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FEMS Yeast Research 5 (2005) 579–593

FEMS Yeast Research

www.fems-microbiology.org

Physiological and genome-wide transcriptional responses of *Saccharomyces cerevisiae* to high carbon dioxide concentrations

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Received 19 August 2004; received in revised form 23 September 2004; accepted 24 September 2004

First published online 25 November 2004

Abstract

Physiological effects of carbon dioxide and impact on genome-wide transcript profiles were analysed in chemostat cultures of *Saccharomyces cerevisiae*. In anaerobic, glucose-limited chemostat cultures grown at atmospheric pressure, cultivation under CO_2-saturated conditions had only a marginal (<10%) impact on the biomass yield. Conversely, a 25% decrease of the biomass yield was found in aerobic, glucose-limited chemostat cultures aerated with a mixture of 79% CO_2 and 21% O_2. This observation indicated that respiratory metabolism is more sensitive to CO_2 than fermentative metabolism. Consistent with the more pronounced physiological effects of CO_2 in respiratory cultures, the number of CO_2-responsive transcripts was higher in aerobic cultures than in anaerobic cultures. Many genes involved in mitochondrial functions showed a transcriptional response to elevated CO_2 concentrations. This is consistent with an uncoupling effect of CO_2 and/or intracellular bicarbonate on the mitochondrial inner membrane. Other transcripts that showed a significant transcriptional response to elevated CO_2 included *NCE103* (probably encoding carbonic anhydrase), *PCK1* (encoding PEP carboxykinase) and members of the *IMD* gene family (encoding isozymes of inosine monophosphate dehydrogenase).

Keywords: Carbon dioxide; Stress; *Saccharomyces cerevisiae*; Transcriptomics

1. Introduction

Carbon dioxide is a common gaseous product of cellular metabolism. It is well established that, at high concentrations, CO_2 can negatively affect microbial metabolism [1]. Indeed, storage of food products and beverages under a CO_2-enriched atmosphere is used to delay microbial spoilage [2,3].

Inhibition of growth and product formation by CO_2 can be a problem in industrial fermentation. During beer fermentation and bio-ethanol production with *Saccharomyces cerevisiae*, the fermentation broth readily becomes saturated with the CO_2 produced during alcoholic fermentation. This effect is augmented in large bioreactors, where hydrostatic pressure may lead to very high dissolved CO_2 concentrations. Effects of CO_2 on *S. cerevisiae* [4] include loss of biomass yield and fermentative capacity [5] as well as inhibition of cell division and bud formation [6]. Furthermore, high partial pressures of CO_2 affect flavour production in beer fermentations and other important fermentation parameters in yeasts and other fungi [7–9].

Despite the industrial relevance of CO_2 effects on yeast physiology, little is known or understood about the molecular mechanisms involved in CO_2 sensitivity in *S. cerevisiae*. Proposed mechanisms for CO_2 toxicity

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1567-1356/$22.00
doi:10.1016/j.femsyr.2004.09.009

include alterations in membrane fluidity (the so-called ''anaesthesia effect''), direct inhibition of certain enzyme activities and internal acidification by the hydration of CO_2 into H_2CO_3, but all of these are essentially hypothetical [1].

The cellular responses of *S. cerevisiae* to many stresses other than elevated CO_2 concentrations have been extensively studied [10,11]. Stress responses in *S. cerevisiae* typically involve signal transduction pathways that trigger transcriptional upregulation of genes encoding the proteins involved in adaptation to the new environment, as well as downregulation of other genes. These mechanisms may be generic (like the General Stress Response), provoking the coordinate response of a number of stress-responsive genes upon exposure to a wide variety of conditions [12], or specific for a certain kind of stress. So far, transcriptional responses of *S. cerevisiae* to CO_2 stress have not been documented.

Knowledge on the genome-wide transcriptional response of *S. cerevisiae* to high CO_2 concentrations may provide a deeper insight into the molecular mechanisms of CO_2 stress. Such insight is essential to develop metabolic-engineering strategies for improving CO_2 tolerance. Furthermore, identification of 'signature transcripts' that uniquely respond to CO_2 stress may be applicable for diagnosing the CO_2 status of industrial fermentations. It has recently been demonstrated that the combination of chemostat cultivation with DNA-microarray-based transcriptome analysis offers a powerful and reproducible approach to identify the transcriptional responses of yeasts to environmental parameters [13–17]. For this reason, in the present study we used chemostat cultures of *S. cerevisiae* to quantify the effect of CO_2 on respiring and fermenting cells, and to determine the genome-wide transcriptional responses of this yeast to high CO_2 concentrations.

2. Materials and methods

2.1. Strains and culture conditions

The prototrophic *S. cerevisiae* strain CEN.PK113-7D [18] was used for this study. Cells were grown at 30 °C in laboratory fermenters (Applikon, Schiedam, The Netherlands) with a working volume of 1 l as described in [19]. Cultures were fed with a defined synthetic medium that was designed to allow for steady-state growth limited by either carbon or nitrogen [14], with all other requirements in excess and at a constant residual concentration. The dilution rate was set to 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 Biocontroler. Stirrer speed was 800 rpm, and the gas flow was 0.5 $lmin^{-1}$.

2.2. Media and gassing

Synthetic media were prepared as described [20] with the following modifications: for carbon-limited cultivation, the medium contained 5.0 gl^{-1} of $(NH_4)_2SO_4$, 3.0 gl^{-1} of KH_2PO_4, 0.5 gl^{-1} of $MgSO_4 \cdot 7H_2O$, and either 7.5 gl^{-1} of glucose or 5.76 gl^{-1} of ethanol. For nitrogen-limited cultures, 1.0 gl^{-1} of $(NH_4)_2SO_4$, 5,3 gl^{-1} of K_2SO_4, 3.0 gl^{-1} of KH_2PO_4, 0.5 gl^{-1} of $MgSO_4 \cdot 7H_2O$, and the necessary glucose to keep the residual glucose concentration at 18 gl^{-1} (59 and 62.2 gl^{-1} for the CO_2-untreated and -treated cultures, respectively). This was done to avoid differences in the degree of glucose repression. For anaerobic cultivation, ergosterol (10 mg l^{-1}) and Tween 80 (420 mg l^{-1}) were added, and the medium vessel was flushed with N_2.

Cells were gassed with air or with N_2 for aerobic or anaerobic cultivation, respectively. For CO_2-enriched anaerobic cultivation, the nitrogen sparging gas was replaced by pure (>99.99%) CO_2 (HoekLoos, Schiedam, The Netherlands). For CO_2-enriched aerobic cultivation, cultures were sparged with a defined gas mixture containing 79% CO_2 and 21% O_2 (HoekLoos, Schiedam, The Netherlands).

2.3. Culture monitoring

Dissolved oxygen was monitored online with an oxygen probe (Ingold model 34-100-3002) and remained above 70% of oxygen saturation in aerobic experiments. The off-gas was cooled by a condenser connected to a cryostat set at 2 °C, and O_2 and CO_2 were measured offline with an ADC 7000 gas analyser (White Rock, BC, Canada). In CO_2-enriched cultures, CO_2 measurement was not possible due to over-ranging of the device. Due to the absence of nitrogen (used as reference gas), respiratory quotients could not be calculated in these cultures. Therefore, oxygen consumption and CO_2 production were estimated by assuming a 100% carbon balance. Dry weight was determined as previously described [21]. Extracellular metabolites were measured by HPLC [14]. Steady-state samples were taken after 7–10 volume changes to avoid strain adaptation due to long-term cultivation [22,23]. Samples for RNA extraction were taken when weight, metabolite concentrations and off-gas analysis differed by less than 2% over a period of two volume changes.

2.4. Fermentative capacity

The maximum fermentative capacity of culture samples was determined by following ethanol production under anaerobic conditions in the presence of excess

glucose [24]. Ethanol was spectrophotometrically determined from the samples using a commercial kit (Roche) and following manufacturer's instructions.

2.5. Total RNA purification, probe preparation and array hybridisation

One hundred millilitres of culture was sampled directly from the fermentor into a beaker containing 200–300 ml of liquid nitrogen and then processed as described [13]. Total RNA was extracted with phenol/chloroform [25]. mRNA extraction, cDNA synthesis and labelling, as well as array hybridisation against Affymetrix YG-S98 GeneChips® was performed as described in the Affymetrix user's manual [26]. Data acquisition was performed using the software packages Microarray Suite v5.0, MicroDB v3.0 and Data Mining Tool v3.0 (Affymetrix, Santa Clara, CA, USA).

2.6. Data processing and analysis

Before comparison, all arrays were globally scaled to a target value of 150 using the average from all the gene features. From the 9335 transcript features on the YG-S98 arrays a filter was applied to extract 6383 open reading frames of which there were 6084 different genes. Signal values below 10 were discarded from the analysis because they were considered below the limit of detection of the system [13]. For statistical analysis, the software packages SAM [27], dChip [28] and Microsoft® Excel were used. Promoter analysis was performed with the web-implemented software RSA Tools [29].

2.7. Enzyme analysis

Two hundred and forty millilitres of the culture was sampled directly from the fermentor, harvested by centrifugation, washed twice with 10 mM potassium phosphate buffer (pH 7.5) containing 2 mM EDTA, concentrated sixfold, aliquoted (4 ml aliquots) and stored at −20 °C until use. Aliquots were thawed, washed, and resuspended in 2 ml of 100 mM potassium phosphate buffer (pH 7.5) containing 2 mM $MgCl_2$ and 1 mM DTT (for phosphoenolpyruvate carboxykinase (PEPCK) and pyruvate carboxylase determinations), or in 2 ml of Tris-barbiturate (pH 8.3) containing 1 μM $ZnSO_4$ and 1 mM DTT (for carbonic anhydrase assays). Crude extracts were prepared by sonication with 0.7-mm-diameter glass beads at 0 °C in a MSE Soniprep 150 sonicator (150 W output, 8 μm peak-to-peak amplitude) for 4 min at 0.5-min intervals. Cell debris was removed by centrifugation (20 min at 36,000*g*) at 4 °C. The supernatant was used as the cell extract. Phosphoenolpyruvate carboxykinase, pyruvate carboxylase and carbonic anhydrase (CA) activity were determined as described [30,31]. Total protein was determined following the Lowry method [32].

3. Results

3.1. Physiological effects of elevated carbon dioxide concentrations

To quantify physiological effects of elevated CO_2 concentrations on yeast physiology, biomass and product formation were studied in chemostat cultures grown in the presence or (virtual) absence of CO_2 in the inlet gas. Three different cultivation conditions were investigated, each resulting in a different mode of glucose dissimilation. A completely fermentative metabolism was obtained in anaerobic, glucose-limited chemostat cultures, and a completely respiratory glucose dissimilation in aerobic, glucose-limited chemostat cultures (Table 1). A mixed respiro-fermentative mode of glucose dissimilation was obtained by nitrogen-limited, aerobic cultivation (Table 1).

The effects of CO_2 on cellular physiology were most pronounced in the aerobic, glucose-limited chemostat cultures. Under these conditions, inclusion of 79% CO_2 in the inlet gas led to a 24% decrease of the biomass yield on glucose (Table 1). Consistent with this reduced biomass yield, respiration rates were higher in the CO_2-enriched respiratory cultures. The dissolved oxygen percentage in the culture broth remained as high as in the non-CO_2-enriched cultures, ensuring that the observed effects were due to the excess of CO_2 and not to O_2 depletion. Conversely, biomass and product yields in the anaerobic, fermentative cultures were not significantly affected when the nitrogen gas used for sparging was completely replaced by CO_2 (Table 1). An intermediate situation (10% decrease of the biomass yield) was observed in the nitrogen-limited, respiro-fermentative cultures (Table 1).

To further explore the relationship between respiratory metabolism and CO_2 sensitivity, we attempted to establish CO_2-enriched ethanol-limited chemostat cultures. However, under these conditions the maximum specific growth rate on ethanol was reduced from $\approx$0.18 h^{-1} [33] to below 0.04 h^{-1} (data not shown). While this precluded steady-state analysis in chemostat cultures, it gives a further indication that detrimental effects of CO_2 are most pronounced in respiring cultures.

In addition to affecting the biomass yield, elevated CO_2 concentrations resulted in changes of the levels of pyruvate and acetate produced by the aerobic, nitrogen-limited and anaerobic, glucose-limited cultures (Table 1). However, the absolute concentrations of these metabolites were low and it is not clear whether their increased concentrations indicated an increased perme-

Table 1
Physiological effects of elevated CO_2 concentrations on glucose-grown chemostat cultures (pH 5, 30 °C, $D = 0.10\ h^{-1}$) of *S. cerevisiae* CEN.PK113-7D

	CO_2 inlet gas (%)	Dissolved CO_2[a]	Yield[b]	qO_2[c]	qGlucose[c]	qEthanol[c]	qAcetate[b]	qPyruvate[b]	qGlycerol[b]	qSuccinate[c]
Anaerobic	<0.01	0.46 ± 0.01	0.096 ± 0.002	–	6.52 ± 0.28	9.95 ± 0.37	0.02 ± 0.00	0.01 ± 0.00	0.87 ± 0.04	0.03 ± 0.00
Anaerobic	100	27.39 ± 0.04	0.091 ± 0.004	–	6.51 ± 0.67	10.39 ± 1.13	0.02 ± 0.00	0.02 ± 0.00	0.68 ± 0.08	0.03 ± 0.00
Aerobic, N-limited	0.05	0.46 ± 0.01	0.095 ± 0.002	2.70 ± 0.10	5.82 ± 0.14	7.99 ± 0.13	0.06 ± 0.01	0.10 ± 0.01	0.08 ± 0.01	0.04 ± 0.02
Aerobic, N-limited	79	21.70 ± 0.19	0.085 ± 0.001	2.56 ± 0.47	6.47 ± 0.24	9.78 ± 0.26	0.16 ± 0.01	0.22 ± 0.01	0.10 ± 0.01	0.18 ± 0.01
Aerobic, C-limited	0.05	0.22 ± 0.00	0.504 ± 0.005	2.71 ± 0.39	1.10 ± 0.07	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Aerobic, C-limited	79	22.30 ± 0.25	0.382 ± 0.045	4.73 ± 0.26	1.41 ± 0.18	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.13 ± 0.03

Results are the average ± standard deviation of three independent cultivations.
[a] Calculated value according to Henry's law (mM).
[b] Yield in biomass (g biomass (g consumed glucose)$^{-1}$).
[c] Expressed in mmol (g of biomass)$^{-1}$ h^{-1}.

ability of the plasma membrane or constraints in primary metabolism.

Fermentative capacity, i.e. the maximum biomass-specific rate of ethanol production under anaerobic conditions in the presence of excess glucose, is a highly relevant characteristic for baker's yeast. This parameter is strongly dependent on cultivation conditions [24,34]. A previous study reported that fermentative capacity was drastically diminished in aerobic fed-batch cultures of *S. cerevisiae* subjected to high CO_2 concentrations [5]. In the present study, this parameter did not change significantly in response to elevated carbon dioxide concentrations (data not shown). This apparent discrepancy may be due to the inherent dynamic nature of fed-batch cultivation, as compared to the steady-state conditions studied here.

3.2. Transcriptional responses to elevated carbon dioxide concentrations

DNA-microarray analysis was performed on all six conditions (three aeration/nutrient limitation regimes, with and without elevated CO_2) to analyse the genome-wide transcriptional responses to elevated CO_2 concentrations. The reproducibility of the data was assessed by calculating the average coefficient of variation (CV = SD/average × 100%) for the transcripts within each set of independent triplicate samples [13]. With the exception of the anaerobic reference cultures (CV = 29%), the CV values for all datasets were below 20%.

To identify CO_2-responsive transcripts, three pairwise comparisons were performed between the CO_2-enriched and reference cultures (one for each aeration/nutrient limitation regime). SAM software [27] was used for a statistical analysis, with a minimum fold change of 2. The false discovery rate (FDR = percentage of called genes that are expected to be false positives) was set to 1%. Under all three aeration/nutrient limitation regimes, only a small fraction of the genome showed a significant transcriptional response to elevated CO_2 concentrations (Tables 2–4). Consistent with the stronger physiological response to CO_2, the largest number of CO_2-responding genes was identified in the aerobic, glucose-limited cultures (104 versus 33 and 34 for the anaerobic, glucose-limited and aerobic, N-limited cultures, respectively, Tables 2–4).

In the aerobic, glucose-limited cultures, almost 50% of the annotated genes that showed an elevated transcript level in the CO_2-enriched cultures encoded mitochondrial proteins (Fig. 1 and Table 4). Some of the encoded proteins are directly involved in oxidative phosphorylation, such as Cox11p, Cox17p and Cox18p, which are implied in the assembly of the cytochrome *c* oxidase complex [35–37]. *ATP11*, which also showed higher transcript levels in the respiratory, CO_2-enriched

Table 2
Genes whose mRNA level changed at least twofold in anaerobic, carbon-limited, CO_2-enriched cultures as compared to reference cultures without CO_2 enrichments

Gene/ORF	Description	Subcellular localization	Control[a]	79% CO_2[a]	Fold change
Upregulated					
AAR2	Splices pre mRNA of the MATa1 cistron	Cytoplasm	1.5 ± 2.2	14.3 ± 3.1	9.77
YAR062W	Putative pseudogene	Cytoplasm	3.2 ± 2.3	19.0 ± 4.0	5.87
YIL059C	Hypothetical protein	Unclassified	22.4 ± 8.3	140.2 ± 32.3	6.26
YFR026C	Hypothetical protein	Unclassified	14.6 ± 4.9	77.6 ± 15.4	5.31
ALR2	Aluminium resistance	Plasma membrane	35.9 ± 11.5	143.1 ± 13.7	3.98
YMR320W	Hypothetical protein	Cytoplasm	24.5 ± 18.1	96.6 ± 11.1	3.94
COS10	Protein with strong similarity to subtelomerically encoded proteins such as Cos5p, Ybr302p, Cos3p, Cos1p, Cos4p, Cos8p, Cos6p, Cos9p	Cytoplasm	14.6 ± 10.3	56.3 ± 5.8	3.86
YDL038C	Similarity to mucin proteins	Unclassified	27.4 ± 13.6	105.2 ± 17.4	3.83
YHR032W	Ethionine resistance protein	Unclassified	32.0 ± 12.4	116.5 ± 6.3	3.64
YFR055W	Strong similarity to β-cystathionases	Unclassified	24.7 ± 11.8	86.7 ± 2.4	3.51
IMD1	IMP dehydrogenase	Unclassified	308.5 ± 117.7	1068.4 ± 100.3	3.46
OPT2	Oligopeptide transporter	Membranes	90.7 ± 44.7	307.3 ± 51.8	3.39
YGL101W	Strong similarity to hypothetical protein YBR242W	Cytoplasm	13.5 ± 7.4	42.1 ± 4.9	3.12
YBR108W	Probable transcription factor	Unclassified	7.7 ± 3.0	22.5 ± 0.5	2.93
FMS1	Multicopy suppressor of fenpropimorph resistance (fen2 mutant), shows similarity to *Candida albicans* corticosteroid-binding protein gene *CBP1*	Cytoplasm	69.0 ± 22.7	191.2 ± 21.5	2.77
YOL031C	Weak similarity to *Y. lipolytica* Sls1 protein precursor	ER	58.8 ± 29.4	162.6 ± 6.1	2.76
YNL158W	Hypothetical protein	Unclassified	49.5 ± 23.9	132.0 ± 8.4	2.67
YPL245W	Weak similarity to human mutL protein homolog	Cytoplasm	37.7 ± 14.7	100.5 ± 5.4	2.66
YIL141W	Questionable ORF	Mitochondria	6.2 ± 3.3	20.4 ± 3.8	2.66
SPC29	Nuclear import protein	Spindle pole body	27.4 ± 11.0	71.4 ± 7.4	2.61
ISU2	NifU-like protein A	Mitochondria	200.7 ± 48.6	497.9 ± 23.8	2.48
MRH1	Strong similarity to putative heat shock protein gene *YRO2*	Bud	419.9 ± 85.5	1035.3 ± 126.1	2.47
BUD31	Involved in bud selection	Cytoplasm	19.5 ± 7.0	47.9 ± 4.5	2.45
YCR087C	Nucleic acid-binding protein	Unclassified	65.7 ± 10.1	158.6 ± 19.2	2.41
PRM7	Pheromone-regulated protein, unknown function	Unclassified	65.6 ± 6.6	156.8 ± 3.3	2.39
YGL041C	Weak similarity to YJL109C	Unclassified	7.6 ± 1.3	17.2 ± 1.6	2.26
KSS1	MAP protein kinase homolog involved in pheromone signal transduction	Unclassified	16.7 ± 3.3	36.3 ± 4.0	2.18
YGL045W	Hypothetical protein	Unclassified	37.9 ± 7.7	82.5 ± 5.2	2.18
YPL095C	Strong similarity to YBR177C	Unclassified	530.9 ± 87.4	1143.3 ± 109.2	2.15
THI3	Positive regulatory factor with thiamin pyrophosphate-binding motif for thiamin metabolism	Nucleus	74.9 ± 16.5	154.1 ± 15.7	2.06
Downregulated					
AHP1	Similarity to *C. boidinii* peroxisomal membrane protein 20 KA	Cytoplasm	889.1 ± 115.5	217.5 ± 64.0	−4.09
CVT19	Protein involved in the cytoplasm-to-vacuole targeting pathway and in autophagy	ER	219.8 ± 15.7	99.7 ± 19.0	−2.20
PDR5	Multidrug resistance transporter	Plasma membrane	262.3 ± 29.4	98.5 ± 19.7	−2.66

[a] Signal values given by the Affymetrix system. Values are expressed as average ± standard deviation of three independent replicates. As a reference, values for *ACT1* (encoding actin) were 3936.0 ± 1189.7 (control cells) and 2974.6 ± 322.6 (CO_2 treated cells).

Table 3
Genes whose mRNA level changed at least twofold in aerobic, nitrogen-limited, CO_2 enriched cultures as compared to reference cultures without CO_2 enrichments

Gene/ORF	Description	Subcellular localization	Control[a]	79%CO_2[a]	Fold change
Upregulated					
COS12	Protein with strong similarity to subtelomerically encoded proteins including Cos2p, Cos4p, Cos8p, YIR040C, Cos5p, Cos9p, and Cos6p	Unclassified	3.9 ± 0.7	19.2 ± 2.0	4.97
MHT1	*S*-methylmethionine homocysteine methyltransferase	Cytoplasm	19.5 ± 4.3	70.6 ± 9.4	3.63
MF(ALPHA)1	Mating factor alpha	Extracellular	305.5 ± 45.4	891.7 ± 81.1	2.92
YHL042W	Similarity to subtelomeric encoded proteins	ER	41.6 ± 7.3	116.1 ± 10.4	2.79
HAC1	bZIP transcription factor that regulates the unfolded-protein response	Cytoplasm	68.1 ± 14.2	160.7 ± 11.8	2.36
YAR075W	Strong similarity to IMP dehydrogenases	Unclassified	556.1 ± 24.7	1209.1 ± 64.7	2.17
Downregulated					
YGR035C	Hypothetical protein	Unclassified	89.0 ± 15.0	3.8 ± 2.4	−23.42
YKL162C	Hypothetical protein identified by SAGE	Mitochondria	15.1 ± 2.8	1.6 ± 1.2	−9.66
DAL4	Allantoin permease	Plasma membrane	238.4 ± 21.2	30.3 ± 3.7	−7.88
POT1	Peroxisomal 3-oxoacyl CoA thiolase	Cytoplasm	65.3 ± 5.3	11.8 ± 2.5	−5.53
YJL213W	Similarity to *Methanobacterium aryldialkylphosphatase* related protein	Unclassified	364.9 ± 73.0	70.0 ± 11.9	−5.22
YAR068W	Potential membrane protein	Unclassified	41.9 ± 6.5	9.7 ± 5.2	−4.32
INO1	L-myoinositol-1-phosphate synthase	Cytoplasm	249.6 ± 29.2	61.9 ± 14.4	−4.03
HXT1	Low-affinity hexose (glucose) transporter	Plasma membrane	313.1 ± 10.8	86.8 ± 20.2	−3.61
PDR12	Multidrug resistance transporter	Cell periphery	523.1 ± 92.3	154.6 ± 30.2	−3.38
NCE103	Putative carbonic anhydrase	Cytoplasm	1006.5 ± 23.7	303.1 ± 76.7	−3.32
YHR140W	Hypothetical protein	Cytoplasm	88.8 ± 3.4	30.7 ± 5.2	−2.89
NDT80	Meiosis-specific gene, mRNA is sporulation-specific	Unclassified	22.4 ± 2.3	7.8 ± 2.2	− 2.89
DIP5	Dicarboxylic amino acid permease	Cell periphery	696.8 ± 44.4	243.1 ± 50.2	−2.87
SPS19	Peroxisomal 2,4-dienoyl-CoA reductase	Cytoplasm	134.0 ± 8.2	48.6 ± 13.5	−2.76
TPO4	Similarity to resistance proteins	Bud	368.4 ± 47.4	134.2 ± 5.2	−2.75
YPL095C	Strong similarity to YBR177C	Unclassified	1032.3 ± 51.9	394.7 ± 16.4	−2.62
DDR2	Multi-stress responsive protein	Unclassified	1288.4 ± 124.2	516.7 ± 33.8	−2.49
STE3	A factor recptor	Plasma membrane	20.0 ± 0.6	8.8 ± 0.4	−2.27
BAT2	Branched-chain amino acid transaminase	Cytoplasm	442.8 ± 57.1	199.8 ± 19.5	−2.22
OYE3	NAD(P)H dehydrogenase	Cytoplasm	177.8 ± 13.1	80.5 ± 12.5	−2.21
YGR150C	Hypothetical protein	Mitochondria	25.2 ± 2.9	11.6 ± 2.0	−2.17
SPS4	Sporulation-specific protein	Unclassified	109.8 ± 6.4	51.8 ± 13.1	−2.12
YJL037W	Strong similarity to hypothetical protein YJL038C	Unclassified	34.5 ± 0.7	16.4 ± 1.1	−2.11
BST1	Protein that negatively regulates COPII vesicle formation, required for proper vesicle cargo sorting	ER	188.8 ± 21.3	90.5 ± 12.2	−2.09
FYV9	Weak similarity *H.influenzae* protoporphyrinogen oxidase (hemK) homolog	Cytoplasm	112.3 ± 6.8	54.5 ± 7.8	−2.06
YLR089C	Strong similarity to alanine transaminases	Nucleus	1008.2 ± 67.2	496.0 ± 10.4	−2.03
FIT2	Involved in the retention of siderophore-iron in the cell wall	Unclassified	25.2 ± 2.6	12.4 ± 2.6	−2.03
YMR041C	Weak similarity to *Pseudomonas* L-fucose dehydrogenase	Mitochondria	99.6 ± 9.9	49.4 ± 4.9	−2.02
KNH1	*KRE9* homolog	Cytoplasm	45.6 ± 3.8	22.8 ± 3.1	−2.00
YHR140W	Hypothetical protein	Unclassified	89.0 ± 15.0	3.8 ± 2.4	−23.42

[a] Signal values given by the Affymetrix system. Values are expressed as average ± standard deviation of three independent replicates. As a reference, values for *ACT1* (encoding actin) were 2265.3 ± 106.0 (control cells) and 2273.2 ± 225.1 (CO_2 treated cells).

Table 4
Genes whose mRNA level changed at least twofold in aerobic, carbon-limited, CO_2 enriched cultures as compared to reference cultures without CO_2 enrichments

Gene/ORF	Description	Subcellular localization	Control[a]	79% CO_2[a]	Fold change
Upregulated					
DIP5	Dicarboxylic amino acid permease	Cell periphery	28.1 ± 9.7	956.3 ± 201.7	34.07
PCK1	Phosphoenolpyruvate carboxykinase	Cytoplasm	202.4 ± 28.2	2133.4 ± 408.2	10.54
COS12	Protein with strong similarity to subtelomerically encoded proteins including Cos2p, Cos4p, Cos8p, YIR040c, Cos5p, Cos9p, and Cos6p	Unclassified	6.7 ± 3.0	46.1 ± 4.7	6.89
IMD2	IMP dehydrogenase	Cytoplasm	262.2 ± 50.9	1545.0 ± 114.5	5.89
IMD1	IMP dehydrogenase	Unclassified	100.2 ± 31.0	587.9 ± 87.4	5.87
YOL162W	Strong similarity to hypothetical protein YIL166C	Unclassified	13.8 ± 3.5	73.4 ± 5.5	5.31
YAR075W	Strong similarity to IMP dehydrogenases	Unclassified	348.5 ± 109.7	1795.0 ± 140.3	5.15
PIC2	Mitochondrial phosphate carrier	Mitochondria	135.5 ± 14.7	575.2 ± 113.5	4.25
MHT1	*S*-methylmethionine homocysteine methyltransferase	Cytoplasm	100.3 ± 17.5	384.8 ± 40.7	3.84
COS2	Protein with similarity to members of the Cos3, Cos5, Cos1, Cos4, Cos8, Cos6, Cos9 family, coded from subtelomeric region	Vacuole	113.2 ± 10.1	426.7 ± 62.7	3.77
COS3	Protein with strong similarity to subtelomerically encoded proteins such as Cos5p, Ybr302p, Cos3p, Cos1p, Cos4p, Cos8p, Cos6p, Cos9p	Vacuole	145.1 ± 26.8	513.7 ± 73.6	3.75
YER187W	YER187W similarity to killer toxin KHS precursor	Unclassified	21.1 ± 9.2	78.1 ± 6.2	3.71
COS3	Protein with strong similarity to subtelomerically encoded proteins such as Cos5p, Ybr302p, Cos3p, Cos1p, Cos4p, Cos8p, Cos6p, Cos9p	Vacuole	145.1 ± 26.8	513.7 ± 73.6	3.54
YFR020W	Hypothetical protein	Unclassified	183.5 ± 19.2	637.5 ± 12.0	3.47
GIC2	Putative effector of Cdc42p, important for bud emergence	Bud	204.8 ± 51.7	655.2 ± 48.4	3.20
YAR029W	Uncharacterised ORF	Unclassified	6.0 ± 1.9	18.8 ± 2.7	3.14
YLR343W	Strong similarity to Gas1p and *C. albicans* pH responsive protein	Cytoplasm	10.9 ± 2.7	32.6 ± 5.4	2.99
SUT1	Involved in sterol uptake	Cytoplasm	37.5 ± 8.7	109.1 ± 6.5	2.91
MRPL3	Mitochondrial ribosomal protein MRPL3 (YmL3)	Mitochondria	131.6 ± 16.0	358.0 ± 12.2	2.72
YIR043C	Member of the COS family of subtelomerically encoded proteins	Unclassified	7.8 ± 1.6	21.2 ± 0.7	2.71
YPS6	GPI-anchored aspartic protease	Unclassified	44.4 ± 2.5	119.1 ± 8.8	2.68
YLR179C	Similarity to Tfs1p	Cytoplasm	279.5 ± 56.5	733.9 ± 90.4	2.63
DUR1 2	Urea amidolyase, contains urea carboxylase and allophanate hydrolase activities fused together in a single polypeptide	Cytoplasm	99.5 ± 27.1	256.0 ± 29.9	2.57
MSE1	Mitochondrial glutamyl-tRNA synthetase	Mitochondria	50.5 ± 2.6	127.9 ± 20.3	2.53
PYC1	Pyruvate carboxylase	Cytoplasm	408.3 ± 127.3	1031.2 ± 109.6	2.53
PNT1	Pentamidine resistance protein	Mitochondria	18.1 ± 1.4	44.7 ± 5.9	2.47
YBL029W	Hypothetical protein	Cytoplasm	17.1 ± 3.8	42.3 ± 5.7	2.47
MRS2	Splicing factor	Mitochondria	36.7 ± 5.6	86.6 ± 7.8	2.36
IFM1	Mitochondrial initiation factor 2	Mitochondria	40.2 ± 6.2	94.2 ± 5.8	2.34
YDL045W	Homologous to Yml37p, component of the 37 S subunit of mitochondrial ribosomes	Mitochondria	197.7 ± 31.7	460.9 ± 49.8	2.33
YDR316W	Hypothetical protein	Mitochondria	139.3 ± 23.7	324.5 ± 19.7	2.33
COX11	Mitochondrial membrane protein required for assembly of active cytochrome c oxidase	Mitochondria	183.2 ± 7.8	424.6 ± 52.4	2.32
YDR010C	Hypothetical protein	Unclassified	6.7 ± 0.2	15.4 ± 2.1	2.31
EDS1	Probable regulatory Zn-finger protein	Unclassified	85.8 ± 13.2	197.2 ± 27.5	2.30
ECM29	Major component of the proteasome	Cytoplasm	25.3 ± 5.2	56.9 ± 5.9	2.25
BNA1	Required for biosynthesis of nicotinic acid from tryptophan	Cytoplasm	114.9 ± 6.1	256.0 ± 25.6	2.23
YDR539W	Similarity to *E. coli* hypothetical 55.3 kDa protein in rfah-rfe intergenic region	Cytoplasm	63.6 ± 13.1	141.4 ± 6.8	2.22

(*continued on next page*)

Table 4 (*continued*)

Gene/ORF	Description	Subcellular localization	Control[a]	79% CO_2[a]	Fold change
RSM18	Mitochondrial ribosomal protein	Mitochondria	173.5 ± 31.4	379.9 ± 21.5	2.19
YNR040W	Hypothetical protein	Cytoplasm	77.7 ± 9.0	167.8 ± 13.1	2.16
YER163C	Weak similarity to *E. coli* cation transport protein	Cytoplasm	84.6 ± 11.2	181.6 ± 19.5	2.15
YKR016W	Weak similarity to mysoin heavy chain proteins	Mitochondria	125.4 ± 29.3	267.7 ± 23.5	2.13
COS5	Protein with similarity to members of the Ybr302p, Ycr007p, Cos8p, Cos9p family, coded from subtelomeric region	Vacuole	171.8 ± 27.2	381.1 ± 68.2	2.12
MUP3	Very low affinity methionine permease	Plasma membrane	65.3 ± 9.2	138.7 ± 15.3	2.12
COX18	Cytochrome oxidase gene 18	Mitochondria	213.4 ± 3.1	451.2 ± 47.2	2.11
COX17	Required for delivery to cytochrome c oxidase	Mitochondria	269.5 ± 29.9	565.8 ± 54.4	2.10
MIP6	PolyA-binding protein	Cytoplasm	64.1 ± 3.5	134.5 ± 10.4	2.10
COS4	Protein with strong similarity to subtelomerically encoded proteins such as Cos5p, Ybr302p, Cos3p, Cos1p, Cos4p, Cos8p, Cos6p, Cos9p	Vacuole	398.2 ± 36.5	832.0 ± 69.9	2.09
ECM22	Regulates transcription of the sterol biosynthetic genes *ERG2* and *ERG3*	Cytoplasm	137.8 ± 12.4	286.3 ± 23.8	2.08
YBL062W	Questionable ORF	Unclassified	10.5 ± 0.9	21.7 ± 2.8	2.07
ATP11	Essential for assembly of a functional F1-ATPase	Mitochondria	340.0 ± 42.2	701.3 ± 63.9	2.06
MRF1	Mitochondrial polypeptide chain release factor	Mitochondria	56.7 ± 3.8	116.7 ± 8.8	2.06
MRPL27	Mitochondrial ribosomal protein MRPL27 (YmL27)	Mitochondria	238.7 ± 8.0	488.5 ± 46.4	2.05
Downregulated					
PRR2	Receptor signaling involved in pheromone response	Cytoplasm	150.5 ± 27.7	4.9 ± 2.7	−30.93
FDH1	Putative formate dehydrogenase	Unclassified	2028.9 ± 529.5	67.0 ± 73.9	−30.28
SIP18	Salt-induced protein	Cytoplasm	954.8 ± 211.1	39.7 ± 7.9	−24.03
HSP26	Heat shock protein of 26 kDa, expressed during entry to stationary phase and induced by osmostress	Cytoplasm	1769.5 ± 317.3	186.2 ± 159.7	−9.50
YML122C	Hypothetical protein	Unclassified	41.1 ± 5.4	4.9 ± 2.3	−8.38
YDR070C	Hypothetical protein	Mitochondria	1056.0 ± 188.3	156.2 ± 158.7	−6.76
GND2	6-phosphogluconate dehydrogenase	Cytoplasm	316.4 ± 69.2	49.4 ± 24.2	−6.41
NCE103	Putative carbonic anhydrase	Cytoplasm	1680.1 ± 102.5	263.4 ± 26.4	−6.38
OYE3	NAD(P)H dehydrogenase	Cytoplasm	198.6 ± 36.9	31.2 ± 12.0	−6.37
YEL041W	Strong similarity to Utr1p	Unclassified	266.4 ± 12.9	55.4 ± 14.3	−4.81
HSP12	Heat shock protein of 12 kDa, induced by heat, osmotic stress, oxidative stress and in stationary phase	Cytoplasm	3088.1 ± 342.0	657.4 ± 299.1	−4.70
SSA4	Member of 70 kDa heat shock protein family	Cytoplasm	405.7 ± 55.8	90.1 ± 21.0	−4.50
SWM1	Spore wall maturation	Nucleus	198.2 ± 39.0	46.3 ± 5.8	−4.28
YNL335W	Similarity to *M. verrucaria* cyanamide hydratase, identical to hypothetical protein YFL061W	Cytoplasm	158.1 ± 20.7	39.3 ± 25.5	−4.02
CPA2	Carbamyl phosphate synthetase	Cytoplasm	441.3 ± 83.0	113.0 ± 10.3	−3.90
AMS1	Vacuolar alpha mannosidase	Vacuole	302.4 ± 49.5	79.8 ± 17.6	−3.79
GPM2	Similar to *GPM1* (phosphoglycerate mutase)	Cytoplasm	66.2 ± 5.6	17.6 ± 4.2	−3.75
YOL153C	Strong similarity to Cps1p	Unclassified	31.9 ± 2.9	9.0 ± 2.8	−3.55
SSE2	*HSP70* family member, highly homologous to Sse1p	Cytoplasm	357.5 ± 59.6	101.7 ± 28.3	−3.51
HOR2	DL-glycerol-3-phosphatase	Cytoplasm	66.5 ± 1.2	19.8 ± 5.6	−3.36
FUN34	Putative transmembrane protein, involved in ammonia production	Nucleus	1617.8 ± 147.9	517.5 ± 101.2	−3.13
KNH1	*KRE9* homolog	Cytoplasm	142.9 ± 22.0	47.4 ± 8.6	−3.02
YNL115C	Weak similarity to *S. pombe* hypothetical protein SPAC23C11	Vacuole	141.7 ± 20.7	47.1 ± 6.3	−3.01
YDL199C	Similarity to sugar transporter proteins	Membranes	68.6 ± 5.9	25.1 ± 9.2	−2.73
ARO10	Similarity to Pdc6p, Thi3p and to pyruvate decarboxylases	Cytoplasm	67.2 ± 3.9	24.8 ± 3.7	−2.71

YJL037W	Strong similarity to hypothetical protein YJL038C	Unclassified	177.1 ± 20.6	65.7 ± 7.3	−2.69
STP2	Transcription factor for amino acid permeases	Cytoplasm	158.6 ± 21.0	60.3 ± 5.9	−2.63
YBL049W	Hypothetical protein	Unclassified	392.0 ± 16.1	149.2 ± 43.0	−2.63
NEJ1	Hypothetical protein	Cytoplasm	33.2 ± 3.9	12.7 ± 3.0	−2.62
PST1	Strong similarity to *SPS2*	Plasma membrane	735.8 ± 113.9	283.6 ± 46.2	−2.59
NHP6A	11-kDa non-histone chromosomal protein	Nucleus	321.1 ± 17.8	124.9 ± 6.4	−2.57
HYR1	Putative glutathione-peroxidase	Cytoplasm	705.3 ± 114.4	280.6 ± 16.7	−2.51
SSK1	Two-component signal transducer that with Sln1p regulates osmosensing MAP kinase cascade(suppressor of sensor kinase)	Cytoplasm	54.9 ± 7.6	22.0 ± 0.7	−2.49
RME1	Zinc finger protein, negative regulator of meiosis.	Cytoplasm	389.4 ± 56.3	157.5 ± 30.4	−2.47
YNL274C	Similarity to glycerate- and formate-dehydrogenases	Cytoplasm	302.5 ± 26.6	124.9 ± 37.6	−2.42
AIP1	Protein localizes to actin cortical patches. Probable binding site on actin lies on front surface of subdomain 3 and 4.	Cytoplasm	420.7 ± 39.7	187.6 ± 9.3	−2.24
YOR215C	Similarity to *M. xanthus* hypothetical protein	Mitochondria	322.5 ± 40.6	144.8 ± 20.5	−2.23
YOR086C	Weak similarity to synaptogamines	cell periphery	111.4 ± 11.4	51.2 ± 3.1	−2.18
YOL107W	YOL107W weak similarity to human PL6 protein	Cytoplasm	69.4 ± 8.1	32.4 ± 5.2	−2.14
PPT1	Serine/threonine phosphatase	Cytoplasm	48.5 ± 1.4	22.7 ± 5.5	−2.14
OSH2	Involved in sterol metabolism	Cytoplasm	176.4 ± 13.8	83.5 ± 17.7	−2.11
MSC3	Protein with unknown function	cell periphery	277.4 ± 27.3	131.5 ± 29.2	−2.11
UME1	Transcriptional modulator	Cytoplasm	36.3 ± 4.8	17.4 ± 0.5	−2.08
YIM1	Mitochondrial inner membrane protease	Cytoplasm	68.7 ± 6.2	33.0 ± 3.8	−2.08
ECM39	α-1,6-mannosyltransferase	ER	97.9 ± 8.8	47.1 ± 9.2	−2.08
DDR48	Flocculent specific protein	Cytoplasm	823.9 ± 52.3	399.7 ± 11.3	−2.06
PEP4	Vacuolar proteinase A	Mitochondria	1555.9 ± 65.4	755.3 ± 76.4	−2.06
YOR152C	Hypothetical protein	Cytoplasm	31.5 ± 2.6	15.3 ± 0.7	−2.06
RHK1	Putative Dol-P-Man dependent alpha(1–3) mannosyltransferase involved in the biosynthesis of the lipid-linked oligosaccharide	Cytoplasm	196.6 ± 8.7	97.1 ± 5.5	−2.02
YKL207W	Hypothetical protein	Unclassified	654.0 ± 52.2	323.2 ± 65.0	−2.02
PST2	Protein secreted by regenerating protoplasts	Cytoplasm	2053.2 ± 115.5	1014.9 ± 221.0	−2.02
DFG5	Protein required for filamentous growth, cell polarity, and cellular elongation	ER	393.2 ± 29.8	196.0 ± 23.6	−2.01

[a] Signal values given by the Affymetrix system. Values are expressed as average ± standard deviation of three independent replicates. As a reference, values for *ACT1* (encoding actin) were 2488.8 ± 81.0 (control cells) and 2329.2 ± 155.4 (CO_2 treated cells).

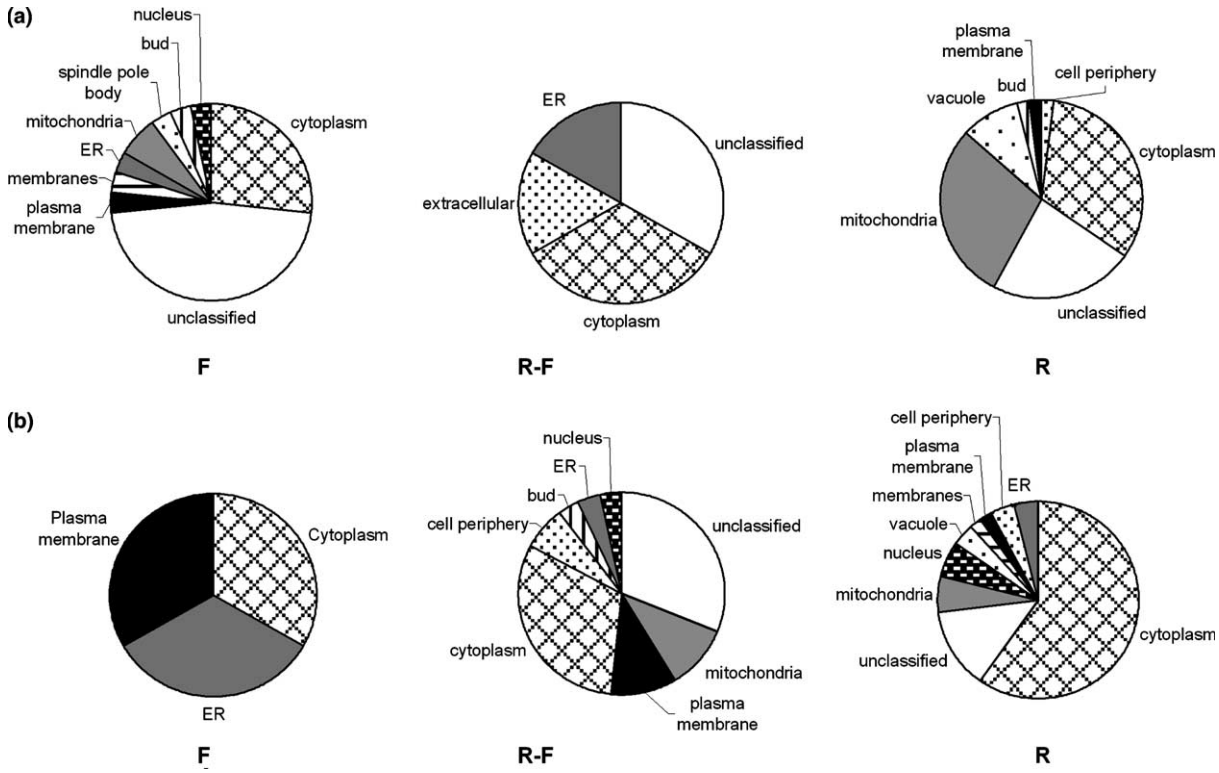

Fig. 1. Subcellular localization of genes whose mRNA levels were increased (a) or decreased (b) in the CO_2-treated cells (According to the MIPS database). **F** = anaerobic (fermentative). **R** + **F** = aerobic, N-limited (respiro-fermentative). **R** = aerobic, C-limited (respirative).

cultures, is necessary for assembly of the ATP-synthase complex [38].

PCK1, which encodes the gluconeogenic enzyme phosphoenolpyruvate carboxykinase [39] was strongly upregulated in the CO_2-enriched, aerobic glucose-limited chemostat cultures. Transcription of this gene is known to be extremely sensitive to glucose availability [40]. However, as other glucose-sensitive genes (including some of the *HXT* genes [41]) did not show clear responses to CO_2, it is unlikely that any changes in the low residual glucose concentrations contributed to the observed up-regulation of *PCK1* in the CO_2-enriched cultures. Consistent with the observed *PCK1* upregulation, PEPCK activity was strongly increased in the CO_2-treated cells with respect to the controls (values of 50.5 ± 13.3 and 5.6 ± 1.2 mU mg^{-1}, respectively). Another important carbon metabolism-related gene with an increased mRNA level in the CO_2-enriched aerobic, glucose-limited cultures was *PYC1*, one of the two genes for the anaplerotic enzyme pyruvate carboxylase. This enzyme is the only source of C-4 intermediates in glucose/ammonia-growing cells, and its absence prevents growth [42]. Pyruvate carboxylase activity was also increased upon CO_2 treatment (141.9 ± 6.1 with respect to 106.5 ± 3.6 mU mg^{-1}), although this minor difference hardly suggests biological significance. A strong transcriptional downregulation by CO_2 was observed for the *FDH1* gene, encoding formate dehydrogenase [43]. The highly similar *FDH2* gene also showed strongly decreased mRNA levels (37-fold, $p < 0.05$ in a *t*-test, although it did not pass the more restrictive SAM analysis). *ARO10*, encoding for a decarboxylase involved in phenylalanine metabolism [44,45] was also downregulated in the presence of elevated CO_2 concentrations. Other decarboxylase-encoding genes (e.g. *PDC1, PDC5* and *PDC6*) did not show a transcriptional response to CO_2.

In an attempt to identify robust CO_2-responsive 'signature transcripts', the three pairwise comparisons were combined (Fig. 2). When applying the robust criteria used in the SAM analysis, no genes were identified that showed a consistent response to carbon dioxide under all three aeration/nutrient limitation regimes (Fig. 2). In view of the relative insensitivity of the anaerobic cultures to CO_2 (Table 1), special attention was subsequently paid to the overlap between the CO_2-responsive gene sets for the glucose- and nitrogen-limited aerobic cultures. This comparison yielded eight genes, of which *DIP5*, encoding a dicarboxylic amino acid permease with high affinity for L-glutamine and L-aspartate [46], was regulated in opposite directions in the nitrogen- and carbon-limited cultures. Of the seven remaining genes, three (YAR075W, *COS12* and *MHT1*) were upregulated, and four (*KNH1, OYE3, NCE103*, and

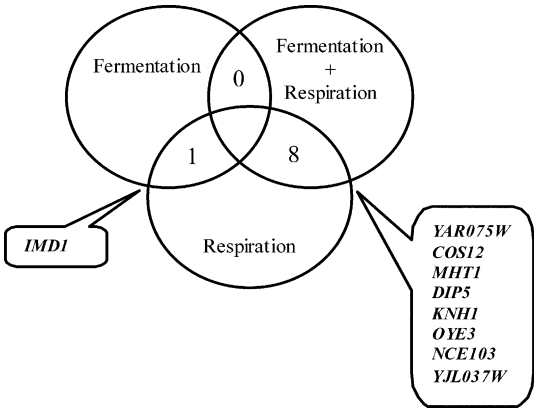

Fig. 2. Strategy for the identification of specifically CO_2-responsive genes. Among the three pools (corresponding to the three different metabolic conditions studied) of genes whose mRNA levels varied significantly at least twofold in the CO_2-treated cells, no genes were found in all three categories. Eight genes were commonly regulated in the aerobic experiments, and only one matched between the two carbon-limited cultures. Further analysis showed that this gene (*IMD1*) is highly homologous to YAR075W, which is in fact a putative pseudogene of the *IMD* family (see text for details).

YJL037W) showed decreased mRNA levels. YAR075W and *COS12* belonged to gene families. As, because the high degree of sequence identity, the Affymetrix microarrays cannot always discriminate accurately between the members of large gene families, the response of all genes of the family was considered, using a *t*-test to investigate significance (Table 5).

YAR075W is probably a pseudogene of the *IMD* family. This family, which further comprises *IMD1*, *IMD2, IMD3* and *IMD4*, is involved in the conversion of inosine mono-phosphate (IMP) into xanthosine-5-phosphate, the metabolic step that redirects flux to dGTP formation in the purine biosynthetic pathway [47]. Three of the four *IMD* genes showed an increased transcript level in response to elevated CO_2 under at least one of the aeration/nutrient limitation regimes (Table 5, $p < 0.05$ in a *t*-test, not all these differences passed the more stringent SAM analysis). A similar situation was observed for *COS12*. Members of the *COS* gene family are subtelomeric genes with unknown function [48]. Again, we found higher signal values of different members of the *COS* family in the CO_2-exposed cultures of the three metabolic situations (Table 5). However, since the expression level of *COS10* and *COS12* were only slightly above the detection limit, the biological significance of this observation is questionable (data not shown).

MHT1 was also transcriptionally upregulated in response to elevated CO_2 in both aerobic cultures. This gene encodes *S*-methylmethionine homocysteine methyltransferase, which is involved in the conversion of *S*-adenosylmethionine (AdoMet) into methionine [49]. AdoMet is the principal methyl donor for methylation of several cellular components, and is essential for cell cycle regulation [50].

With respect to the downregulated genes in the CO_2-enriched aerobic cultures (both under glucose limitation and under nitrogen limitation), *KNH1* has been implicated in cell wall synthesis, because its overexpression restored the low levels of β-1,6-glucan found in a *kre9* mutant [51]. *OYE3* is an intriguing gene. Together with its homologue *OYE2*, it encodes for an NADPH oxidoreductase (known as 'old yellow enzyme'). In spite of the existence of OYE proteins in several species [52], its physiological role is still unknown. *NCE103* is of special interest as it has sequence identity with carbonic anhydrase genes. Although its physiological role was initially enigmatic [31], a recent study reported that the Nce103 protein does indeed have carbonic anhydrase activity [53]. However, consistent with an earlier report [31],

Table 5
mRNA levels of *IMD* and *COS* genes in response to high CO_2

Gene name	Aerobic, N-limited		Aerobic, C-limited		Anaerobic, C-limited	
	Fold change	*p* value	Fold change	*p* value	Fold change	*p* value
IMD1	3.1	0.015	5.9	0.006	3.46	0.001
IMD2	2.0	0.004	5.9	0.001	1.50	0.007
IMD3	1.2	0.002	1.8	0.001	1.21	0.139
IMD4 (probe I)	1.7	0.008	2.4	0.04	1.86	0.059
IMD4 (probe II)	2.9	0.115	3.6	0.15	5.30	0.017
COS2	1.6	0.012	3.8	0.011	1.17	0.506
COS3	1.6	0.002	3.5	0.007	1.04	0.859
COS4	1.2	0.093	2.1	0.002	0.88	0.04
COS5	1.4	0.163	2.2	0.022	1.30	0.236
COS6	1.3	0.134	1.5	0.125	1.13	0.666
COS7	1.4	0.272	3.0	0.075	1.34	0.653
COS8	1.3	0.425	1.2	0.558	1.37	0.5
COS9	0.9	0.663	1.7	0.066	1.66	0.121
COS10	3.5	0.14	3.5	0.162	3.86	0.008
COS12	5.0	0.002	6.9	0.001	1.31	0.357

we were unable to detect carbonic anhydrase activity in cell extracts. This may indicate that published procedures for assaying this enzyme activity are not suitable for crude extracts of *S. cerevisiae*. The mRNA level of *NCE103* was also significantly reduced in the anaerobic CO_2-exposed cultures ($p < 0.05$ in a *t*-test, rejected by the more stringent SAM analysis). With respect to YJL037W, little is known about its biological role. Genome-wide analysis revealed a transcriptional up-regulation of this ORF in respiratory-deficient *petite* mutants [54], and also in cells defective in NAD^+ synthesis [55], suggesting a possible role in mitochondrial redox metabolism.

3.3. Promoter analysis

With the aim to find possible regulatory sequences involved in CO_2-dependent regulation of transcription, promoter sequences of CO_2-responsive genes were analysed via two approaches. First, RSA Tools software [29] was used to identify over-represented motifs in the promoter sequences. We analysed the 800 bp upstream of the start codon (avoiding possible overlaps with preceding genes) in the CO_2 up- and downregulated genes all together and separately. This yielded a six-base sequence (ACTCTA) present at least once in five of the matching CO_2-responsive genes (Table 6). This sequence was also found in the *IMD1* and *IMD3* promoters, but not in the promoters of the other *IMD* and *COS* genes. Secondly, promoter sequences were aligned to identify homologous regions. We found a sequence of eight nucleotides (TTCCTCCC) at −192 and −185 base pairs of the start codon of YJL037W and *NCE103*, respectively. This sequence has a very low genomic coverage in *S. cerevisiae* promoter regions: only 0.84% of the yeast ORFs have this sequence within the 300 bp preceding the start codon (Table 6). This sequence does not match any described regulatory consensus sequence and was not found in the promoters of other genes downregulated by CO_2.

4. Discussion

4.1. Physiological responses to CO_2

Exposure of aerobic fed-batch cultures to elevated CO_2 concentrations has been previously reported to have strong negative effects on *S. cerevisiae* [5]. In another study, CO_2 only weakly affected biomass yield in oxygen-limited chemostat cultures [7]. The present study supports the notion that, under atmospheric pressure, CO_2 saturation does not have a strong impact on fermentative growth and metabolism of *S. cerevisiae*. From an evolutionary perspective, this is not surprising. Natural and man-made environments in which *S. cerevisiae* exhibits a fermentative metabolism are likely to become saturated with CO_2. Conversely, environments in which *S. cerevisiae* exhibits a completely respiratory metabolism are fully aerobic [56], which requires efficient gas transfer, thus making CO_2 saturation less likely. In view of the higher oxygen consumption rate in the aerobic, glucose-limited chemostat cultures exposed to high CO_2, the effects of CO_2 could be described as metabolic uncoupling: a decrease of the biomass yield on glucose coinciding with an increased flux through dissimilatory glucose metabolism. Together with the transcriptional responses of several genes involved in mitochondrial respiration, this suggests that energy coupling of respiration may be the primary target of CO_2 in respiring yeast cultures. A molecular mechanism consistent with our observations is bicarbonate activation of ATP hydrolysis by the mitochondrial F_1/F_0 ATPase/synthase, a phenomenon that has been extensively investigated in vitro [57–59].

The decrease of biomass yield observed in the aerobic, glucose-limited chemostat cultures grown at elevated CO_2 concentrations (≈24%) was not as pronounced as the yield decrease previously reported for fed-batch cultures exposed to CO_2 excess [5]. While effects of strain background or experimental details cannot be ruled out, this suggests that steady-state chemo-

Table 6
Common sequences found in the promoter regions of CO_2-responsive genes

Sequence	Gene	Position	Genome coverage[a]
ACTCTA	YAR075W	−255	19%
	DIP5	−10 −119 −554	
	COS12	−627	
	NCE103	−144 −704	
	YJL037W	−128 −266	
	IMD1	−730	
	IMD3	−760 −177	
TTCCTCCC	*NCE103*	−195	1.4% (−800 to 0)[b]; 0.84% (−300 to 0)[c]
	YJL037W	−192	

[a] Percentage of yeast genes holding the sequence in the promoter (according to RSA Tools, 6450 ORFs considered).
[b] Considering as promoter the 800 nucleotides upstream the start codon (unless overlapping with the preceding ORF).
[c] Considering as promoter the 300 nucleotides upstream the start codon (unless overlapping with the preceding ORF).

stat cultivation allows for a better physiological adaptation to excess CO_2 than the dynamic conditions of fed-batch cultivation. A similar phenomenon has been observed in *Aspergillus* [60].

4.2. Transcriptional responses to CO_2

In comparison with other changes in environmental parameters in chemostat cultures, such as oxygen availability [13,61] and nature of the growth-limiting nutrient [14], increasing the CO_2 concentration had a relatively small impact on the *S. cerevisiae* transcriptome. The number of CO-responsive genes correlated well with the physiological effects of elevated CO_2 levels, being highest in aerobic, respiratory cultures. This observation supports the notion that, under anaerobic, glucose-limited conditions and at atmospheric pressure, 'CO_2 stress' does not exist in *S. cerevisiae*. Further research should address the question to what extent and how CO_2 affects physiological performance and transcriptional regulation of anaerobic yeast cultures under other nutrient limitation regimes and at hyperbaric pressures.

For most of the eight genes that showed a consistent transcriptional response to CO_2 in the aerobic cultures, we were unable to establish a clear link with carbon dioxide. A clear exception was *NCE103*, which has originally been reported to encode a protein involved in non-classical protein secretion [62]. Recent sequence comparisons and heterologous complementation studies [53] have indicated, however, that *NCE103* encodes carbonic anhydrase, the enzyme that catalyses the interconversion of $CO_2 + H_2O$ and H_2CO_3. In spite of this, carbonic anhydrase activity could not be detected in crude extracts of wild-type cells or *NCE103* overexpressing strains [31], which is consistent with our results. Cells lacking *NCE103* are unable to grow aerobically on glucose, but are not pH-sensitive, suggesting that this gene may also be involved in protection against oxidative stress [31]. *NCE103* is transcriptionally induced by a variety of natural stresses, including high pH [12,63], as well as in respiratory-deficient mutants [54]. A possible physiological role of carbonic anhydrase is the provision of HCO_3^- for the anaplerotic pyruvate carboxylase reaction. This would be consistent with the observed upregulation of *NCE103* under low-CO_2 conditions, where spontaneous bicarbonate formation may be too slow to meet metabolic demands. It remains to be investigated whether the common sequence motif found in the promoters of *NCE103* and YJL037W is indeed involved in CO_2 sensing.

An unexpected result from the transcriptome analysis was the strong upregulation, in aerobic glucose-limited cultures grown with elevated CO_2, of *PCK1*, the gene for the gluconeogenic enzyme phosphoenolpyruvate carboxykinase. This enzyme activity was also increased in these cultures. The physiological direction of the reaction catalysed is towards phosphoenolpyruvate [64], but the reaction is reversible in vitro [65]. Based on our observations, it is tempting to speculate that PEPCK may function as an alternative anaplerotic enzyme under high-CO_2 conditions.

The transcriptional response of several of the members of the *IMD* gene family, involved in purine biosynthesis, was remarkable. Lack of a functional member of this gene family results in guanine auxotrophy [66]. *IMD1* has been reported to be transcriptionally silent, but the expression of *IMD2, IMD3* and *IMD4* is repressed by guanine. In addition, *IMD2* transcription is increased by the addition of mycophenolic acid (MPA), a drug that inhibits IMDp activity [47,66]. A clear relationship between purine biosynthesis and CO_2 has been reported in the literature: the adenine auxotrophy of *ade2* null mutants can be complemented by incubation with high CO_2 concentrations [67]. However, since Ade2p functions in the common branch of purine metabolism, it is unclear whether and how this CO_2 effect is related to the transcriptional upregulation of IMD genes.

Most of the CO_2-responsive transcripts identified in this study have previously been shown to respond to other cultivation conditions as well. Still, when used in combination, they may be applicable for diagnosing the CO_2 status of aerobic *S. cerevisiae* cultures. However, a more detailed analysis of the physiological role of the encoded proteins under high-CO_2 conditions is required before true 'signature transcripts' for CO_2 stress can be identified.

Acknowledgements

We thank Jean-Marc Daran for his helpful advice on data handling. The research group of J.T.P. is part of the Kluyver Centre for Genomics of Industrial Fermentation, which is supported by The Netherlands Genomics Initiative. J.A. is supported by a Marie Curie postdoctoral fellowship.

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