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Homeostatic Adjustment and Metabolic Remodeling in Glucose-limited Yeast Cultures[D]

Matthew J. Brauer,* Alok J. Saldanha,† Kara Dolinski,* and David Botstein*

Department of Genetics, Stanford University School of Medicine, Stanford, CA 94122

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We studied the physiological response to glucose limitation in batch and steady-state (chemostat) cultures of *Saccharomyces cerevisiae* by following global patterns of gene expression. Glucose-limited batch cultures of yeast go through two sequential exponential growth phases, beginning with a largely fermentative phase, followed by an essentially completely aerobic use of residual glucose and evolved ethanol. Judging from the patterns of gene expression, the state of the cells growing at steady state in glucose-limited chemostats corresponds most closely with the state of cells in batch cultures just before they undergo this "diauxic shift." Essentially the same pattern was found between chemostats having a fivefold difference in steady-state growth rate (the lower rate approximating that of the second phase respiratory growth rate in batch cultures). Although in both cases the cells in the chemostat consumed most of the glucose, in neither case did they seem to be metabolizing it primarily through respiration. Although there was some indication of a modest oxidative stress response, the chemostat cultures did not exhibit the massive environmental stress response associated with starvation that also is observed, at least in part, during the diauxic shift in batch cultures. We conclude that despite the theoretical possibility of a switch to fully aerobic metabolism of glucose in the chemostat under conditions of glucose scarcity, homeostatic mechanisms are able to carry out metabolic adjustment as if fermentation of the glucose is the preferred option until the glucose is entirely depleted. These results suggest that some aspect of actual starvation, possibly a component of the stress response, may be required for triggering the metabolic remodeling associated with the diauxic shift.

INTRODUCTION

Budding yeast *Saccharomyces cerevisiae* is well adapted to life in a wide range of chemical and nutritional conditions. Because growing cells generally alter their immediate environment, the ability to adapt smoothly and efficiently to environmental changes confers a selective advantage over evolutionary time. *Saccharomyces* has evolved a particularly effective mechanism for adapting its physiology to the changing concentration and composition of the carbon-containing nutrients that provide both the carbon and energy required for growth. In the initial phases of a growing batch culture, Crabtree positive yeast such as *Saccharomyces* oxidatively metabolize glucose to some extent but generally prefer to metabolize glucose by using the high-flux, fermentative Embden-Meyerhof pathway, and produce ethanol even when oxygen is abundant (Dickinson, 1999). When the glucose is exhausted, cells undergo a "diauxic shift," in which they switch to a fully respiratory metabolism, catabolizing carbon compounds via the tricarboxylic acid (TCA) cycle and oxidative phosphorylation in the mitochondria (Dickinson and Schweizer, 1999). This pathway allows the oxidation of the accumulated fermentation products and is highly efficient as a mechanism for generating ATP.

Respiration of glucose in yeast, however, operates at a much lower flux than does fermentation, and it supports a considerably lower growth rate than does the fermentative pathway. Also, because fermentation and respiration occupy central positions in the network of metabolic pathways, the redirection of carbon flux from the fermentative to the respiratory pathway affects cellular processes beyond those related to the extraction of energy. Switching to respiratory metabolism comes with an additional cost, because cells that primarily respire must defend themselves against oxidative damage. The "oxidative stress response," itself is part of a more general "environmental stress response," involves the change in expression of many hundreds of genes (Gasch *et al*., 2000).

It follows from all these considerations that the transition from the anaerobic fermentation of glucose to ethanol to the oxidative respiration of ethanol requires a large-scale remodeling of the metabolic apparatus (Boy-Marcotte *et al*., 1996, 1998; Parrou *et al*., 1999; Pedruzzi *et al*., 2000; Puig and Perez-Ortin, 2000; Diderich *et al*., 2001; Haurie *et al*., 2001). In fact, the metabolic reprogramming that takes place during the diauxic shift is associated with a dramatic change in the pattern of gene expression (DeRisi *et al*., 1997). Before the shift, expression is maximal for those genes involved in the high-flux and low-efficiency metabolic pathway leading to fermentation. At the same time, the low-affinity, high-flux transporters and hexokinases are at maximum transcript abundance. In contrast, the many genes subject to glucose repression are expressed minimally (Carlson, 1999). When the diauxic shift is completed, the expression pattern has

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Present addresses: *Lewis-Sigler Institute for Integrative Genomics, Carl Icahn Laboratory, Princeton University, Princeton, NJ 08544; †Department of Biology, California Institute of Technology, 1200 E. California, Pasadena, CA 91125.

Address correspondence to: Matthew J. Brauer (mbrauer@princeton.edu).

changed to favor the high-affinity transporters and kinases (Herrero *et al*., 1995; Ye *et al*., 2001) and the genes of the high-efficiency, low-flux oxidative respiratory pathway. As glucose in the growth medium becomes depleted, this physiological switch into the oxidative respiratory mode theoretically maximizes the efficiency of energy extraction. In addition to the expression response directly attributable to the change in carbon source (i.e., those genes with a carbon source response element in their upstream regions), changes in metabolic response also include genes with a stress response element as well as genes responsive to the *HAP2,3,4,5* complex (DeRisi *et al*., 1997).

In a chemostat (Monod, 1950; Novick and Szilard, 1950), cells are maintained in a condition of steady-state exponential growth, wherein they use all available limiting nutrient immediately upon addition. The residual concentration of the limiting nutrient thus approaches zero. The nutritional state that results has been likened in bacteria to "hunger"—the condition of being neither starving nor in nutrient-excess (Ferenci, 2001). In a related study (Saldanha *et al*., 2004), we showed, using the global pattern of gene expression as a sensitive indication of the physiological state of the cells, that cells growing in chemostats limited for phosphate or sulfate are in essentially the same state as they are when coming to the end of a batch growth limited for the same nutrient. Cells seem to adjust their growth rate to nutrient availability and maintain homeostasis in the same way in batch and steady-state conditions. We observed no stress response or cell cycle arrest in the chemostat, and concluded that the cells are "poor" but not "starving."

For the case of glucose-limitation, where there are two potential growth regimes, we sought once again to understand the relationship between the physiology of cells in the chemostat relative to batch growth. Specifically, how does the homeostatic mechanism deal with glucose limitation: do cells behave like fermenting batch cultures until the glucose is exhausted, or do they move at least partially into an aerobic regime? Does controlling the dilution rate (and hence the growth rate and residual glucose concentration) substantially change the balance between fermentation and respiration? Is there any sign of a stress response?

To provide a basis for comparison of gene expression in such studies to the diauxic shift in *Saccharomyces*, we examined the gene expression changes accompanying the shift at a relatively fine temporal scale in media and strains equivalent to those used in a chemostat culture. We found that, as measured by global gene expression, cultures in the chemostat most closely resemble batch growth cultures just before the glucose is exhausted, i.e., where cells are poor and exhibit the hunger response but are not yet starving. The chemostat cultures do not show many of the dramatic changes in gene expression characteristic of the diauxic shift; we found only minimal indication of the remodeling associated with aerobic metabolism after the diauxic shift in the chemostat even when the dilution rate was reduced fivefold to a rate similar to that found for cells growing in batch on ethanol after the diauxic shift.

MATERIALS AND METHODS

Strain and Growth Media

We used DBY10085 [*Mata; URA; LEU; HIS; TRP; MAL2-8^C; SUC2*], a wild-type haploid derivative of CEN.PK122 (van Dijken *et al*., 2000). DBY10085 was isolated as a wild-type spore from a dissection of the diploid strain CEN.PK122.

Cultures were grown in minimal defined (MD) medium supplemented with glucose. MD media were prepared by autoclaving a solution containing 0.1 g/l $CaCl_2{\cdot}2H_2O$, 0.1 g/l NaCl, 0.5 g/l $Mg_2(SO_4)_2{\cdot}7H_2O$, 1 g/l K_2HPO_4, and 5 g/l $(NH_4)_2SO_4$. This salt solution was supplemented with metals and vitamins as detailed previously (Saldanha *et al*., 2004) and added via sterile filtration. Glucose was added via sterile filtration to a final concentration of 2.4 g/l.

Batch and Continuous Culture Conditions

Batch cultures were grown either in multiple 250-ml flasks each containing 50-ml each culture volume and shaken at 225 rpm or in 500-ml fermenter vessels containing 300-ml culture volume, stirred at 400 rpm, and sparged with 5 standard liters per minute (slpm) humidified and filtered air. Cultures grown in the fermenter were monitored for dissolved oxygen tension, pH, and temperature. All cultures were maintained at 30°C. Batch cultures were inoculated to $A_{600} = 0.05$ with a saturated culture grown in the corresponding medium. To reduce variability in batch replicates, 20 250-ml flasks were prepared each containing 50-ml aliquots of the same 1-liter starting culture at the appropriate density and then incubated separately. Three separate time-course experiments were analyzed.

The culture used for common microarray reference RNA was grown in a Sixfors (ATR, Laurel, MD) fermenter vessel adapted as a chemostat. Growth was in MD medium with 2.4 g/l glucose, with 5 slpm airflow, stirring at 400 rpm, and a constant 30°C temperature. The dilution rate of the chemostat was held at ~0.25 vol/h. Growth conditions were maintained and culture parameters recorded by the microprocessor controller in the Sixfors fermentation unit. This reference culture was inoculated with 0.5 ml of a saturated culture of DBY10085, allowed to grow in batch mode (i.e., at a dilution rate of 0.0 vol/h) for 24 h, and switched to chemostat conditions. After 48–72 h (~15–20 culture generations), the entire 300-ml culture was harvested for RNA isolation. A second chemostat culture was grown under conditions identical to the reference culture, except that the dilution rate was held at 0.05 vol/h.

Measurement of Culture Growth Parameters

At each time-point, 2 ml of culture was withdrawn and sonicated for 10 s., just enough to break up all clumps of cells, as confirmed in the light microscope. Sonicated cultures were examined under a light microscope at 200× magnification for identification of the proportion of cells with no buds, with small buds or with large buds. Culture density was measured both by absorbance at 600 nm and with a Coulter counter (model Z2; Beckman Coulter, Fullerton, CA), set to count cells with volumes between 8.0 and 250.0 fL; data describing the distribution of cell volumes also was recorded.

Online measurements of dissolved oxygen tension and pH were taken from a 300-ml batch culture, identical to the flask cultures but grown in a Sixfors fermenter vessel. Readings were taken at 10-s intervals. The rate of oxygen use by the culture was calculated from the dissolved oxygen tension. In addition, direct estimates of respiratory rate were made by stopping the aeration for 30 s and monitoring the drop in dissolved oxygen tension. The rate of change of this value is the rate at which the cells remove oxygen and thus estimates the culture respiratory rate. Specific respiratory rates were estimated by normalizing this value to the cell density (Coulter count) or the culture density (klett).

Cell and Media Harvest

Cultures (50 ml, prepared as described above) growing in 250-ml flasks were harvested for RNA by vacuum filtration of one to three flasks onto nylon filters. Filters were immediately placed into tubes containing liquid nitrogen and stored at −80°C until extracted for RNA. Fermenter cultures were harvested by siphoning through a drop-tube and similarly filtered, frozen, and stored at −80°C.

Metabolite Assays

Residual glucose and ethanol concentrations were assayed in the growth medium by enzyme-coupled NADH oxidation reactions (assay kits from R-Biopharm, Darmstadt, Germany). One milliliter of culture was first centrifuged at high speed to remove cells, and supernatant was stored at −20°C until the assays were performed.

RNA Isolation, Labeling, Microarray Hybridization, and Data Analysis

RNA for microarray analysis was extracted from culture filtrate by the acid-phenol method. The poly(A)+ fraction was purified from total RNA by oligo(dT) resin, as provided by Oligotex midi kit (QIAGEN, Valencia, CA). One to 2 μg of poly(A)$^+$ RNA was labeled by direct incorporation of Cy5- (experimental) or Cy3-dCTP (reference) by using reverse transcription. The labeled cDNA was mixed and hybridized overnight to cDNA arrays containing all known and predicted *S. cerevisiae* open reading frames. Microarrays were washed, scanned with a GenePix 4000B laser scanner (Axon Instruments, Foster City, CA), and analyzed using GenePix Prosoftware. Resulting microarray data were submitted to the Stanford Microarray Database for analysis.

Results for each gene and time point were expressed as the $\log_2$ of the sample signal divided by the signal in the reference channel. Spots flagged as "bad" or "missing" during gridding were discarded, as were those with a

red:green pixel regression correlation of <0.6. Batch experiment 1 had an additional filter applied. The expression ratio for each spot was calculated in terms of the SD of noise present on an array. Spots with a signal of <2 standard deviations of noise (2 "noise units") were discarded.

Expression data for the remaining spots in each experiment were clustered by gene, by using a UPGMA algorithm (Gollub *et al*., 2003). Significant or interesting clusters were identified by eye and labeled according to the most significant gene ontology (GO) annotations, as determined by GO Finder (Gollub *et al*., 2003) or by the GO-TermFinder perl module (Sherlock, 2003). Ontologies used were downloaded 10 August 2004 from www.geneontology.org, whereas the GO gene associations for the yeast genome were obtained from the Saccharomyces Genome Database (www.yeastgenome.org).

Estimation of Total Transcript Abundance across the Batch Time Course

Eight 5-ml samples of cells were taken from a batch culture for RNA extraction and analysis of transcript abundance by quantitative PCR (qPCR). Before extraction, samples were spiked with 0.076 pg of RNA transcribed in vitro from a plasmid containing the *Bacillus subtilis* ThrB gene. This transcript, poly(A)$^+$ labeled and comprising the full ThrB sequence, was then used as an internal standard for qPCR. RNA samples were used as templates for reverse transcriptase reactions with *Taq*Man Gold (Applied Biosystems). After first strand cDNA synthesis, samples were analyzed in triplicate reactions with an ABI7700 sequence detector and seven PCR primer and fluorogenic probe sets (Supplementary Table S1). For each gene sequence detected by qPCR, the absolute per cell transcript abundance was calculated, by using Coulter counter data and scaling to the value obtained for the spiked bacterial sequence. Complete data sets and supplemental materials are archived at http://genomics-pubs.princeton.edu/DiauxicRemodeling/home.shtml.

RESULTS

We compared the genome-wide transcription patterns of cells growing at steady state in glucose-limited continuous culture to the transcription patterns of cells undergoing the shift from fermentative to oxidative metabolism (the diauxic shift) as glucose was exhausted in batch culture. Unlike many previous studies, the cells were grown in a minimal medium. We prepared RNA samples at roughly 15-min intervals from a large batch culture during the diauxic shift. We also followed growth (A_{600} and cell numbers) as well as the concentrations of glucose and ethanol in the medium from this culture. In batch culture the cells grew exponentially at a fast rate until the glucose was exhausted. After the shift, they again grew exponentially at the expense of the ethanol they produced but at a much slower rate (Figure 1). The residual glucose level in the batch culture fell below the level found in the reference chemostat (0.17 g/l) some time after 9.25 h, and below the level of the lower dilution rate chemostat (0.025 g/l) no more than 15 min later, i.e., exactly at the time of the diauxic shift as estimated from the intersection of the extrapolated exponential growth curves (Figure 1).

We prepared these RNA samples for microarray analysis, ultimately labeling the cDNA made from them with Cy5. As a common reference we used Cy3-labeled cDNA made from RNA that had been collected from a steady-state chemostat culture, by using the same medium. At the relatively high dilution rate chosen (D = 0.25 vol/h), cell growth rate is ~60% of the exponential growth rate in fermenting batch cultures (Figure 1). We also studied a second chemostat culture grown at a lower dilution rate, D = 0.05 vol/h, chosen to approximate the growth rate of respiring cultures growing on ethanol, after the diauxic shift (see below). The cDNA from RNA of this chemostat also was labeled with Cy5, and mixed with the same Cy3-labeled common reference. Data from the hybridization of these samples to yeast DNA microarrays were analyzed together by hierarchical clustering (Eisen *et al*., 1998). The ratios of RNA abundance for each gene are presented as the $\log_2$ [(Cy5)/reference chemostat (Cy3)].

Because all the subsequent analysis is based on these ratios, we checked that the absolute abundance of the mRNA of six genes whose ratio changed little remained within a twofold range through the entire batch time course (see *Materials and Methods*). Changes in the ratios should therefore reliably reflect changes in the actual mRNA levels.

Gene Expression during the Diauxic Shift in the Batch Culture: Overview

The genome-wide expression analysis showed clusters of genes with distinct transcriptional behaviors over the course of the diauxic shift (Figure 2); these results largely recapitulate the results of DeRisi *et al*. (1997), but in minimal medium, at higher temporal resolution and with the chemostat as a common reference. Seven clusters that showed some biological coherence and had fairly high correlations were examined more closely. The gene ontology annotations for these clusters revealed several significant trends in the gene expression program of the diauxic shift (Table 1). These most strongly enriched ($p < 0.0001$) GO annotations in each cluster and all the gene names and annotations can be viewed in the online Supplementary Materials and the Web site.

Cluster I (Protein Biosynthesis/Ribosome Biogenesis). This cluster includes many genes encoding ribosomal proteins and translation factors. It showed strong expression during the early growth exponential phase of the batch culture, noticeably decreased mRNA levels as glucose was depleted, and then, at 9.25 h, when the glucose was depleted, gene expression of this cluster of genes was already strongly reduced.

Cluster II (Amino Acid Metabolism). This cluster, which is enriched in genes encoding enzymes of amino acid and nucleotide metabolism and other anabolic functions, was expressed maximally during the later stages of fermentative growth before being strongly reduced in expression after the glucose exhaustion.

Clusters IV and VII (Oxidative Phosphorylation/Energy Pathways). These clusters join many genes that are involved in stress responses or aerobic metabolism (notably including oxidative phosphorylation), or both, and were increased in expression during the latter part of the fermentation phase of growth. Unlike cluster II, their expression continued (with a modest decrease at ~9 h) through the diauxic shift into the respiratory phase of growth. Cluster IV genes were very strongly increased in expression at 9.25 h onward, whereas cluster VII genes continued to be expressed at elevated but not rising levels. Several subsets of cluster IV genes (marked by letters) differ mainly by how persistent the elevation of expression remained during the respiratory growth phase.

Cluster VI. This cluster contains genes expressed during all the active growth phases in the batch culture but most strongly during the latter part of the fermentative phase of batch growth.

Other clusters are less interpretable in terms of the diauxic shift: cluster III consists of genes expressed more strongly in the reference chemostat than at any time in the batch cultures. Cluster V contains genes expressed better during batch growth phases (and somewhat surprisingly, many genes involved in basic cell biology) than in the chemostat. We did not study these clusters further.

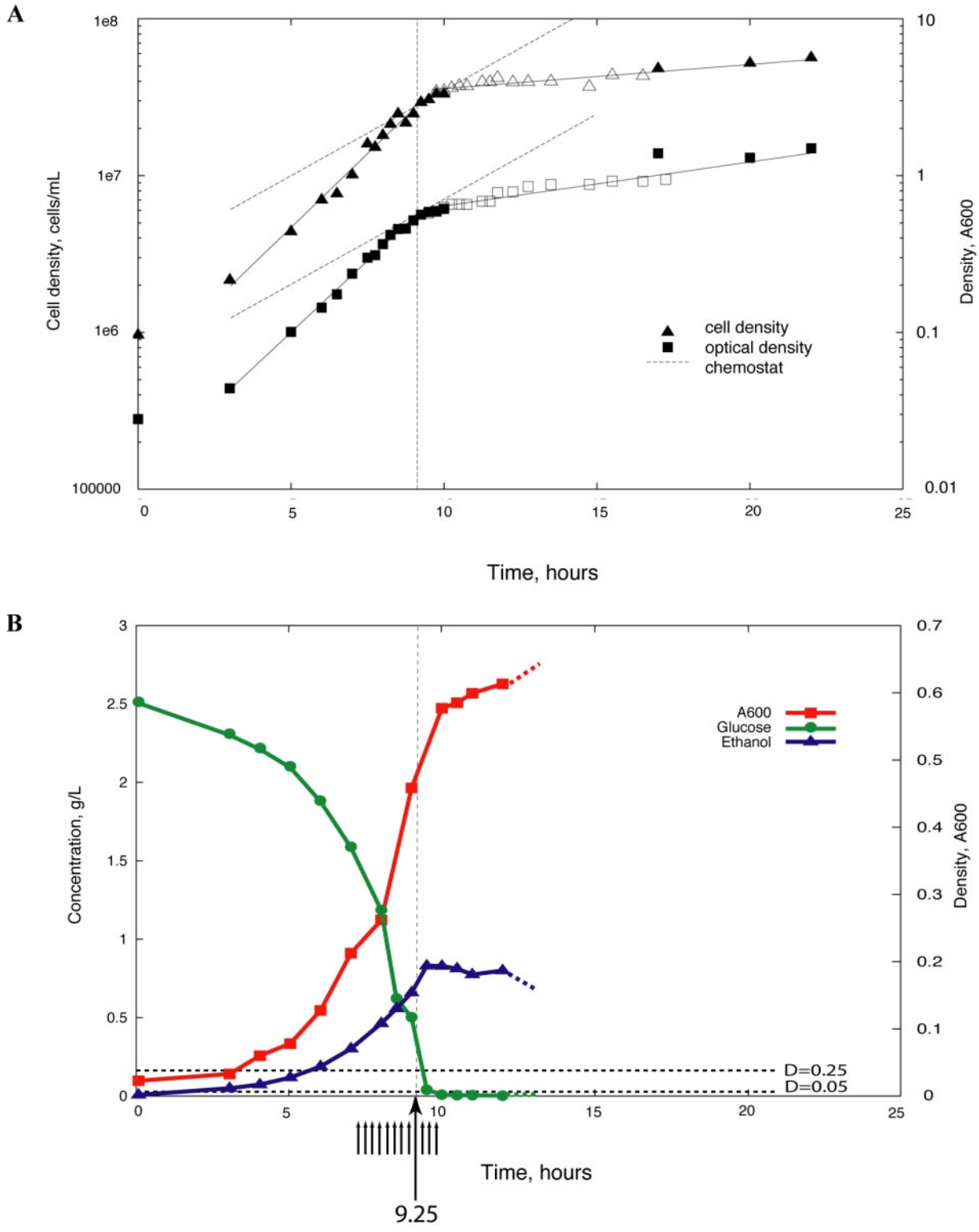


Figure 1. Culture characteristics across the diauxic shift. (A) Cell density (measured by scattering at 600 nm and by Coulter particle count) is plotted on a logarithmic scale versus the time at which the sample was harvested. The exponential parameters for each phase of growth were calculated by fitting an exponential curve to the data and are represented by solid lines. From optical density measurements, the parameter r = 0.4211 (R^2 = 0.996) for exponential growth before the diauxic shift and r = 0.0646 (R^2 = 0.8828) for exponential growth after the diauxic shift. From particle count measurements before the diauxic shift, r = 0.4342 (R^2 = 0.982). Subsequent to the diauxic shift, r = 0.0354 (R^2 = 0.8608). The chemostat growth rate corresponding to r = 0.25 is shown for comparison as a dotted line tangent to each growth curve. The vertical dotted line marks the point in the culture at which the glucose becomes exhausted. Open and closed symbols represent data taken from different culture replicates. (B) Culture density and residual glucose and ethanol concentrations. Arrows indicate time points at which the culture was sampled for gene expression, and horizontal lines show residual glucose concentrations in chemostat cultures with different dilution rates (D = 0.05, D = 0.25).

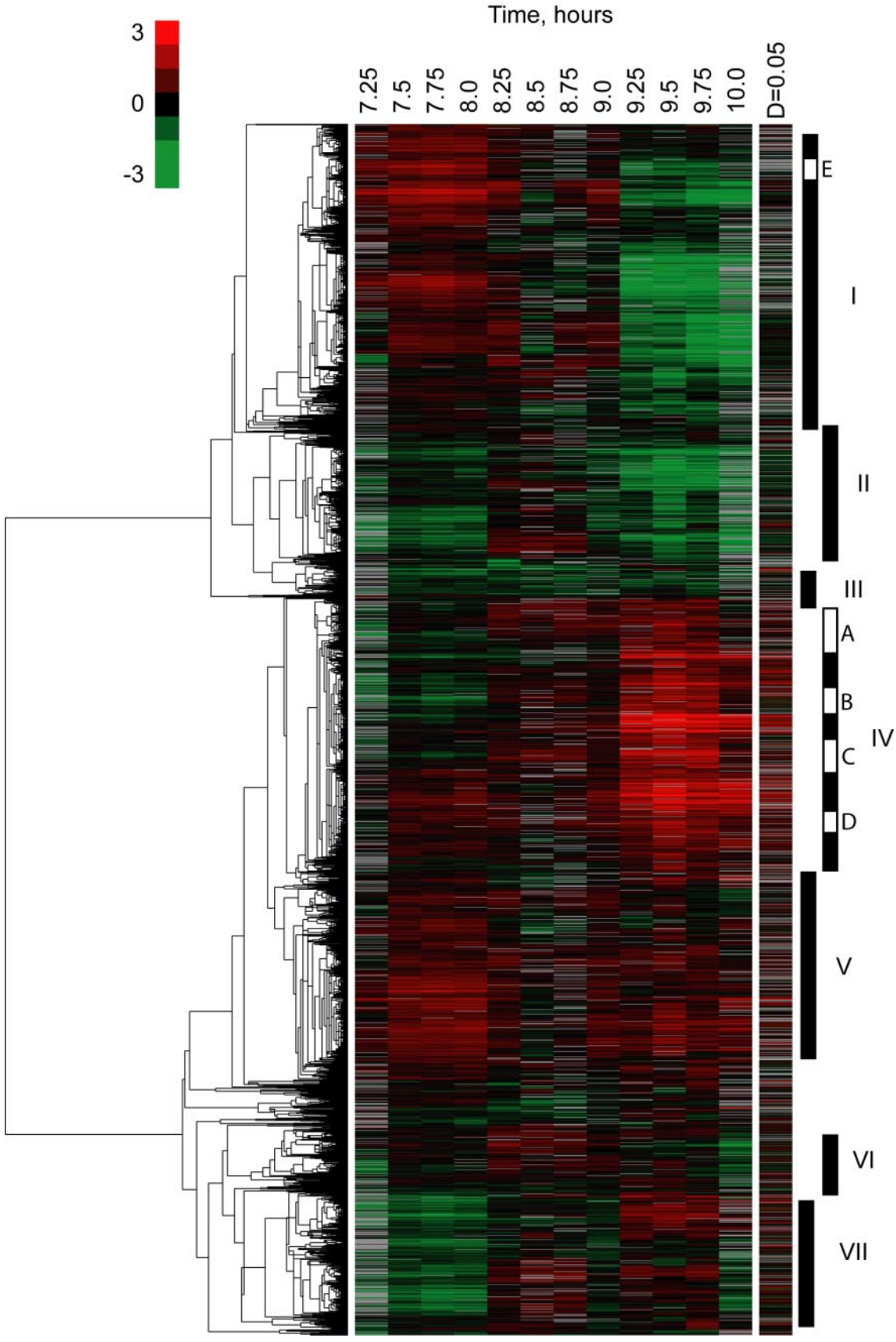


Figure 2. Global gene expression across the diauxic shift. Gene expression values are $\log_2$ of the ratio of batch culture to chemostat (D = 0.25) culture expression. Hierarchical clustering by gene was done for expression in the batch time points (columns headed with the time of harvest). Data from the low dilution rate chemostat were subsequently added (column labeled D = 0.05). Clusters identified for subsequent analysis are shown with vertical bars. In several instances groups of genes with transient expression are shown with a white bar and labeled A–E.

Table 1.

GO annotation of genes in major clusters

Cluster	GO process annotation	p value
I	Protein biosynthesis	1.0×10^{-27}
	Ribosome biogenesis and assembly	4.6×10^{-27}
II	Amine biosynthesis	4.9×10^{-25}
	Amino acid and derivative metabolism	1.2×10^{-22}
III	Siderochrome transport	8.9×10^{-5}
IV	Oxidative phosphorylation	1.5×10^{-9}
	Energy pathways	2.2×10^{-8}
V	Intracellular transport	3.4×10^{-8}
	Secretory pathways	4.8×10^{-7}
	Vacuole organization and biogenesis	7.0×10^{-5}
VI	Hexose catabolism	5.8×10^{-5}
	Monosaccharide catabolism	7.0×10^{-5}
VII	None significant	

Most of the significant clusters show discontinuities in the level of gene expression both at the 8-h time point (toward the end of exponential growth on glucose) and at the 9-h time point (Figure 2). In what follows, we dissect the latter discontinuity in some detail. The earlier discontinuity also may be physiologically significant but remains to be studied.

The Genome-Wide Transcription Pattern of Cells in Continuous Culture Resembles That of Cells at the Onset of the Diauxic Shift in Batch Culture

Our experimental design contrasts with other recent whole genome analyses of chemostat cultures (Boer *et al*., 2003) in that we make explicit the comparisons between the chemostat and batch culture time points. In this design, cultures with identical levels of transcript for all of their genes will produce an array in which all the $\log_2$ ratios will be close to zero and will be visualized as black, rather than strongly green or red. It seems that the patterns of gene expression from the three or four time points between 8 and 9 h were closest to the chemostat reference (Figure 2). At this time, the cells in the batch culture were still growing. The measured residual glucose was still above the levels found in the reference chemostat; the residual concentration of glucose fell below this level at ~9.25 h (Figure 1). The growth rate in the reference chemostat matched the nominal growth rate at ~9 h in the batch culture.

We quantified the extent to which this overall similarity in gene expression extended to the behavior of the independent major clusters. For each time point in the batch cultures, we calculated the average deviation from $\log_2 = 0$ (i.e., equal expression to the chemostat) for the major clusters (I, II, IV, and VII) obviously relevant to the diauxic shift. This deviation was minimal during the three time points just before the glucose was exhausted, at about the time that the growth rate was comparable with the rate in the reference chemostat (Figure 3A).

Lowering the Dilution Rate Does Not Significantly Alter the Pattern of Gene Expression

When a chemostat's dilution rate is lowered, the specific growth rate of the cells decreases correspondingly. To test whether a sufficiently low dilution/growth rate (and correspondingly low residual glucose concentration) might by itself trigger the transcriptional changes associated with the diauxic shift, we examined a chemostat culture grown at a rate approximating the growth rate in the late stages of batch culture.

The growth rate of cells in the respiratory phase of a batch culture was estimated by fitting an exponential curve to the cell count and optical density data from this phase of growth (Figure 1). A dilution rate of 0.05/h was chosen on this basis. The resulting low-dilution rate chemostat at steady state had a residual glucose concentration of 0.025 g/l and a residual ethanol concentration of 0.0018 g/l. These values were substantially different from the residual metabolite concentrations of the D = 0.25/h chemostat (0.166 g/l glucose and 0.16 g/l ethanol). Nevertheless, the gene expression profile of the chemostat run at D = 0.05 most closely resembled the chemostat at D = 0.25. The overall correlation coefficients between gene expression in the D = 0.05 chemostat and the patterns in the batch culture time points were strongly positive only in the samples taken between 8 and 9 h (Figure 3B), suggesting an overall similarity in transcription pattern only in that interval. Thus, although the chemostats were running at very different rates, cells in both displayed similar patterns of gene expression and most closely resembled the patterns seen in batch cultures just before the glucose was exhausted.

Some striking differences emerged between the chemostats; however, when genes were filtered by difference in expression ratio. To identify these genes, a threshold was set at a twofold change above and below the mean $\log_2$(ratio) of expression (corresponding to <2 standard deviations from the mean). Among the genes with increased expression at low dilution rate were those with GO annotations mapping to the TCA cycle, metal ion response, and cation transporters (Table 2; clusters IV and VII in Figure 2). This result suggests that at least some cells in the D = 0.05 chemostat have experienced oxidative stress and possibly that some significant level of aerobic metabolism of the glucose may have been taking place. This is evident despite the lack of a large-scale shift at the lower dilution rate to the fully aerobic metabolism characteristic of postdiauxic shift growth.

Cells in Continuous Culture Generally Avoid the Stress Response Characteristic of the Diauxic Shift

The diauxic shift typically includes an increase in transcript abundance of the genes involved in the generic stress response (DeRisi *et al*., 1997). Table 3 summarizes the numbers of previously characterized environmental stress-response genes that occur in each of the clusters. Clusters I, II, IV, and VII all have representatives of this class in them. However, the biggest representation of induced stress response genes is in cluster IV, which is the cluster that is most strongly induced after the diauxic shift. Because the reference for all these measurements is the chemostat, we can infer that there is a relatively minimal stress response in the chemostat, because the ratio of expression is nearly 1 (i.e., the same) as the values found in batch before the diauxic shift, when the cells still have abundant glucose.

It also is worth noting that cluster IV is particularly heterogeneous with respect to the longevity of the increase in expression (marked as open and filled bars in Figure 2); the low-dilution chemostat tended to express the less persistent subset much less than the rest of cluster IV. The low dilution rate chemostat showed an increase in expression of some explicitly stress-related genes (particularly of *HSP26*, *GRE1*, *YGP1*, and *HSP104*). Despite the complexity of the results, it seems clear that widespread, coordinated induction of the stress response seen in the batch cultures is largely lacking in both chemostats.

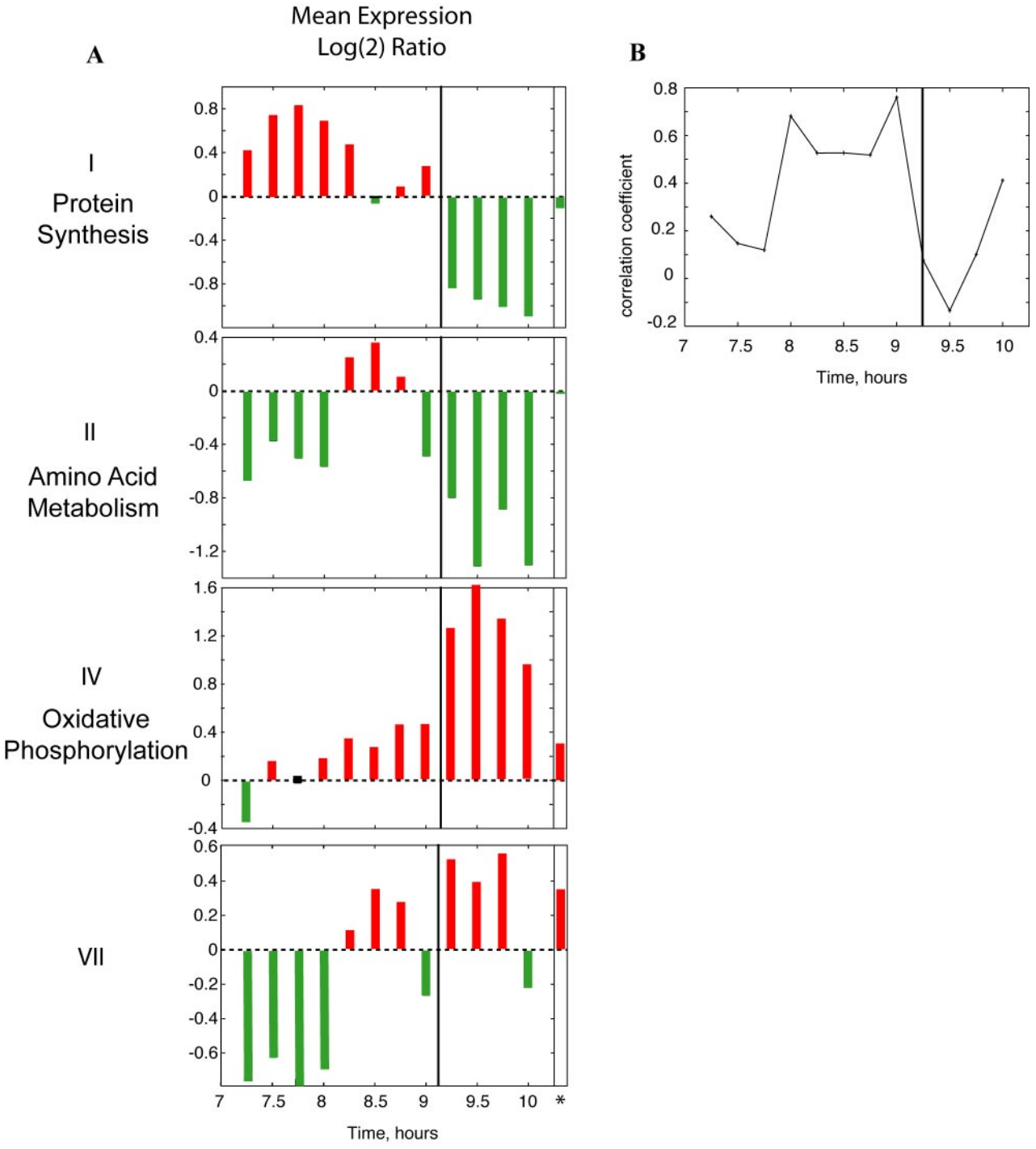


Figure 3. Composite gene expression of clusters I, II, IV, and VII. (A) Mean expression of each cluster. (B) Mean deviation of batch expression from the high dilution rate (D = 0.25) chemostat. Values for low dilution-rate (D = 0.05) chemostat are given in columns marked with an asterisk (*).

It also is the persistent subclass of the genes in cluster IV that contains the greatest number of genes found to be increased in their expression in strains that have been derived by experimental evolution in glucose-limited chemostats (Table 3; Ferea *et al*., 1999). These genes are ones whose expression seems to be associated with more efficient glucose use as opposed to stress response per se, suggesting again that the increased expression of many of the genes in cluster IV in the low-dilution chemostat is associated with metabolism as opposed to stress.

Metabolism in the Chemostat Remains Largely Fermentative

To better understand glucose metabolism in chemostats, we carried out a second experiment in which we concentrated on time points well before and after the diauxic shift. We

Table 2.

GO annotation of genes showing either increased or decreased expression in a low dilution rate chemostat, relative to the high-dilution-rate reference culture

Response	GO process annotation	p value
Increase 428 genes	Tricarboxylic acid cycle intermediate metabolism	4.1×10^{-11}
	Response to metal ion	1.2×10^{-7}
	Cation transport	1.3×10^{-7}
	Telomerase-independent telomere maintenance	2.6×10^{-6}
Decrease 66 genes	No significant terms	

followed expression of specific genes whose role in fermentative and respiratory metabolism is relatively well understood. Two of these genes, *PDC1* and *PDC5*, control the flow of metabolites to ethanol during fermentation (Figure 4C). As expected, *PDC1* and *PDC5* show a strong reduction in expression after the diauxic shift, whereas the low-dilution and reference chemostats both have a level of expression characteristic of fermentative metabolism (Figure 4A), suggesting that carbon is still flowing out of glycolysis and into ethanol.

This pattern contrasts with that of the TCA cycle enzymes (Figure 4B), all of which are increased twofold or more in their expression after the shift, and also in the chemostats (the $\log_2$ expression during batch fermentation lies well below zero). Thus, it seems that the TCA cycle gene expression was elevated in the chemostats despite the continuation of flux of carbon into ethanol production.

The TCA cycle plays a central role in amino acid metabolism, which is largely repressed (or diverted into catabolism) after the diauxic shift. For this reason, we also examined the patterns of gene expression of *GDH1*, *GDH2*, *GLN1*, and *GDH3* (Figure 5, C and D). *GDH1* and *GLN1*, which take carbon (α-keto-glutarate) out of the TCA cycle and into amino acids, are decreased in expression after the diauxic shift relative to the chemostats. In contrast, *GDH2* and *GDH3*, which take carbon out of the amino acids into the TCA cycle after the diauxic shift are elevated. These results are consistent with continued fermentative metabolism despite the elevation of the levels of the TCA cycle enzymes themselves.

We found, as expected, that the levels of several genes that function in nitrogen assimilation and catabolism of amino acids (*PUT1*, *GAP1*, *ASP3*, and *MEP2*) were increased in expression after the diauxic shift relative to the chemostats (Figure 5A). This result, like the *GDH* results, is consistent with fermentative metabolism in the chemostats. Interestingly, the vacuolar proteases *PRB1*, *PEP4*, and *CPS1* are all strongly elevated in expression after the diauxic shift relative to the reference chemostat (Figure 5B). However, in the low-dilution-rate chemostat there are apparently divergent results. This may be an indication that although there has not yet been a real diauxic shift, catabolism of some proteins and amino acids may have begun. The potential significance of indications in the chemostats of oxidative stress and elevation of TCA cycle enzymes during apparently fermentative metabolism is addressed in *Discussion*.

We studied the expression of the genes encoding hexose transporters, hexokinases, and the enzymes that function in gluconeogenesis (Figure 6). The several transporters and *HXK2* showed expression levels roughly consistent with the glucose levels: as glucose concentration diminished in the batch cultures, the genes encoding higher affinity transporters were expressed more strongly (and vice-versa for the lower affinity transporter *HXT2*). The low-dilution-rate chemostat still had high expression of *HXT2* but also had elevated levels of the low-affinity group. These results are consistent with homeostatic regulation at the level of the transporters themselves.

Two glycolytic enzymes (*ENO2* and *PGK1*) that control flux of glucose during fermentation and that also function in gluconeogenesis during respiration were examined. These enzymes are sharply reduced in expression after the diauxic shift (Figure 6C). However, both chemostats showed expression close to the respiratory pattern. As discussed below, we see this result as more indicative of a homeostatic regulatory response to reduced flux of carbon through the glycolytic pathway than as an indication of a switch to respiration.

Finally, it should be noted that many genes important in mitochondrial function after the diauxic shift (cytochrome oxidases, genes annotated to ATP generation) are minimally elevated in the low-dilution-rate chemostat, although they all come up very strongly in the diauxic shift. This is particularly true for genes associated with each of the protein complexes in the mitochondrial inner membrane associated with the respiratory electron chain (Figure 7). On average, the increase in gene expression increased is less than twofold ($\log_2 = 1$) for the genes encoding the components of each of the complexes. (Note, however, that expression of these genes is elevated in both chemostats relative to the early time points of the batch cultures.)

Table 3.

Gene clusters identified as having significance in function and expression pattern

Cluster	Total genes	Stress[a]		Adaptation[b]	
		Induced	Repressed	Induced	Repressed
I	581	0	97	2	1
II	238	0	28	1	3
III	68	0	1	2	0
IV (transient)	202	21	0	7	1
IV (persistent)	286	62	0	23	3
V	368	2	8	3	2
VI	117	0	2	2	5
VII	270	10	0	7	5

[a] From 519 genes identified by Gasch et al. (2000) as responsive to stress. No data were available for 288 of these genes.

[b] From 84 genes identified by Ferea et al. (1999) as adapting to glucose limitation. No data were available for 38 of these genes.

Changes in Cell Volume, Dissolved Oxygen, and Bud Index during the Diauxic Shift

We followed cell morphology and the level of dissolved oxygen as the cells underwent the diauxic shift. These data reveal an interesting dichotomy between the continuous adjustment in cell volume and the abrupt change in budding pattern (indicative of a change in cell cycle regulation) after the glucose has been exhausted (Figure 8). The level of dissolved oxygen initially declined gradually until the end of fermentative growth. This was followed by a short pause, a spike, and a further decline as the new aerobic growth regime became established. Estimates of respiratory rate mirror this pattern: the initial respiratory rate of the culture is low and then increases at the onset of the diauxic shift (see

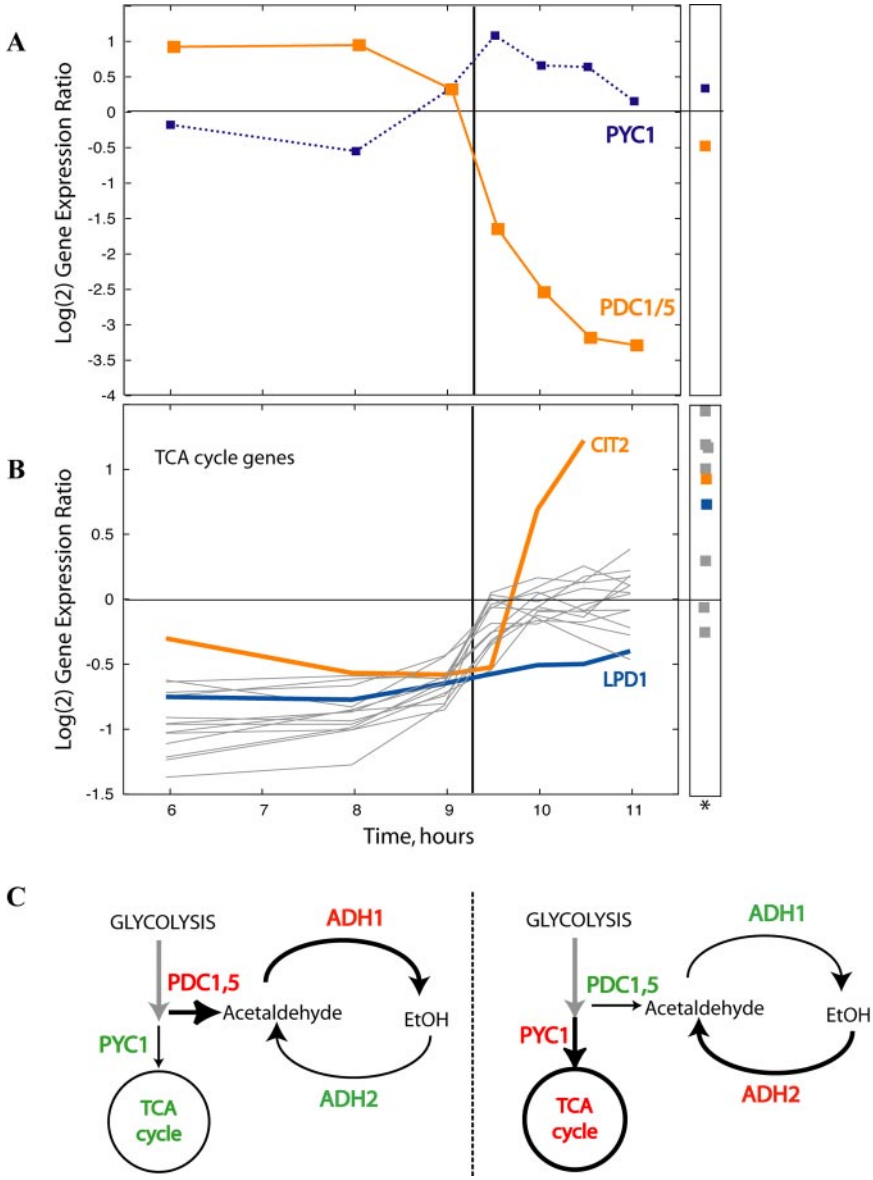


Figure 4. Expression of individual genes related to the fermentative and respiratory pathways. Gene expression profiles in Figures 4–6 are from the second batch culture. Data are uncentered and relative to the expression of the chemostat (D = 0.25) reference culture. Data for the low dilution rate (D = 0.5) chemostat are shown, when available, in the column marked with an asterisk (*). (A) Genes involved in the direction of carbon flux toward fermentation are coordinately expressed over the course of the diauxic shift. (B) Genes involved in the TCA and glyoxylate cycles become highly expressed after glucose depletion. Genes shown are *FUM1*, *SDH1-4*, *MDH1*, *MDH3*, *CIT1-2*, *ACO1*, *KGD1-2*, *LPD1*, and *IDH1-2*. For clarity, only the genes showing most (*CIT2*) and least extreme changes (*LPD1*) are labeled. (C) Inferred effect of gene expression changes is to direct carbon metabolic flux from the fermentative into the respiratory pathway. Qualitative changes in gene expression are given by the color of the gene labels (red for increase and green for decrease) and by the thickness of the line describing the step in the pathway.

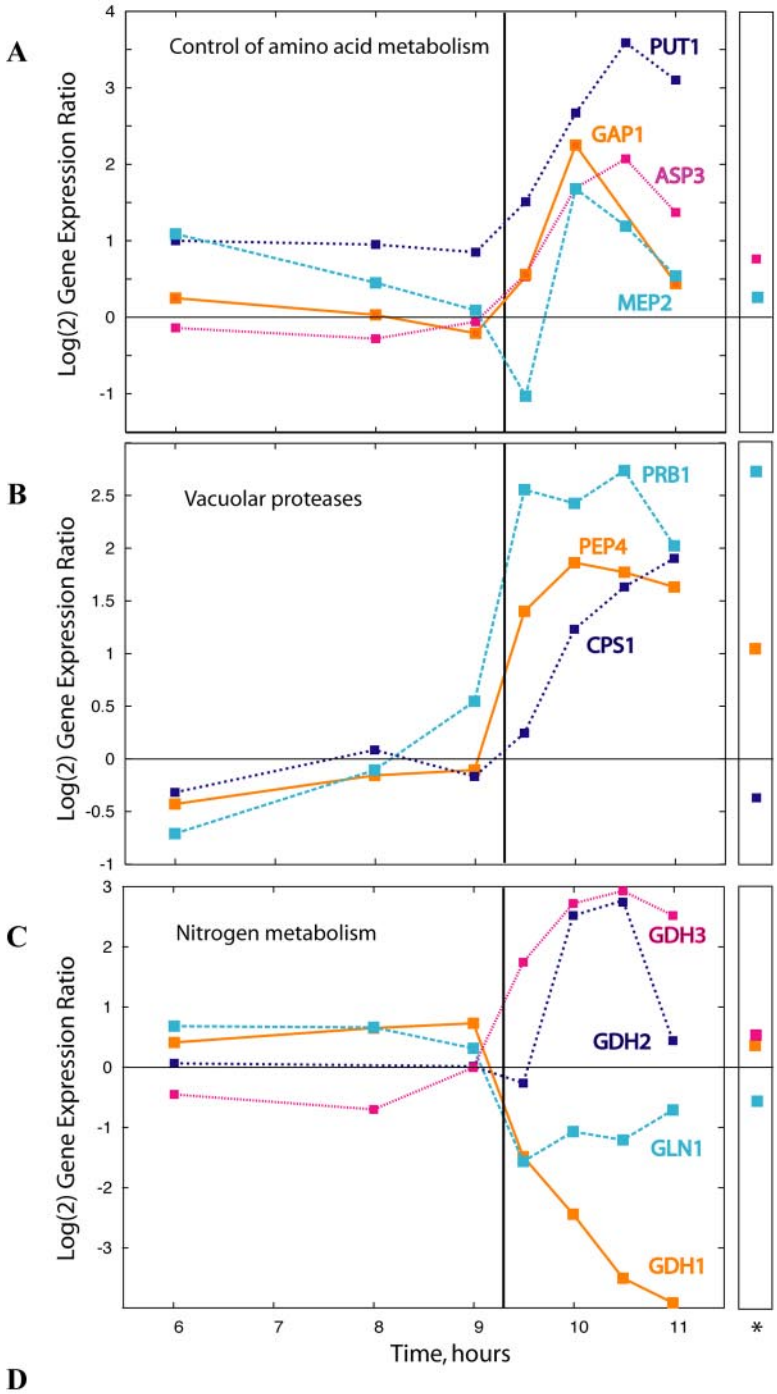
supplemental figures). Taken on a per cell or per cell mass basis, the specific respiratory rate decreases to a minimum at the point of glucose exhaustion and then increases rapidly. Both chemostats show specific respiratory rates well below the minimum level seen during the diauxic shift in batch cultures.

The significant observation is that the fraction of cells with buds decreases abruptly just as the pause in oxygen consumption begins. This result is strongly reminiscent of our previous results with phosphate and sulfate starvation. Under these conditions cells accumulate in the unbudded state, but only when the limiting nutrient is fully exhausted and homeostatic mechanisms can no longer adjust to produce balanced growth, i.e., when the cells face starvation (Saldanha *et al.*, 2004). Here, it seems that a similar event is triggered. However, in conditions of glucose exhaustion the cells, instead of ceasing growth altogether, begin to grow fully aerobically but with a different characteristic fraction of cells in the G_0/G_1 (unbudded) phase of the cell cycle. These results support an interpretation (see below) that there is a regulatory signal given at the onset of starvation that sets in train the remodeling of metabolism and altered distribution of cells in the phases of the cell cycle at the onset of starvation. Very possibly this signal is part of the environmental stress response.

DISCUSSION

Based on the genome-wide pattern of gene expression, we conclude that cells in glucose-limited chemostats occupy a metabolic state that corresponds closely to a point in a glucose-fed batch culture just before the glucose is completely exhausted. As we have found previously for cultures growing in chemostats under limiting sulfate or phosphate (Saldanha *et al.*, 2004), glucose-limited chemostat cultures do not induce a full-blown stress response. In limiting glucose, as in limiting phosphate or sulfate, the cells in chemostats are poor but not yet starving.

Our interpretation of the events during the diauxic shift combines the results given above with previous studies of batch and steady-state growth in limiting phosphate and sulfate. We suggest that whenever cells face a declining



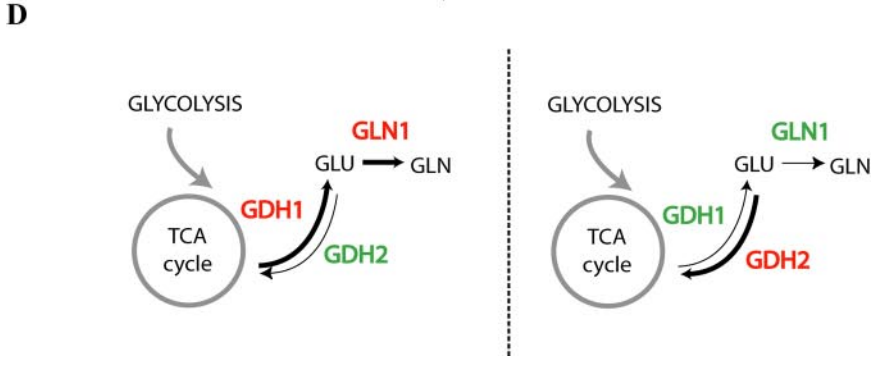


Figure 5. Expression of individual genes related to nitrogen use and amino acid metabolism. (A) Amino acid synthesis and utilization genes. (B) Vacuolar protease genes. (C) Genes involved in directing the flux of carbon into the ammonium assimilation pathway. (D) Inferred effects of expression changes on the ammonium assimilation pathway. Labels are as for Figure 4D.

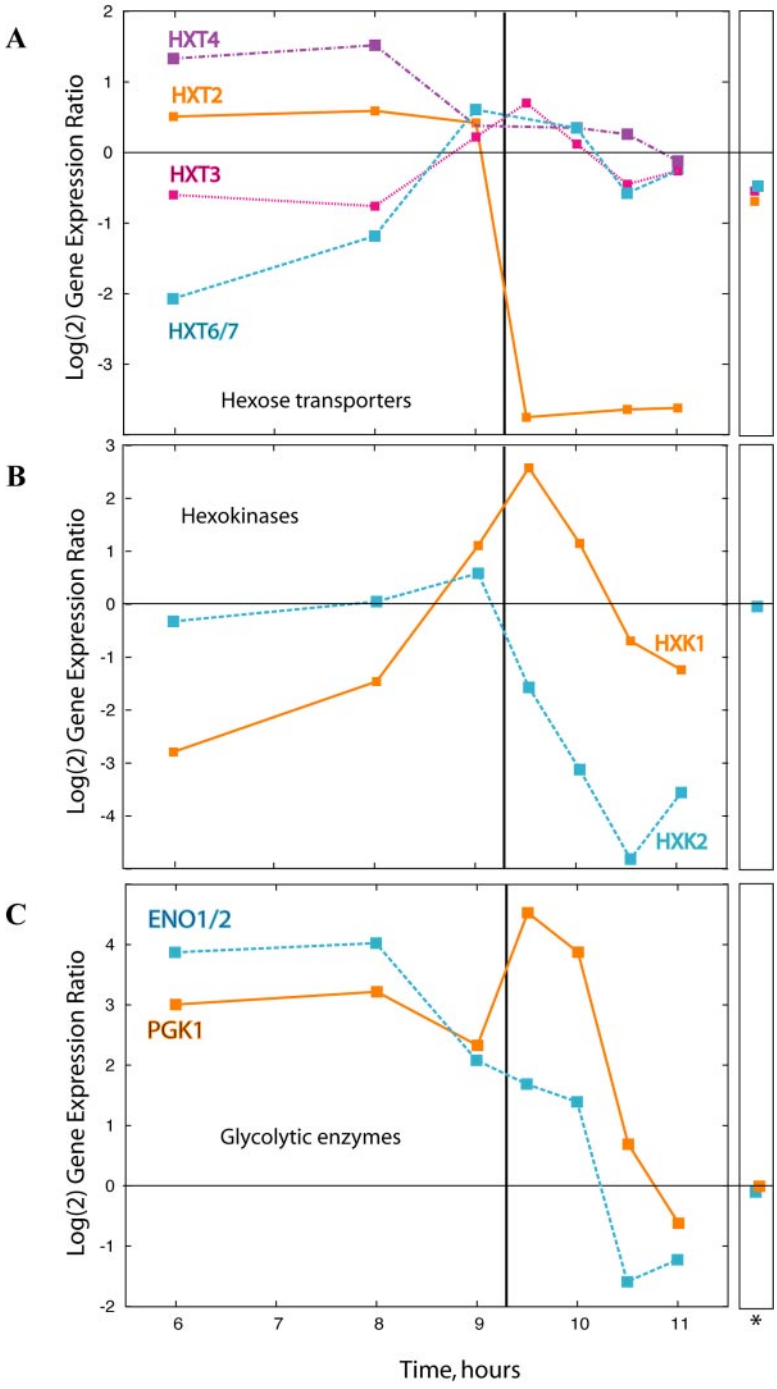


Figure 6. Expression of individual genes related to glycolysis and respiratory metabolism. (A) Genes involved in hexose import. (B) Two hexokinase genes. (C) Genes involved in glycolysis and gluconeogenesis.

concentration of limiting nutrient, they use a homeostatic "metabolic adjustment" to maintain balanced growth but stop short of a full-blown environmental stress response with concomitant interruption of the cell division cycle (Figure 9). In each limiting nutrient studied, the bud index remained the same until the nutrients were exhausted. However, when starvation was actually at hand, the cells accumulated in the G_0/G_1 (unbudded) phase of the cell cycle, awaiting renewed opportunities for growth.

In the diauxic shift, the renewed opportunity is already present. However, this condition apparently cannot be perceived or exploited without a real cessation in growth, stress response, and, we suppose, some extensive remodeling of metabolism. We suggest that the complexities in gene expression patterns during the diauxic shift regime can be more easily understood if one accepts that there are two different types of regulatory mechanism in play: one (the aforementioned homeostatic metabolic adjustment) involves continuous changes that include substitution of transporters of higher affinities, changes in cell volume, changes in gene expression in response to changed flow of metabolites, etc. For glucose, we suggest that the adjustments include decreased expression of genes specifying the enzymes of glycolysis and increased expression of the genes encoding the enzymes of the TCA cycle, and some of the other apparently continuous gene expression changes described above. We

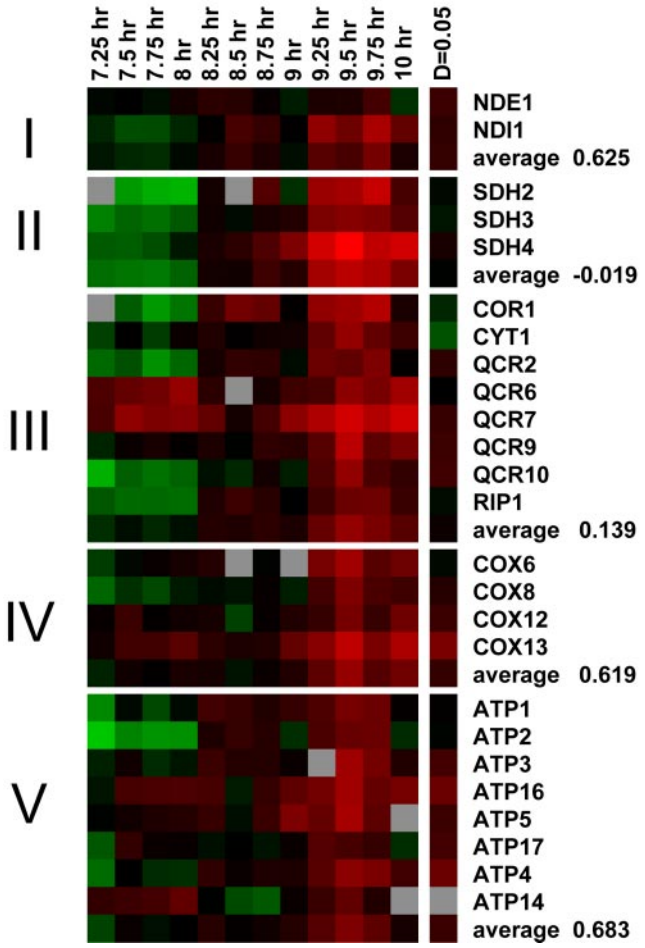


Figure 7. Changes in gene expression for components of the respiratory enzyme complexes. Genes were organized by identity in the mitochondrial oxidative phosphorylation complexes. Complex 1, NADH-dehydrogenase; complex II, succinate dehydrogenase; complex III: cytochrome bc_1; complex IV, cytochrome oxidase; and complex V, ATP synthase. Mitochondrial complex identity follows Ohlmeier *et al*. (2004).

propose that these continuous homeostatic adjustments account for the changes in gene expression that are shared between cells ending the fermentative phase of the diauxic shift in batch culture, and the low-dilution chemostat (Figure 9, metabolic adjustment I). (A similar process, denoted metabolic adjustment II, presumably coordinates the physiological changes that occur in the second, purely respiratory growth phase that precedes the transition into stationary phase.)

The second type of regulatory mechanism we postulate is a "metabolic remodeling." Within this process, we specifically include changes that result in an altered cell division cycle, which we observe as different distributions between cells that are in the G_0/G_1 (unbudded) phase relative to actively cycling cells. In complete starvation (as observed for phosphate and sulfate), all the cells accumulate as unbudded (Saldanha *et al*., 2004). In the diauxic shift, we suggest that the cells, instead of arresting completely, initiate the metabolic remodeling, cell cycle changes, and accompanying stress responses that result in the new, lower rate with fully aerobic growth on ethanol.

Growth in Glucose-limiting Chemostats Is Still Basically Fermentative

In steady-state glucose-limited growth, we found that yeast cells exhibit an unexpectedly strong preference for fermentative metabolism of glucose over respiration; they behave as if fermentation of the glucose is their best option. This finding is consistent with a limited respiration capacity model of the Crabtree effect (Alexander and Jeffries, 1990). In this model, respirofermentative metabolism occurs in cultures growing above a rate (D_{CRIT} for chemostat cultures) at which the respiratory capacity becomes saturated. In contrast to the noncarbon-source limitations, glucose-limited batch cultures are not really on the verge of a transition to stationary phase. Instead, they will, when the appropriate signals are given, undergo the diauxic shift to respiratory growth on ethanol at a lower exponential growth rate. Even at very low dilution rates, corresponding to the batch respiratory growth rate, chemostat cultures have a specific respiratory rate substantially less than the minimum seen during the diauxic shift. Thus, it is a significant new finding that these cultures do not seem to be able to undergo the full shift to respiration (Figure 7). As long as there is any glucose present in the media, the respiratory capacity of the cells remains saturated, and consequently fermentation continues. This means that the mechanisms of homeostatic adjustment, that regulate growth rate according to nutrient availability, ensure balanced growth, and stave off the stress response, seem to be functioning similarly under these conditions as under conditions of phosphate and sulfate limitation (Saldanha *et al*., 2004).

Many if not all of the mechanisms underlying metabolic adjustment effect their functions directly, for example by regulating the expression of transporters of graded affinity in response to the concentrations of nutrients (Ozcan and Johnston, 1999; Forsberg and Ljungdahl, 2001; Flick *et al*., 2003; Moriya and Johnston, 2004). We would interpret the other gene expression changes that occur before the diauxic shift and in the low-dilution-rate chemostats similarly, and expect to find such direct control mechanism in each case. A significant target for further study in this respect is the apparent discontinuity in patterns of gene expression found at 8 h (Figure 2); clearly, this occurs well before the diauxic shift but may well reflect another point of control based on assessments of metabolite concentrations.

It is interesting that the well studied catabolite repression mechanisms seem not to have very strong effects as glucose concentration falls during the late stages of growth before the diauxic shift. Indeed, it would seem that the cells are not freed of dependence on aerobic fermentation of glucose until the metabolic remodeling we postulate has been done. For example, genes (including the high-affinity hexose transporters *HXT2*, *HXT3*, and *HXT4*) known to be controlled by well studied regulator *MIG1* (Klein *et al*., 1998; Carlson, 1999) are largely unchanged in gene expression until after the shift (Figure 2 and Supplementary Table S1). Thus, it seems that the main effect of the catabolite repression systems is not in what we call metabolic adjustment but that these are actually part of the metabolic remodeling systems. This line of reasoning is entirely consistent with the many observations, in yeast as well as bacteria, of failure to metabolize alternative carbon sources until glucose is fully exhausted (Jacob and Monod, 1961). It also accords with the understanding that the Crabtree effect is not itself related to glucose repression (Alexander and Jeffries, 1990; Sierkstra *et al*., 1992).

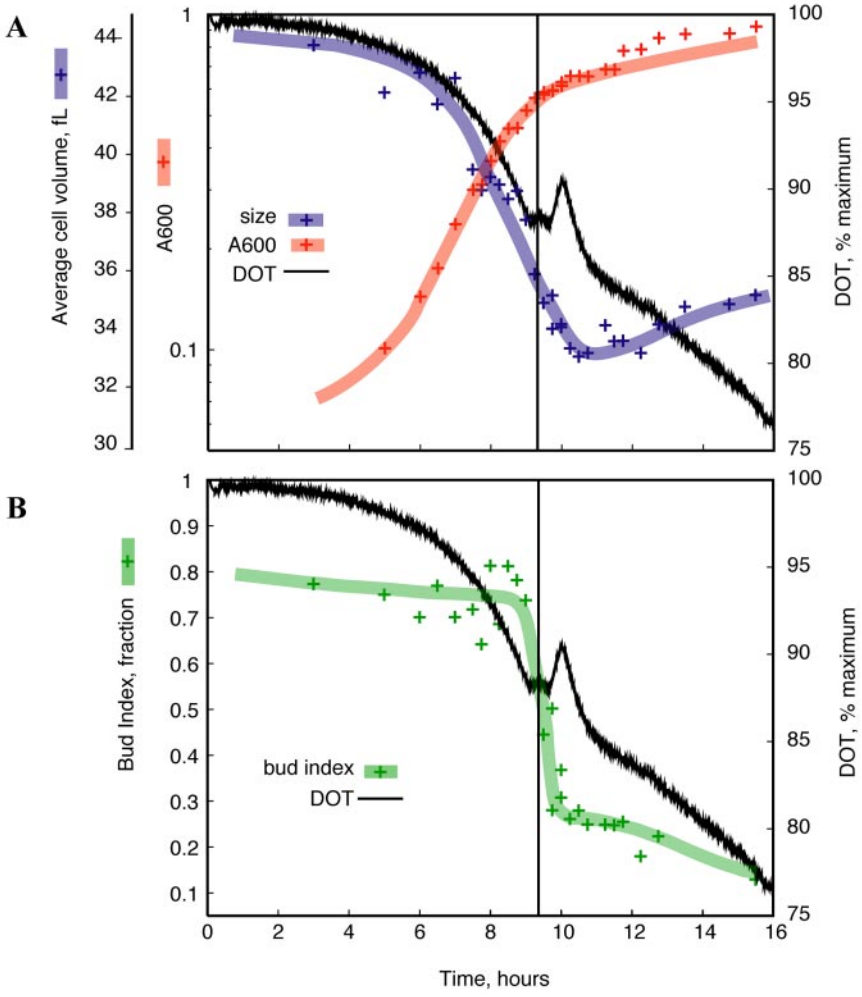


Figure 8. Changes in cell volume, culture density, dissolved oxygen, and bud morphology across the diauxic shift. (A) Culture density and average cell volume as determined by Coulter particle analyzer are plotted across the course of the batch time course. (B) Proportion of cells with microscopically identifiable buds (bud index) plotted across the time course. In both A and B, the dissolved oxygen tension (DOT) is indicated, expressed as percentage of saturation. The vertical dotted line indicates the point at which residual glucose concentration drops below the sensitivity of the assay. Light colored lines were estimated by eye to denote the approximate trends in the data.

What Triggers Metabolic Remodeling?

It has always been tempting to think of the physiological state of cells in the chemostat as being similar to the state of cells at a "balancing point," at the cusp of a seamless transition between two modes of metabolism. Our finding that cells in steady-state glucose limitation do not move beyond this point indicates that the chemostat growth conditions—even at low dilution rate—are sufficient to prevent the cells from fully entering a state of carbon starvation and that some aspect of this starvation may be an essential prerequisite to fully triggering the diauxic shift from largely fermentative to fully respiratory metabolism. Thus, it would seem that there is no seamless transition of fermentation to respiration; the process is more complicated or elaborate than that. This idea is consistent with our findings of fluctuations in dissolved oxygen, cell morphology, and cell size that, like the strong stress response, occur during the diauxic shift but not in chemostats, even at low dilution rate.

We are led to believe that at very low dilution rates the cells are able to persist in the fermentative phase simply by extracting even more of the residual glucose from the medium. The behavior of the chemostat cultures supports the idea of a regulatory switch that must be turned on to initiate the metabolic remodeling at the diauxic transition. As the dilution rate is reduced, the cells in chemostats move ever closer to activation of this switch without actually triggering it.

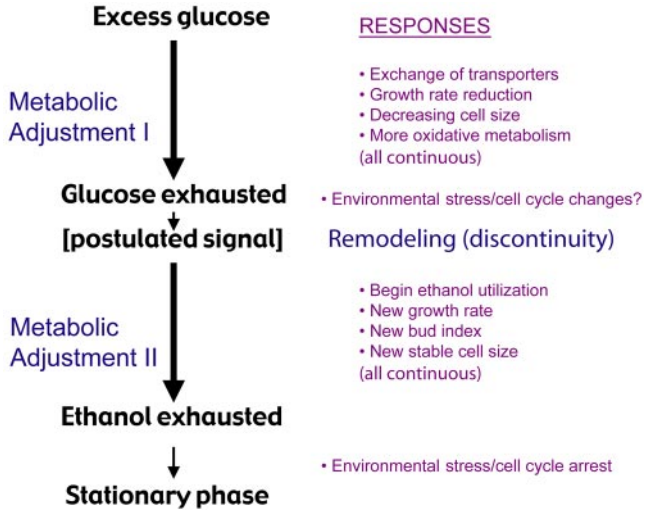


Figure 9. Model for metabolic changes during the diauxic shift. The cell's response to changes in carbon source availability is composed of two kinds of mechanisms: the continuous metabolic adjustments to declining residual metabolite concentration and the discontinuous remodeling that accompanies a switch between metabolic modes.

The gene expression results we have described above, combined with those of the previous studies of phosphate and sulfate limitation, suggest strongly that the cell cycle changes may require some aspect of the environmental stress response attending starvation. This is because in all cases that we have examined, neither the cell cycle changes nor the stress response occurs in chemostats, despite the very low level of residual limiting nutrients. For the diauxic shift, we propose that a similar situation obtains: that some aspect of the environmental stress response (which includes response to carbon starvation; Gasch *et al*., 2000; Saldanha *et al*., 2004) may be required for the cells to enter the program of metabolic remodeling. We propose that, just as the signal for cell cycle arrest cannot be given in phosphate and sulfate limitation until the cells actually begin to starve (as indicated by the stress response), so, too, the signal for the metabolic remodeling phase of the diauxic shift cannot be given until the cells actually begin to starve for lack of glucose. It is not ruled out that this remodeling might actually be only fully executed when cells are in, or passing through, the "start" or G_0/G_1 stage of the cell cycle, as is the case for cell-type mating pheromone response or sporulation (Hartwell *et al*., 1974).

In our model (Figure 9), the many genes annotated to aerobic metabolism and oxidative stress response (including all the genes specifying the enzymes of the TCA cycle) that are elevated both toward the end of the fermentative growth phase in batch and also in the chemostats are part of the metabolic adjustment system. The conclusion cannot be escaped that the cells in these circumstances, although they are not yet starving, are experiencing some oxidative stress, suggesting that at least some of the cells are metabolizing some of the remaining glucose aerobically. However, it seems clear that the cells have not undergone the full diauxic shift, and it seems from the patterns of gene expression that they are not growing on the ethanol available to them. It is notable in this regard that many genes important in mitochondrial function after the diauxic shift (most of the cytochrome oxidase components, many genes annotated to ATP generation) are not elevated in their expression in the low-dilution-rate chemostat.

Understanding Gene Expression Changes in Experimental Evolution

The chemostat has been used in studies of experimental evolution to apply a constant directional selection to yeast populations limited in their growth by glucose (Dykhuizen and Hartl, 1983; Dykhuizen, 1993; Ferea *et al*., 1999; Notley-McRobb and Ferenci, 1999). Such studies have shown that cultures adapt by modifying their regulation of carbon metabolism so as to use glucose more efficiently. One of the motivations for undertaking this study was to try to better understand the nature of the gene expression changes associated with the manifestly better performance, in glucose-limited steady-state culture, of strains obtained through experimental evolution in such chemostats (Paquin and Adams, 1983a,b; Adams *et al*., 1985; Ferea *et al*., 1999; Dunham *et al*., 2002). We suggest that the results of experimental evolution studies can now best be interpreted as changes that allow more efficient metabolic adjustment, as opposed to metabolic remodeling, for the following reasons.

First, many of the genes expressed at increased levels in a previous study (Table 3; Ferea *et al*., 1999) are increased in their expression not only during the diauxic shift but also in the chemostats. These include many of the genes in clusters IV and VII discussed above and associated with aerobic metabolism and oxidative stress. This is in accord with the previous inference that increased oxidative metabolism of glucose is the likely basis for the improved growth in chemostats and supports the idea that there may be significant oxidative metabolism in aerobic fermentations in general and in chemostats specifically, especially at low dilution rates.

Second, the genes that indicate continued fermentative metabolism and failure to pass through the diauxic shift (namely, *PDC1*, *PDC5*, *GDH1*, *GDH2*, and *GLN1*) were unchanged in the "evolved" strains of Ferea *et al*. (1999). Conversely, *HXK2*, which in our experiments was strongly reduced in expression after the diauxic shift and unchanged in the chemostats (Figure 6) was increased in expression in the evolved strains of Ferea *et al*. (1999). Using the same reasoning, we are led to conclude that the evolved strains are still in a physiological state resembling a batch culture before the diauxic shift. However, the evolved strains are using glucose more efficiently, possibly by oxidative routes already available to the metabolic adjustment system without resorting to the metabolic remodeling characteristic of the diauxic shift.

To conclude, we propose on the basis of our gene expression study that some aspect of actual starvation (as indicated by the environmental stress response) is required before yeast cells will switch from fermentation of glucose to respiration of ethanol. In steady-state growth on limiting glucose chemostats, there is no actual starvation, no stress response, and no diauxic shift.

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