yeast by differential gene expression has been observed in response to sulfur demand induced by cadmium stress (40). Our results corroborate these reports because we found a (2-fold) overrepresentation of genes that code for proteins with low methionine content (below 1%) among those elevated specifically during sulfur limitation. This differential regulation was most dramatically illustrated by the *PDC6* transcript that increased some 10–50-fold when compared with the other three conditions. The protein encoded by this gene has 6 sulfur-containing amino acids, in contrast to its isozymes Pdc1p and Pdc5p, which contain 17 and 18 sulfur-containing amino acids, respectively. However, in contrast to the report of Fauchon *et al.* (40), we only found the relationship to be true for the methionine content in proteins and not for cysteine. It is possible that this is a function of the importance of cysteine to protein stability. This important distinction may only be detectable in our experiments since we used steady-state sulfur-limited chemostat cultures and not stress-induced sulfur depletion.

In addition to these regulatory events that reflect adaptation to nutrient limitations, we observed disproportionately low biomass yields on glucose of the nitrogen-, phosphorus-, and sulfur-limited cultures for the rates of respiration. The significantly lower yield per ATP has previously been explained for nitrogen-limited chemostat cultures by increased uncoupling between ATP synthesis and biomass production (24). This in-

TABLE IV

List of diagnostic genes that are indicative of growth limited by either carbon, nitrogen, phosphorus, or sulfur

Genes are represented here whose average abundance in one condition differed by more than 10-fold from its abundance in all three other conditions.

Gene name	Systematic name	Growth-limiting nutrient			
		Carbon	Nitrogen	Phosphorus	Sulfur
Specific identifiers for carbon-limited growth					
	YBR116C	235.1 ± 101[a]	<12[b]	19.2 ± 5	<12
TKL2	*YBR117C*	138.5 ± 67	<12	<12	<12
	YCR010C	1032.0 ± 149	<12	<12	<12
	YDL085W	307.1 ± 90	22.8 ± 3	18.5 ± 5	<12
CTA1	*YDR256C*	1239.3 ± 241	<12	<12	<12
	YDR384C	1543.4 ± 62	35.5 ± 7	51.4 ± 4	23.2 ± 3
	YER024W	713.8 ± 172	53.9 ± 11	54.7 ± 12	70.6 ± 7
POX1	*YGL205W*	246.7 ± 27	13.6 ± 2	15.6 ± 5	12.4 ± 2
	YGR043C	310.8 ± 136	19.6 ± 0	12.2 ± 2	14.8 ± 2
	YGR236C	351.5 ± 161	<12	<12	<12
HXT5	*YHR096C*	1207.9 ± 342	39.1 ± 6	25.5 ± 4	67.3 ± 17
	YIL057C	1984.0 ± 150	<12	<12	<12
POT1	*YIL160C*	1083.7 ± 365	65.3 ± 5	75.3 ± 14	34.4 ± 6
SFC1	*YJR095W*	507.1 ± 30	<12	<12	<12
JEN1	*YKL217W*	1943.4 ± 143	39.5 ± 4	42.0 ± 13	18.2 ± 8
IDP2	*YLR174W*	669.0 ± 87	51.8 ± 9	34.3 ± 15	41.0 ± 5
FBP1	*YLR377C*	214.4 ± 30	<12	<12	<12
	YMR107W	2622.7 ± 320	<12	<12	15.9 ± 1
	YMR118C	325.4 ± 181	17.4 ± 2	<12	<12
SIP18	*YMR175W*	954.8 ± 211	<12	24.0 ± 6	<12
	YMR206W	349.8 ± 49	29.1 ± 9	15.0 ± 3	<12
ADH2	*YMR303C*	3255.2 ± 1249	22.5 ± 4	52.1 ± 15	37.3 ± 0
	YNL195C	381.7 ± 134	35.4 ± 13	35.7 ± 10	21.9 ± 3
FUN34	*YNR002C*	1617.8 ± 148	42.9 ± 14	87.2 ± 6	77.2 ± 9
	YOL101C	141.9 ± 39	<12	<12	<12
FDH1	*YOR388C*	2028.9 ± 529	<12	15.0 ± 2	<12
	YPL201C	501.8 ± 123	<12	<12	<12
	YPL276W	1041.7 ± 307	<12	<12	<12
CIT3	*YPR001W*	376.3 ± 66	<12	<12	<12
HXT3	*YDR345C*	83.9 ± 20	2359.7 ± 143	1931.0 ± 97	1488.4 ± 79
HXT1	*YHR094C*	14.1 ± 3	313.1 ± 11	532.6 ± 157	432.9 ± 28
Specific identifiers for nitrogen-limited growth					
DAL4	*YIR028W*	20.5 ± 2	238.4 ± 21	23.6 ± 9	<12
CPS1	*YJL172W*	32.4 ± 14	333.4 ± 150	25.3 ± 8	23.1 ± 5
DAL5	*YJR152W*	90.8 ± 18	2436.8 ± 168	102.3 ± 15	105.5 ± 7
DAL80	*YKR034W*	12.7 ± 3	829.6 ± 180	44.4 ± 7	<12
	YMR088C	102.6 ± 23	1280.1 ± 28	81.6 ± 12	95.1 ± 6
	YPR194C	<12	1426.6 ± 114	91.4 ± 29	<12
Specific identifiers for phosphorus-limited growth					
PHOL1	*YAR071W*	55.2 ± 37	68.2 ± 12	2357.2 ± 114	44.7 ± 4
GIT1	*YCR09SC*	13.3 ± 8	12.7 ± 4	983.3 ± 118	<12
	YDR281C	28.2 ± 26	49.8 ± 12	960.1 ± 90	22.4 ± 6
SPL2	*YHR136C*	60.8 ± 85	<12	1267.3 ± 84	<12
SUL1	*YBR294W*	1035.2 ± 67	487.2 ± 31	42.0 ± 8	1885.4 ± 397
Specific identifiers for sulfur-limited growth					
AGP3	*YFL055W*	31.6 ± 5	<12	<12	773.8 ± 111
PDC6	*YGR087C*	66.0 ± 44	161.1 ± 30	37.4 ± 7	1874.3 ± 236
	YIL166C	22.3 ± 3	21.7 ± 2	24.3 ± 4	1650.4 ± 271
	YLL056C	45.5 ± 9	40.1 ± 7	53.7 ± 11	588.3 ± 27
	YLL057C	<12	<12	<12	2232.7 ± 354
	YLL058W	25.0 ± 2	25.6 ± 3	26.8 ± 4	596.0 ± 9
	YOL162W	13.8 ± 4	32.4 ± 3	13.2 ± 6	614.7 ± 39
	YOL163W	<12	21.9 ± 4	<12	638.0 ± 20
	YOL164W	25.9 ± 12	40.8 ± 8	41.6 ± 12	1738.9 ± 193
YRO2	*YBR054W*	451.3 ± 170	375.6 ± 49	606.2 ± 83	19.2 ± 5
SCS3	*YGL126W*	369.3 ± 290	438.9 ± 206	602.7 ± 251	<12
CTR3	*YLR411W*	1039.9 ± 139	439.0 ± 29	345.3 ± 57	26.6 ± 5

[a] Represents the average signal from three independent biological replicates ± S.D.

[b] <12, genes whose expression were not reliably detectable.

teresting observation is under further investigation in our laboratory.

The Activities of Specific Transcriptional Regulons during Nutrient-limited Growth—Genome-wide regulation of transcription in response to environmental change is an essential component of the rearrangement of metabolic fluxes. However, because of the introduction of microarray technologies, it is apparent that the functional relationships of many changed transcripts are difficult if not impossible to explain. One factor that often contributes to this ambiguity is experimental design since the changes observed for a given experimental condition are defined relative to an unavoidably imperfect reference. Our experiments illustrate this point clearly since for each experimental culture (carbon-, nitrogen-, phosphorus-, or sulfur-limited) each of the other three cultures could serve as a reference condition because they contained each others' nutrients in excess at a constant residual concentration. The most informative group of genes for specific regulatory information, therefore, are the 484 genes identified here that were specifically up- or down-regulated in response to change of only a single nutrient.

We found several over-represented sequences in the promoters of the co-regulated genes; however, the relationship between these putative transcription factor binding sites and gene regulation was imperfect. The explanation for this is 2-fold; (i) changes in transcript abundance can be achieved indirectly through the combined actions of other regulatory events, and (ii) for most transcription factors, the sequence(s) of their binding site is poorly described. We have tried to minimize the occurrence of the former possibility by using multiple comparisons as described above; however, this can be further improved by the addition of more reference situations (*e.g.* trace element or vitamin growth limitations) and selecting the specifically regulated gene sets. Improvement on the issue raised by the second possibility is, however, more complicated, as shown by a study of the *DAL1*/*DAL4* promoter that showed active promoter elements were extremely difficult to predictably identify (18). The promoter analysis data presented here therefore holds its greatest value in simply identifying over-represented sequences to indicate which transcription factors are likely to be active. This can be used to indicate what regulons underlie the observed metabolic adaptations to nutrient-limited growth. This knowledge can be useful for the metabolic engineering of global gene regulation.

Transcriptional Information to Identify Nutrient-limited Growth—Development of array-based diagnostic approaches requires clear definitions of the nature of gene changes in response to environmental conditions. Our experimental approach forms the basis for this information by studying growth that was limited by each of four nutrients that are assimilated by the cell to form the principle components of biomass. Although each of the 484 genes identified above is subject to regulation that is specific for one nutrient limitation, further refinement of this list can provide identifier genes whose changes are clearly recognizable even in the absence of reference situations. We therefore searched these genes for specific increases or decreases whose magnitude was at least 10-fold in comparisons with each others' nutrient limitation. This resulted in 54 transcripts, of which 48 were specifically higher, and 6 were specifically lower in a single limitation (Table IV). With the ease of custom array design and array technologies that hugely reduce the time from sample to signal, it is conceivable that transcript signals using these open reading frames can be used in a control loop to ensure successful fermentations when media composition is unknown.

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