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Response of *Saccharomyces cerevisiae* to Stress-Free Acidification

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Genome-wide transcriptional analysis of a *Saccharomyces cerevisiae* batch culture revealed that more than 829 genes were regulated in response to an environmental shift from pH 6 to pH 3 by added sulfuric acid. This shift in pH was not detrimental to the rate of growth compared to a control culture that was maintained at pH 6 and the transcriptional changes most strikingly implicated not up- but down-regulation of stress responses. In addition, the transcriptional changes upon acid addition indicated remodeling of the cell wall and central carbon metabolism. The overall trend of changes was similar for the pH-shift experiment and the pH 6 control. However, the changes in the pH 6 control were much weaker and occurred 2.5 h later than in the pH-shift experiment. Thus, the reaction to the steep pH decrease was an immediate response within the normal repertoire of adaptation shown in later stages of fermentation at pH 6. Artificially preventing the yeast from acidifying the medium may be considered physiologically stressful under the tested conditions.

***Keywords*:** stress response, *Saccharomyces cerevisiae*, acidification, fermentation, pyruvate decarboxylase

Saccharomyces cerevisiae is used in the food and biotechnology industries and naturally acidifies its environment while maintaining the intracellular pH around neutral. Nevertheless, stress responses have been observed when yeast were artificially exposed to weak organic acids like acetic, lactic, succinic or citric acid (Causton *et al.*, 2001; Lawrence *et al.*, 2004; Kawahata *et al.*, 2006), food preservatives such as sorbic acid (Schuller *et al.*, 2004) or when the pH was lowered by addition of hydrochloric acid (Kawahata *et al.*, 2006). Responses to acidification that were highlighted by these authors included, for example, decreased growth, a global Msn2/Msn4 induced transcriptional change that is observed upon many environmental challenges, down-regulation of ribosome synthesis, up-regulation of an ATP-dependant efflux pump encoded by *PDR12*, up-regulation of intracellular metal metabolism genes under the control of Aft1p, and cell wall remodelling. These studies help to understand how yeast cells adapt to and survive stressful changes in the environment.

Scientific studies and commercial bioprocesses are often conducted under firmly controlled conditions including the control of pH at a constant value. However, our group found that the production of the biocatalyst pyruvate decarboxylase increased 2.7-fold when the pH control was switched off after 13 h cultivation, allowing *Candida utilis* to naturally acidify the fermentation broth from pH 6 to pH 2.9 (Chen *et al.*, 2005). A similar result was obtained when the pH was abruptly lowered from pH 6 to pH 3 by the addition of sulfuric acid. In contrast, cultivation at constant pH 3, 4, 5, or 6 did not result in such improved enzyme production. Thus, the transitional pH change rather than the absolute pH value was responsible.

The effect of physiologically stressful acidification treatments on yeast cells has been well characterised, particularly for *Saccharomyces cerevisiae* (Causton *et al.*, 2001; Piper *et al.*, 2001; Kawahata *et al.*, 2006). In such studies, treatment conditions are intended to be physiological challenges and usually result in impaired growth rates (e.g. Kawahata *et al.*, 2006). However, in both cases of acidification for *C. utilis* described in our previous publication (Chen *et al*., 2005), the rates of cellular growth and glucose consumption remained mostly unaffected, as would be preferred for many industrial bioproccesses. Thus, these acidification conditions did not appear to be very stressful.

In order to better characterise the response of yeast to acidification under favourable growth conditions, the present study investigated the genome-wide transcriptional response to a shift from pH 6 to pH 3. *S. cerevisiae* was chosen as a model organism rather than *C. utilis* since it is used in many commercial processes, and because of the availability of extensive *S. cerevisiae* microarray resources and published studies for comparison.

Materials and Methods

Cultivation conditions and metabolite assays

Pre-seed cultures of *Saccharomyces cerevisiae* strain Y-156

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(ATCC 2389) were grown in an orbital shaker at 30°C and 250 rpm in 500 ml baffled Erlenmeyer flasks with 25 ml defined medium containing 20 g/L glucose, 5 g/L ammonium sulfate, 1.7 g/L yeast nitrogen base without amino acids (YNB) (Difco) and 200 mM 2-[*N*-morpholino]ethanesulfonic acid (MES) at pH 6.0. Seed cultures were grown as for the pre-seed culture, but in 50 ml defined medium. Three hundred mililiters of the exponentially growing seed culture was transferred to a 5 L bioreactor with a working volume of 3 L. The bioreactor cultivation medium had higher glucose, ammonium sulfate, and YNB concentrations (100 g/L, 10 g/L, and 8.5 g/L, respectively) and was not buffered with MES. Temperature, aeration, and stirrer speed were maintained at 30°C, 0.1 vvm and 300 rpm. The dissolved oxygen concentration remained above 45% air saturation throughout the cultivation. Since *S. cerevisiae* is a Crabtree-effect positive yeast, the high glucose concentration would have suppressed respiratory metabolism. The pH was controlled by the addition of 5 M sodium hydroxide or 5 M sulfuric acid. For the control experiment, the pH was maintained at 6.0. For the 'pH 3 shift' experiment, the pH was maintained at 6.0 until approximately 20 g/L glucose were consumed (10 h). The culture pH was then decreased to 3.0. Analysis of metabolites, dry cell mass (DCM) and preparation of cells for enzyme activity were carried out according to Chen *et al.* (2005). Pyruvate decarboxylase activity of permeabilized cells was determined as the rate of phenylacetyl carbinol formation from pyruvate and benzaldehyde, as described in Rosche *et al.* (2002).

Cell harvesting and RNA extraction

Samples were harvested from both the constant pH 6 and the pH 3 shift cultures after 10 h of growth (microarray time point zero) and then at 20, 40, 60, 90, 115, 180, and 240 min after time point zero. Cells from 40 ml of culture were collected by centrifugation, washed with sodium acetate buffer (50 mM sodium acetate, 10 mM EDTA, pH 5.5) and frozen at -80°C. Total RNA was extracted using the AE-phenol procedure described in Schmitt *et al.* (1990). This was followed by on-column DNase I digestion and clean-up using an RNeasy kit from QIAGEN (Australia). RNA purity and integrity were checked by UV spectrophotometry and denaturing agarose-gel electrophoresis.

cDNA labeling and microarray hybridization

Spotted oligonucleotide microarrays were obtained from the Ramaciotti Centre for Gene Function Analysis (University of New South Wales, Sydney, Australia). These carried 40-mer oligonucleotide probes to 6,250 *S. cerevisiae* open reading frames (MWG Biotech, Germany) printed in duplicate on epoxy-coated glass microarray slides (Eppendorf, Germany). RNA labeling and hybridization were carried out as described in Gelling *et al.* (2004). Two-colour labelling was used, with all samples from each cultivation being co-hybridised against one pooled time-zero reference sample from the same cultivation. Each slide was prepared in at least duplicate, using reciprocal or 'dye-swap' labeling.

Microarray data collection and analysis

The microarray slide image was obtained and processed using GenePix Pro 6.1 software (Axon Instruments). The signal of a gene was deemed present if the signal intensity of the spot was above the background and there was no artefact associated with the spot. The processed data were then normalized and statistically analysed using GeneSpring 7.1 software (Silicon Genetics, USA). Fluorescence intensities were normalized by the LOWESS method and Welch's analysis of variance (with a Benjamini-Hochberg multiple testing correction) was applied in order to determine which genes showed significantly changed expression during either time course (Supplementary data Table 1).

829 genes showed a significant change in expression relative to zero time in either or both of the cultivations with an estimated 3% false discovery rate. The average expression data for these genes was then hierarchically clustered using the programme Cluster 3.0 (Eisen *et al.*, 1998) using single linkage clustering of the centered Pearson's correlation co-efficient. The output of a 6×6 self-organising map was used to determine leaf order of the dendrogram.

In order to compare the data to two other studies (Causton *et al.*, 2001; Kawahata *et al.*, 2006), the data sets were combined in Genespring 7.1. Since replicate values were not available for the data in Kawahata *et al.* (2006), expression data from those genes that showed a change greater than 1.5-fold in any experiment were selected as representative of statistically significant changes. Clustering was performed as above, except an 8×8 self-organising map was used for leaf order determination. Groups of co-expressed genes from selected dendrogram nodes were analysed using the FunSpec web server (Robinson *et al.*, 2002), which uses the hypergeometric distribution to determine whether any functional annotations are statistically over-represented amongst a group of genes. The reader should refer to the SGD database (www.yeastgenome.org) for gene specific information when references are not cited in the text. The microarray data associated with this paper has been deposited with the EMBL/EBI ArrayExpress database, under the accession E-MEXP-1602 (http://www.ebi.ac.uk/microarray-as/ae).

Results

Pyruvate decarboxylase activity and glucose consumption increased following a shift from external pH 6 to pH 3

Two *S. cerevisiae* cultivations were performed in parallel in medium containing 100 g/L glucose. In the first experiment, the pH was maintained at pH 6 throughout the experiment, and in the second the pH was maintained at pH 6 for the first 10 h of cultivation and was then adjusted to pH 3 with sulfuric acid and maintained at that pH for the remainder of the experiment (referred to as the pH 3 shift experiment). Figure 1A~C shows the growth profile of the pH 6 experiment, which reached final concentrations of 36.1 g/L ethanol, 13.1 g/L glycerol, and 7.0 g DCM/L biomass after 21 h. In the pH 3 shift experiment (Fig. 1D~F), similar final concentrations were reached (37.4 g/L ethanol, 10.5 g/L glycerol, and 6.6 g DCM/L biomass) some 3 h earlier. Accordingly, the rates of glucose consumption, ethanol production and increase in biomass over the 4 h following acid addition were statistically significantly higher in the pH 3 shift ex-

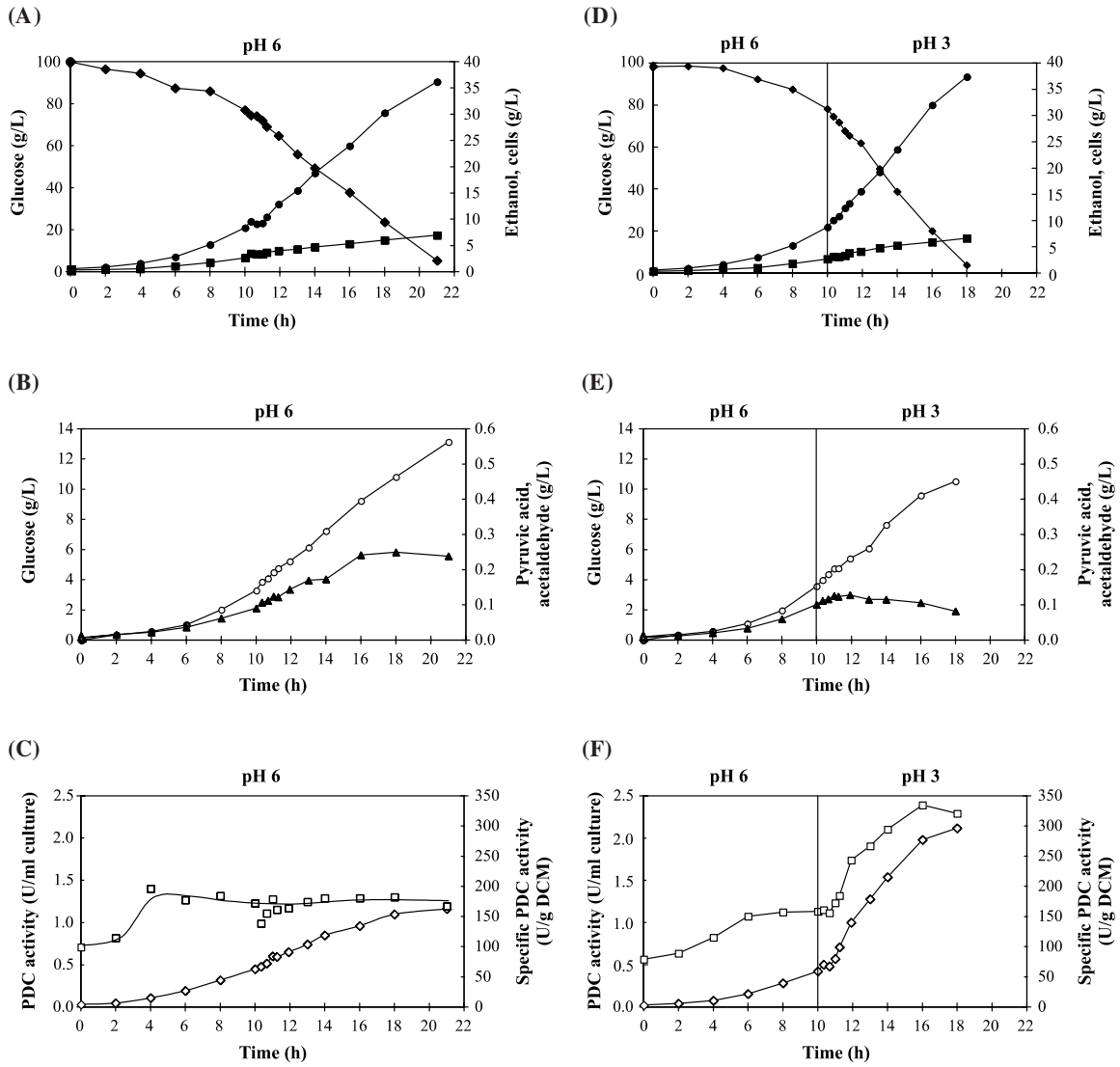

Fig. 1. *S. cerevisiae* cultivation profiles at pH 6 (A~C) and at pH 6 with a shift to pH 3 after 10 h (D~F). (A) and (D), Growth profile: (□) glucose, (■) cell concentration, and (●) ethanol. (B) and (E), Fermentation by-products: (○) glycerol and (▲) pyruvic acid. (C) and (F), Pyruvate decarboxylase (PDC) production: (◊) volumetric PDC activity, and (□) specific PDC activity. All data are the average of duplicate measurements.

periment than in the equivalent period of the pH 6 experiment (Table 1). The average specific rates of glucose consumption (i.e. per g biomass) were 1.9 g/g/h±0.58 at pH 6 and 2.6 g/g/h±0.57 in the pH 3 shift experiment (±95% confidence intervals), although the variance introduced into the Figures by performing the biomass correction was too high to draw firm conclusions from this. Thus it is not clear whether the observed increase in cell growth in the pH 3 shift experiment was sufficient to explain the increased rate of volumetric glucose consumption.

Table 1. Rates of cell growth and product formation

	pH 6 experiment[a]	pH 3 shift experiment[a]	*P*-value[b]
Biomass accumulation rate (g/L/h)	0.441	0.640	7.7×10^{-4}
Glucose consumption rate (g/L/h)	7.16	9.60	4.3×10^{-7}
Ethanol accumulation rate (g/L/h)	2.63	3.64	8.8×10^{-8}
Glycerol accumulation rate (g/L/h)	0.933	0.930	0.94

[a] calculated by linear regression over the data points between 10 h and 14 h of the time-course
[b] estimated probability that the slopes of the regression lines obtained from the two experiments would differ by the observed amount by chance

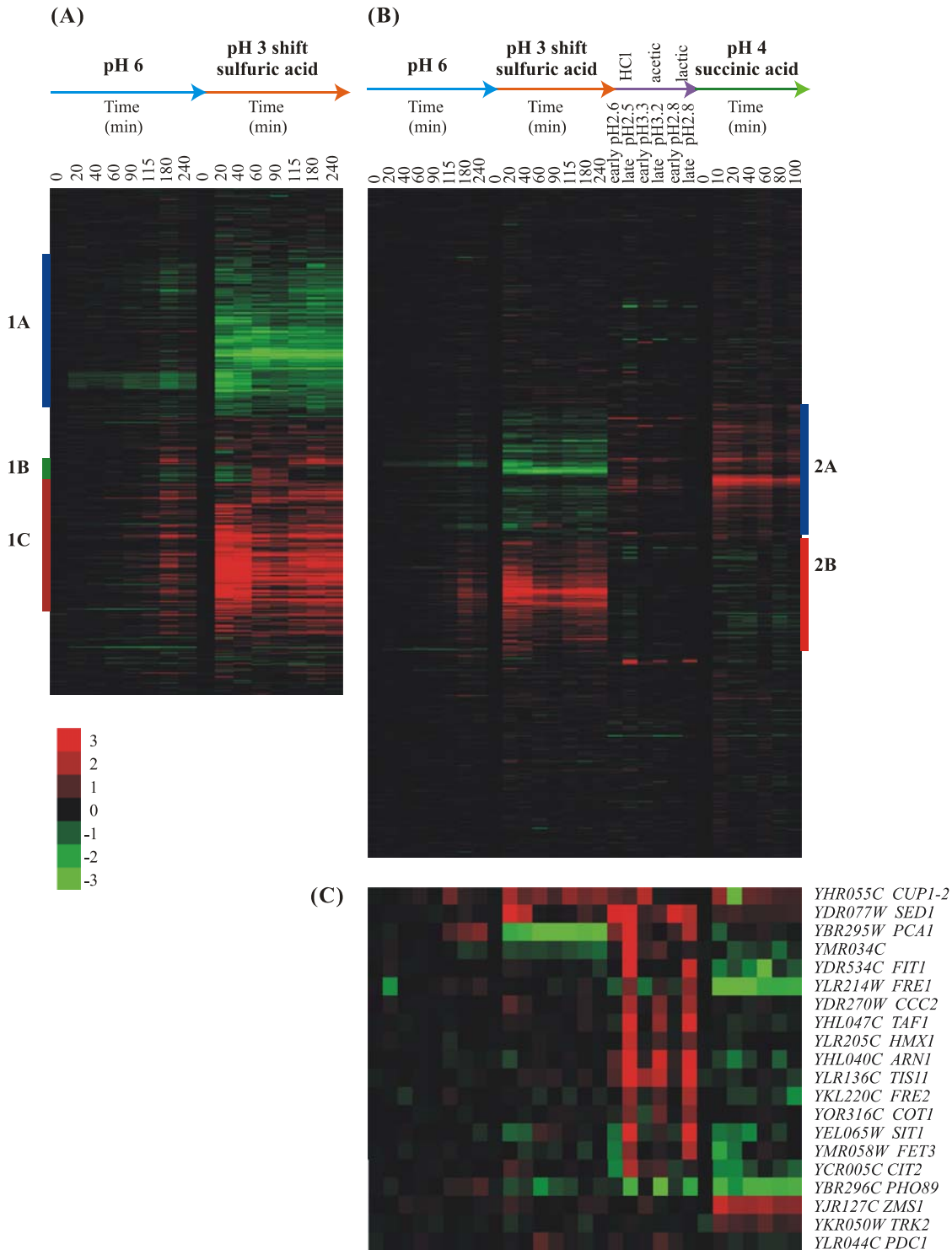

Fig. 2. Cluster analyses of gene expression following acidification of the growth medium. Color intensity is proportional to the $\log_2$ fold-change compared to the zero time sample or control. (A) Clustered expression data from cells maintained at pH 6 and those shifted from pH 6 to pH 3. Only data for those genes with statistically significant expression changes are shown. (B) Clustered expression data from experiments in (A) in combination with other published data. The succinic acid data is from Causton *et al.* (2001) and the hydrochloric, acetic, and lactic acid data are from Kawahata *et al.* (2006). 'Early' and 'late' refer to the experiments described as 'shock' and 'adaptation' in Kawahata *et al.* (2006). Only genes that changed by greater than 1.5 fold in at least one condition are shown. (C) Expression data of selected genes. The first 15 are those connected with metal metabolism identified in Kawahata *et al.* (2006) as being induced by longer-term acid treatment. The next four genes were identified in Causton *et al.* (2001) as having pH-modulated expression levels.

In addition, after the shift to pH 3, there was a sharp increase in the pyruvate decarboxylase (PDC) activity (Fig. 1F), as previously observed for *Candida utilis* (Chen *et al.*, 2005). Accordingly, the accumulation of extracellular pyruvic acid ceased shortly after the pH shift, although the total accumulated under both conditions was low (Fig. 1B and E). Even at pH 6 (Fig. 1B), *S. cerevisiae* accumulated much less pyruvate than *C. utilis* (less than 0.3 g/L versus 11 g/L reported by Chen *et al*., 2005), but *S. cerevisiae* produced more than 10 g/L glycerol which was not detected in the *C. utilis* culture. However, it is important to note that different cultivation media have been used for the two organisms.

Taken together, these data indicate that there is some metabolic adjustment in response to the pH 3 shift and that the decreased pH was not deleterious to yeast growth under these conditions, but instead slightly beneficial.

The genome-wide transcriptional response to a shift from pH 6 to pH 3 indicates remodelling of central carbon metabolism and the cell wall

Samples were taken from the experiments described above over a four-hour time-course following the tenth hour of the cultivation (i.e. time zero of the pH shift) and two-colour microarray analysis was performed. Each sample from the two different pH regimes was compared to a time zero reference control from the corresponding time course. This allowed the transcriptional changes occurring with time in the pH 6 culture to be identified, as well as the effect of acidification on these changes. 829 genes showed a statistically significant expression change at any time-point relative to the corresponding time zero sample. The expression data from these genes were hierarchically clustered as depicted in Fig. 2A. These data revealed that some progressive transcriptional change did indeed occur with time in the pH 6 experiment. The most obvious difference between the two conditions was largely the magnitude and timing of transcriptional responses. That is, similar groups of genes were up- or down-regulated with time for both pH regimes but the magnitude of transcriptional responses was generally much higher for the pH 3 shift than the pH 6 experiment and highest at the beginning of the time-course rather than the end. This indicates that the major transcriptional response to acidification of the external pH was an amplification of the basal transcriptional programme occurring during fermentation at a pH fixed at 6.

To examine the functions of the regulated genes, three cluster nodes were selected that captured the major expression patterns observed and the genes from these nodes were analysed for significantly over-represented functional annotations (Table 2). Cluster 1A contained mostly genes that were down-regulated after time zero in both experiments, and included an over-representation of gluconeogenic and TCA cycle genes. Cluster 1B contained mostly genes that were up-regulated in the pH 6 experiment but in the pH 3 shift experiment were initially down-regulated and subsequently up-regulated. This cluster had an over-representation of genes with functions required for RNA transcription and processing. Cluster 1C contained mostly genes that were up-regulated after time zero in both experiments. It included several genes involved in cell wall biogenesis, including *SED1*, a gene previously demonstrated to be induced under acidic conditions but to impart acid resistance when deleted (Kawahata *et al.*, 2006) (Fig. 2C).

There were no indications from our data that the Aft1p-regulated iron regulon was induced in the pH 3 shift experiment, a phenomenon observed by Kawahata *et al.* (2006) during longer-term but not short-term acid treatment (Fig. 2C). In fact, *PCA1*, a cadmium transporter gene identified in that experiment as being co-induced with the iron regulon, was strongly down-regulated in the pH 3 shift experiment. Interestingly, the sodium/phosphate transporter gene *PHO89*, which is directly upstream on the opposite strand to *PCA1* (and which may therefore potentially share promoter elements) was also down-regulated in the pH 3 experiment (Fig. 2C). *PHO89* expression has been shown to be pH-dependent, being down-regulated at low pH and up-regulated at high pH (Causton *et al.*, 2001; Serrano *et al.*, 2002).

These data also revealed that the increased PDC activity that occurs after the shift to pH 3 is not the result of up-regulation at the level of transcription. None of the three genes encoding a pyruvate decarboxylase enzyme (*PDC1*, *PDC5*, *PDC6*) showed overall expression changes that passed the test for statistical significance (Fig. 2C).

The shift from pH 6 to pH 3 resulted in an alleviation of the transcriptional stress response

Induction of stress-responsive genes after the shift to pH 3 was not evident from the data described above. This is in contrast to the previously observed acid activation of the Msn2/4p-mediated stress response reported by others (Causton *et al.*, 2001; Serrano *et al.*, 2002; Lawrence *et al.*, 2004; Schuller *et al.*, 2004; Kawahata *et al.*, 2006). In order to compare our observations of the response to medium acidification with other published acid responses, our complete dataset was combined with the acid shock and adaptation datasets published by Kawahata *et al.* (2006) and the organic acid treatment experiment from Causton *et al.* (2001). Hierarchical clustering across all the experiments was performed on those genes that showed a greater than 1.5-fold change in any experiment (Fig. 2B). This revealed that few genes were consistently up- or down-regulated across all three datasets, but there was a clear inverse correlation between the expression of 1062 genes in the pH 6 and pH 3 shift experiments and the acid treatments described in Causton *et al.* (2001) and Kawahata *et al.* (2006) (nodes 2A and 2B of Fig. 2B). That is, a large group of genes that were up-regulated in our experiments were down-regulated in the other acid treatments and *vice versa.*

Analysis of the functions over-represented amongst this group of co-regulated genes revealed that many are associated with the response to stress (Table 2). For instance, genes annotated as stress-responsive were down-regulated in the pH 3 shift experiment and the later stages of the pH 6 experiment, but up-regulated in the other acid treatment experiments. Genes involved in trehalose biosynthesis and ubiquitination showed a similar expression pattern to the 'stress-responsive' genes and have previously been shown to be up-regulated in response to a large number of environmental stresses (Gasch *et al.*, 2000; Causton *et al.*, 2001). Genes involved in ribosome biogenesis and assembly, RNA

modification and processing, which are often observed to be down-regulated in response to stress (Gasch *et al.*, 2000; Causton *et al.*, 2001), were also down-regulated by the acid treatments described in Causton *et al.* (2001) and Kawahata *et al.* (2006) but were up-regulated in the present pH 3 shift experiments.

In addition to changes in these stress-related genes, genes involved in aspects of respiratory metabolism, including the TCA cycle, the electron transport chain and mitochondrial protein synthesis were down-regulated in the pH 3 shift experiment and up-regulated in the other acid-treatment experiments. Up-regulation of respiratory genes is not usually considered a feature of the common environmental stress response, but has been observed in response to several individual stresses, including heat shock (Gasch *et al.*, 2000).

In summary, these results have shown that a shift of culture pH from 6 to 3 that had increased the yield of pyruvate decarboxylase in *C. utilis*, also increased the yield of pyruvate decarboxylase in *S. cerevisiae*, and resulted in slightly improved *S. cerevisiae* growth and volumetric glucose consumption rates compared to a culture maintained at pH 6. The transcriptional changes occurring during this pH shift indicated down-regulation of the environmental stress response, some cell wall remodelling and a down-regulation of genes involved in respiratory metabolism.

Discussion

Several previous studies have identified a large number of genes that are similarly transcriptionally regulated in res-

Table 2. Over-represented functional categories amongst clustered genes (Nodes of dendrograms for clusters in Fig. 2)

Node	Gene ontology category	*P*-value	Genes in node from functional category
1A	Tricarboxylic acid cycle [GO:0006099]	2.45×10^{-3}	*KGD2 ACO1 IDH1 CIT3*
	Gluconeogenesis [GO:0006094]	8.90×10^{-3}	*PYC2 ENO2 SIP4 FBP26*
1B	Transcription, DNA-dependent [GO:0006351]	1.58×10^{-3}	*NHP6B RAI1 CBF5 TAF4 RPC31 DIS3 ESA1*
1C	Cell wall organization and biogenesis [GO:0007047]	1.63×10^{-3}	*ECM8 KTR3 LRE1 SED1 ECM12 CAP2 SDP1 CIS3 PIR1 ROT1 GAS1 ECM3*
2A	Response to stress [GO:0006950]	2.70×10^{-5}	*PIM1 UBC4 IST2 TPS1 YCL033C GRX1 TRX3 GPD1 YPD1 NTH1 TPS2 SAC6 HSP42 HSP78 DPL1 TTR1 SSA4 HSP12 HAC1 CPR2 GRE3 CAP1 MSN4 MYO3 MCR1 HSP104 UBI4 TSL1 MSN2 TPS3 YKU70 YGP1 CRS5 GCY1 YDC1 HSP82*
	Electron transport [GO:0006118]	2.33×10^{-5}	*SDH4 RIP1 CYC7 QCR8 CYC1 SDH1 MCR1 NDI1 CYT1*
	Alpha, alpha-trehalose-phosphate synthase complex (UDP-forming) [GO:0005946]	6.31×10^{-5}	*TPS1 TPS2 TSL1 TPS3*
	Mitochondrial ribosome [GO:0005761]	8.38×10^{-5}	*MRPS9 IMG1 MRPL11 MRPL28 RSM23 MRPL25 RSM27 MRPL9 MRPL6 RSM7 MRP17 MRPL38 YMR188C MRPL19 MRPL10 MRP51 MRPL40*
	Proton-transporting ATP synthase complex, catalytic core F(1) [GO:0045261]	2.93×10^{-4}	*ATP1 ATP3 ATP16 ATP2*
	Tricarboxylic acid cycle [GO:0006099]	1.22×10^{-3}	*KGD2 SDH4 LSC2 MDH1 SDH1 CIT1*
	Polyubiquitination [GO:0000209]	8.38×10^{-3}	*UBC4 PIB1 UBC8 RSP5 HUL5 UBI4*
2B	RNA processing [GO:0006396]	3.19×10^{-4}	*PRP6 TRM7 MUD1 MAK5 PRP5 PTC1 DBP10 PRP11 TRM8 NHP2 CWC2 CTH1 SNU13 PRP22 SPB4 CEG1 PRP31 MTR3 PUS6 NAM8 IMP3 UTP9 MRS1 GCD14 LSM8 ISY1 UTP11 CFT2 DUS1 UTP15 STO1 CTL1 RLP7 NOP2 GCD10 PRP2 SMM1 RAT1 FHL1*
	Ribosome biogenesis [GO:0007046]	5.61×10^{-3}	*MAK16 MAK5 KRR1 DBP10 NHP2 SNU13 SPB4 NOP7 MTR3 IMP3 UTP9 MAK11 UTP11 UTP15 RLP7 NOP2 RIA1 RAT1 FHL1*
	Nucleus [GO:0005634]	2.43×10^{-3}	*MAK16 REB1 PRP6 RDH54 MUD1 MAK5 RPB5 PRP5 ENP1 DPB3 KRR1 TAF2 HCM1 MED2 CDC7 DBP10 PRP11 TSR1 UGA3 LYS20 NHP2 CWC2 HO MSH6 INO2 CTH1 EBS1 MET32 MSN5 SNU13 PRP22 MOT2 SPB4 NIC96 NAB2 CEG1 RAD54 NMA2 PRP31 NOP7 SRB5 MTR3 BRF1 NAM8 SET1 IMP3 UTP9 SKN7 MET30 MET18 SSL2 CTK2 RRN7 NUP192 NUP82 ARP4 HPR5 GCD14 LSM8 POL32 ISY1 MOG1 RGT1 NUP100 UTP11 SLD2 RPC25 DBR1 MLP1 NSE1 POM34 RIC1 CFT2 ACE2 SLX4 SEC13 NBP1 FPR3 GTR1 MCM1 UTP15 STO1 NUP53 CTL1 HAS1 RLP7 NOP2 GCD10 NIS1 NOP13 PRP2 GAL11 HIR2 DBP5 RAT1 NUP1 RPB8 SNF2 REV1 ULP1 NAB3 SPO69 FHL1 RPC40*

ponse to many different kinds of stresses, a phenomenon variously referred to as the common environmental response or the environmental stress response (Gasch *et al.*, 2000; Causton *et al.*, 2001). The fact that following the shift to pH 3 many genes from the environmental stress response were regulated in the opposite direction to that seen under stressful acidification conditions indicates that this response was in an activated state before the pH shift (i.e. before time zero).

This in turn indicates that some form of environmental stress was acting upon the cells during cultivation at pH 6, and that the subsequent decrease in the pH relieved this stress. Because most of the changes observed following the pH shift also occurred in the later time-points of the culture maintained at pH 6, it seems that the cells were eventually able to adapt to the stress, but that acidification increased the speed of this process. Since activation of the environmental stress response is common to most stresses, these data do not reveal the precise nature of the stress experienced by the cells when the pH was maintained at pH 6. However, it is possible to speculate that perhaps some form of impaired nutrient uptake is involved since the uptake of many nutrients and ions is driven by the plasma membrane proton gradient (Sigler and Hofer, 1991). Another possibility is that at the high glucose concentrations used in these experiments, and in other industrial settings, yeast may be subjected to a degree of osmotic stress. This would be consistent with the slight relief of the stress response in the later time-points of the pH 6 experiment, which occurred despite increasing ethanol concentrations. It would be interesting to investigate whether osmotic stress may be relieved by alterations to ion fluxes induced by external acidification.

There was a small increase in growth rate following the shift to pH 3, compared to the equivalent period in the pH 6 experiment. This observation is entirely consistent with the more rapid up-regulation of genes involved in ribosome function in the pH 3 shift conditions, since increased growth rates are associated with the transcriptional induction of ribosome production (Warner, 1999). That the shift to pH 3 did not result in environmental stress is not surprising, since the conditions were deliberately chosen to optimise production of PDC, and stressful acidification would be likely to reduce final yields. Indeed the acidification experiments to which our data are compared in Fig. 2B used more severe conditions, with treatments either at pH lower than 3 or with weak organic acids (Causton *et al.*, 2001; Kawahata *et al.*, 2006). Treatment with weak organic acids has physiological effects that are distinct from simply altering external pH, since the undissociated acid can diffuse across the cell membrane and then dissociate and accumulate in the relatively neutral cytosol, thereby disproportionately decreasing the intracellular pH (Piper *et al.*, 2001). Because the weak acid response transcription factor War1p senses only moderately lipophilic organic acids (Hatzixanthis *et al.*, 2003), induction of the War1p regulon, including Pdr12p was not observed in the pH 3 shift experiment.

It should be appreciated that it is not our contention that the transcriptional stress response observed by both Causton *et al.* (2001) and Kawahata *et al.* (2006) was in error. Rather, because the specific conditions studied here were less severe than those used by Causton *et al.* (2001) and Kawahata *et al.* (2006), and did not result in growth inhibition it is likely that both 'stressful' and 'stress-free' acidification is possible. Experimental conditions other than the nature of the acid and the pH were also different between the compared datasets, including glucose and nitrogen concentrations, aeration, strain backgrounds, and treatment duration, which in this study were chosen for their relevance to an industrial, rather than a laboratory setting.

Aside from relief of the stress-response, another likely effect of lowering the pH in this experiment was remodelling of the cell wall, as indicated by the up-regulation of certain genes involved in cell wall biogenesis. Re-modelling of the cell wall and up-regulation of some genes encoding cell wall biogenesis and cell wall-located proteins is known to occur in response to low pH (Kapteyn *et al.*, 2001; Kawahata *et al.*, 2006). However, any adjustment to cell wall composition under these conditions was unlikely to be stimulated by cell wall damage, since this would have in turn stimulated the transcriptional stress response (Boorsma *et al.*, 2004). Instead, some genes (such as *SED1*) may be regulated by another signal of external acidification, rather than in response to acid-induced damage. Changes in transcription of the members of the Aft1/2p iron regulon were not observed under these conditions, however, this phenomenon appears to be specific for acid 'adaptation' conditions (Kawahata *et al.*, 2006), which were not used here.

In the present experiments, the down-regulation of genes involved in various aspects of respiration (the TCA cycle, the ETC, and mitochondrial protein synthesis), as well as genes involved in gluconeogenesis, was inverse to the two other acid treatment data sets (Causton *et al.*, 2001; Kawahata *et al.*, 2006). This down-regulation may also be related to the observed relief of the transcriptional stress response in our experiment. For instance, environmental challenges that slow growth and glucose uptake have been correlated with TCA cycle up-regulation even in the presence of extracellular glucose (Blank and Sauer, 2004). It is thus possible that the slower growth and glucose consumption and the slower down-regulation of respiratory genes in the pH 6 compared to the pH 3 shift experiment also reflect that the maintained pH 6 presents an environmental challenge.

Acidification of the medium is the inevitable result of fermentation by yeast, and may partly contribute to its ecological strategy of competitive exclusion of other microorganisms. Hence, yeast carry extensive physiological adaptations to acidic environments. Artificially preventing the yeast from acidifying the medium may become physiologically stressful under some conditions and therefore, the fixed pH conditions frequently used in bioreactors may not be optimal for bioprocesses using yeast.

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References

Blank, L.M. and U. Sauer. 2004. TCA cycle activity in *Saccharomyces cerevisiae* is a function of the environmentally determined specific growth and glucose uptake rates. *Microbiology* 150, 1085-1093.

Boorsma, A., H. De Nobel, B. Ter Riet, B. Bargmann, S. Brul, K.J. Hellingwerf, and F.M. Klis. 2004. Characterization of the transcriptional response to cell wall stress in *Saccharomyces cerevisiae*. *Yeast* 21, 413-427.

Causton, H.C., B. Ren, S.S. Koh, C.T. Harbison, E. Kanin, E.G. Jennings, T.I. Lee, H.L. True, E.S. Lander, and R.A. Young. 2001. Remodeling of yeast genome expression in response to environmental changes. *Mol. Biol. Cell.* 12, 323-337.

Chen, A.K., M. Breuer, B. Hauer, P.L. Rogers, and B. Rosche. 2005. pH shift enhancement of *Candida utilis* pyruvate decarboxylase production. *Biotechnol. Bioeng.* 92, 183-188.

Eisen, M.B., P.T. Spellman, P.O. Brown, and D. Botstein. 1998. Cluster analysis and display of genome-wide expression patterns. *Proc. Natl. Acad. Sci. USA* 95, 14863-14868.

Gasch, A.P., P.T. Spellman, C.M. Kao, O. Carmel-Harel, M.B. Eisen, G. Storz, D. Botstein, and P.O. Brown. 2000. Genomic expression programs in the response of yeast cells to environmental changes. *Mol. Biol. Cell.* 11, 4241-4257.

Gelling, C.L., M.D. Piper, S.P. Hong, G.D. Kornfeld, and I.W. Dawes. 2004. Identification of a novel one-carbon metabolism regulon in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 279, 7072-7081.

Hatzixanthis, K., M. Mollapour, I. Seymour, B.E. Bauer, G. Krapf, C. Schuller, K. Kuchler, and P.W. Piper. 2003. Moderately lipophilic carboxylate compounds are the selective inducers of the *Saccharomyces cerevisiae* Pdr12p ATP-binding cassette transporter. *Yeast* 20, 575-585.

Kapteyn, J.C., B. Ter Riet, E. Vink, S. Blad, H. De Nobel, H. Van Den Ende, and F.M. Klis. 2001. Low external pH induces HOG1-dependent changes in the organization of the *Saccharomyces cerevisiae* cell wall. *Mol. Microbiol.* 39, 469-479.

Kawahata, M., K. Masaki, T. Fujii, and H. Iefuji. 2006. Yeast genes involved in response to lactic acid and acetic acid: acidic conditions caused by the organic acids in *Saccharomyces cerevisiae* cultures induce expression of intracellular metal metabolism genes regulated by Aft1p. *FEMS Yeast Res.* 6, 924-936.

Lawrence, C.L., C.H. Botting, R. Antrobus, and P.J. Coote. 2004. Evidence of a new role for the high-osmolarity glycerol mitogen-activated protein kinase pathway in yeast: regulating adaptation to citric acid stress. *Mol. Cell. Biol.* 24, 3307-3323.

Piper, P., C.O. Calderon, K. Hatzixanthis, and M. Mollapour. 2001. Weak acid adaptation: the stress response that confers yeasts with resistance to organic acid food preservatives. *Microbiology* 147, 2635-2642.

Robinson, M.D., J. Grigull, N. Mohammad, and T.R. Hughes. 2002. FunSpec: a web-based cluster interpreter for yeast. *BMC Bioinformatics* 3, 35.

Rosche, B., N. Leksawasdi, V. Sandford, M. Breuer, B. Hauer, and P. Rogers. 2002. Enzymatic (*R*)-phenylacetylcarbinol production in benzaldehyde emulsions. *Appl. Microbiol. Biotechnol.* 60, 94-100.

Schmitt, M.E., T.A. Brown, and B.L. Trumpower. 1990. A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. *Nucleic Acids Res.* 18, 3091-3092.

Schuller, C., Y.M. Mamnun, M. Mollapour, G. Krapf, M. Schuster, B.E. Bauer, P.W. Piper, and K. Kuchler. 2004. Global phenotypic analysis and transcriptional profiling defines the weak acid stress response regulon in *Saccharomyces cerevisiae*. *Mol. Biol. Cell.* 15, 706-720.

Serrano, R., A. Ruiz, D. Bernal, J.R. Chambers, and J. Arino. 2002. The transcriptional response to alkaline pH in *Saccharomyces cerevisiae*: evidence for calcium-mediated signalling. *Mol. Microbiol.* 46, 1319-1333.

Sigler, K. and M. Hofer. 1991. Mechanisms of acid extrusion in yeast. *Biochim. Biophys. Acta.* 1071, 375-391.

Warner, J. 1999. The economics of ribosome biosynthesis in yeast. *Trends Biochem. Sci.* 24, 437-440.