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Slow Growth Induces Heat-Shock Resistance in Normal and Respiratory-deficient Yeast

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Yeast cells respond to a variety of environmental stresses, including heat shock and growth limitation. There is considerable overlap in these responses both from the point of view of gene expression patterns and cross-protection for survival. We performed experiments in which cells growing at different steady-state growth rates in chemostats were subjected to a short heat pulse. Gene expression patterns allowed us to partition genes whose expression responds to heat shock into subsets of genes that also respond to slow growth rate and those that do not. We found also that the degree of induction and repression of genes that respond to stress is generally weaker in respiratory deficient mutants, suggesting a role for increased respiratory activity in the apparent stress response to slow growth. Consistent with our gene expression results in wild-type cells, we found that cells growing more slowly are cross-protected for heat shock, i.e., better able to survive a lethal heat challenge. Surprisingly, however, we found no difference in cross-protection between respiratory-deficient and wild-type cells, suggesting induction of heat resistance at low growth rates is independent of respiratory activity, even though many of the changes in gene expression are not.

INTRODUCTION

One of the major challenges faced by free-living unicellular organisms is that their external environment is not stable, but their internal environment must maintain stability in order for orderly growth to be possible. Sudden and drastic changes in the environment have the potential to disrupt the internal system of cells, affect critical cellular functions, and thereby prevent normal growth (Gasch and Werner-Washburne, 2002). *Saccharomyces cerevisiae* (budding yeast) is typical of many such organisms in its responses to potentially disruptive environmental change, or "stress." It generally slows or ceases growth, induces genes specifying a variety of proteins involved in repair of intracellular damage, and then resumes growth only when the damage is repaired and internal conditions return to conditions consistent with further growth.

The advent of microarray technology made it convenient to follow, on the genomic scale, how cells change their expression pattern in response to different conditions. Although each type of environmental disruption results in a characteristic gene expression profile, comparative analysis of stress-induced gene expression reveals a common pattern (Gasch *et al*., 2000; Causton *et al*., 2001). This common response (referred to by Gasch *et al*. (2000) as the environment stress response [ESR]) involves >900 genes, two thirds of which show significantly decreased expression, and one third of which show significantly increased expression, regardless of the type of stress applied. Functional roles of the 600 genes whose expression decreases after stress include various aspects of cell growth, RNA metabolism, and protein synthesis, conspicuously genes encoding ribosomal proteins. Of the 300 genes that increased in expression in response to stress, only 40% are functionally characterized. These include genes that participate in diverse processes, including carbohydrate metabolism, detoxification of reactive of oxygen species (ROS), protein folding and degradation, DNA damage repair, and cell wall modifications (Gasch *et al*., 2000).

Recently, Brauer *et al*. (2008) and Castrillo *et al*. (2007) shed new light on the set of generic ESR genes defined by Gasch *et al*. (2000). Taking advantage of the ability to control steady-state growth rate in a chemostat (Novick and Szilard, 1950), Brauer *et al*. (2008) observed that the expression of as many as a third of all yeast genes strongly correlates with growth rate. When ESR genes in particular were examined for their relationship to this parameter two categories emerged. Genes whose expression rises in response to stress are negatively correlated with the steady-state cellular growth rate, whereas down-regulated ESR genes are positively correlated with growth rate. The extensive correspondence between ESR genes and growth rate-correlated genes strongly suggests the possibility that what Gasch *et al*. (2000) interpreted as a direct response to stress is actually a growth rate response that generally accompanies stress response. The direct effect of stress may be to slow or halt growth, and the reduction in growth rate causes, indirectly, gene expression changes. Alternatively, it may be that slowing of growth, like starvation itself, is somehow perceived by cells as "stress."

To try to disentangle the direct effects of stress from the changes in growth rate that are part of stress response, we designed experiments in which a particular stress (heat shock) was applied to cultures growing at different steady-state growth rates in chemostats. This procedure allowed us to partition genes whose expression responded to a mild heat pulse into those that also showed correlation with steady-state growth rate, and those that did not. We found

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that cells growing more slowly in glucose are more resistant to heat stress than faster growing cells, suggesting that the functions that allow cells to survive heat-stress better when induced are among those that respond to both heat and growth rate. We also performed such experiments using respiratory-deficient *[rho°]* mutants where we found that the trend between slow growth and higher heat tolerance also holds. However, when we equalized the growth rate between wild type and *[rho°]* mutants, we observed that survivorship was virtually identical between the two, even though the expression of known stress genes is lower in *[rho°]* than wild type. These results suggest that induction of heat-resistance at low growth rates is independent of respiratory activity, even though many of the changes in gene expression are not.

MATERIALS AND METHODS

Strains

We used DBY11092 (Mat**a** MAL2-8^{C}), a prototrophic CEN.PK derivative described by Van Dijken *et al.* (2000), and DBY 11338, a spontaneous *[rho°]* derived from DBY11092 by Amy Caudy (Lewis-Sigler Institute, Princeton University), who verified that this mutant lacks mitochondrial DNA, as verified by Comparative Genomic Hybridization (Pinkel *et al.*, 1998; Pollack *et al.*, 1999).

Chemostats

All chemostat media protocols are found in Supplemental Table 1. Yeast cultures were cultured in chemostats under phosphate limitation (final concentration, 10 mg/l potassium phosphate) at five different dilution rates. The dilution rates (equal to the population growth rate at steady state) range from 0.05 h^{-1} (14 h doubling time) to 0.25 h^{-1} (2.8 h doubling time). The dilution rate was estimated by direct measurement of the effluent volume over time. Chemostats were established in 500-ml fermenter vessels (Sixfors; Infors AG, Bottmingen, Switzerland) containing 300 ml of culture volume. They were stirred at 400 rpm and sparged with five standard liters per minute humidified and filtered air. The chemostats were monitored as described previously (Brauer *et al.*, 2005); the temperature probes were individually calibrated with an electronic thermometer.

Heat-Pulse Experiments

A heat pulse was applied to cultures growing at steady state at 28°C by heating the block around each chemostat vessels. Temperature was raised to 36°C for all vessels in ~6.5 min. The vessels were immediately allowed to cool back down to 28°C, which took ~15 additional minutes. Culture samples were taken at steady state (preheat pulse), the height of the heat pulse (36°C), and postheat pulse (5, 10, 15, 30, 60, and 120 min after reaching 36°C). RNA isolation, labeling, and microarray hybridization of collected samples were carried out as described by Brauer *et al.* (2008). The reference RNA for all samples was taken from the phosphate-limited chemostat grown to steady state at a dilution rate of 0.1 h^{-1} (6.9-h doubling time).

Analysis of Gene Expression Data

Expression data (available in supplemental data) were clustered by gene, according to Eisen *et al.* (1998). Because each of these experiments is a time course over quite short times, and because all the genes behaved continuously and consistently, we did not carry out extensive replicates, although individual experiments were repeated from time to time. The data shown here are from a single set of experiments carried out at the same time. Growth rate response genes were identified by modeling each gene as a linear function of the growth rate. For the six subsets in Figure 1 and Table 1, growth rate response genes were picked based on the overall correlation and steepness of the linear slope requiring that the magnitude of expression difference between the slowest and fastest growth rate be twofold or more. Heat-pulse–responsive genes were identified to have at least a twofold change in either direction in at least one of the chemostats. We chose this cutoff arbitrarily; a brief analysis of more or less stringent criteria did not change any of our conclusions even though a few more or fewer genes were represented in each subset.

Functional Annotation

Subsets of genes were assigned functional annotation from the Gene Ontology (GO) (Ashburner *et al.*, 2000). The significant representation of the GO terms was evaluated using the GO-Term Finder (Boyle *et al.*, 2004) implemented at the Lewis-Sigler Institute for Integrative Genomics, Princeton University (Princeton, NJ).

Prediction of Relative Instantaneous Growth Rates

Data were analyzed using the tools described by Brauer *et al.* (2008). Briefly, 72 genes that were found to be best correlated with changes in growth rate were picked to calibrate a linear model that estimates the relative instantaneous growth rates. The model is then used to estimate the instantaneous growth rate of the culture under various conditions; a complete description of the algorithm can be found in Airoldi *et al.* (2008).

Thermotolerance Assays

To test the relationship between growth rate and heat resistance, the following experiments were carried out. For the chemostat experiments, four DBY11092 cultures were grown to steady state at 30°C at the following dilution rate (0.05, 0.1, 0.15, and 0.3 h^{-1}). In addition, one vessel (0.15 h^{-1}) was grown to steady state at 36°C. In the batch culture experiments, the same strain was grown with shaking in three chemostat-based media each with a different nitrogen source (proline, asparagine, or urea) at 30°C. The media were modified to contain equal final molar concentration of nitrogen (8 mM). All cultures were inoculated at very low density and grown until they were in the exponential phase of the growth curve (Klett value of 70).

For the actual heat shock, a sample from each culture was promptly diluted into a preheated tube of water at 50°C. Samples were taken from the 50°C mixture at each time point and promptly put on ice. Each sample was serially diluted in water and spread on rich yeast peptone dextrose (YEPD; 10 g of Bacto-Yeast extract, 20 g of Bacto-peptone, 20 g of Bacto-agar, and 20 g of glucose in 1000 ml of water) plates to measure viability. Colonies were counted after 48-h incubation at 30°C. For each time point, a minimum of 200 colonies were counted from two to three serially diluted plates; in most cases, the results are averages from two plates. The percentage of survival was calculated by comparing to samples treated similarly but not exposed to the heat shock.

For comparison between wild type and *[rho°]* mutants, wild type (DBY11092) and *[rho°]*(DBY11338) were grown to steady state at 30°C at both 0.05 and 0.1 h^{-1}. Survival during heat shock was assessed as described above.

RESULTS

To try to distinguish the effects of growth rate from the effects of heat shock on global gene expression, we grew a prototrophic CEN.PK strain in five chemostats limited for phosphate, each at a different steady-state growth rate. We adjusted the dilution rate, defined as the rate at which fresh media flows into the vessel, over a broad range such that the lowest growth rate culture has a doubling time of ~14 h, whereas the fastest culture has a doubling time of roughly 2.8 h. After steady state was reached at 28°C, we subjected each culture to a nonlethal heat pulse, during which the temperature climbed to 36°C in ~6 min. Then each culture was cooled to the starting temperature, which took ~15 min. Samples were taken before the heat pulse, at the height of the heat pulse, and up to 2 h after reaching 36°C (cooling phase). For most of the genes that responded to the heat pulse, there is a distinct rise or fall in expression level in all chemostats with the maximum change observed 5 min after the pulse (Figures 1–3). This is followed by a recovery phase where we observed that the expression level for many of the genes tends to overshoot the starting level before returning to the starting state. Typically, all expression levels returned to the normal state 60 min after the heat shock. This behavior has also been observed in other transcriptional pulse perturbation experiments (Gasch *et al.*, 2000; Causton *et al.*, 2001; Ronen and Botstein, 2006).

Distinguishing the Effect of Heat Pulse from Growth Rate on Gene Expression

Figure 1 shows hierarchical clustering of the gene expression changes during heat-shock time course from all five chemostats. The relatively mild heat perturbation elicited a profound change in the expression profile across all chemostats, consistent with what was found previously by Gasch *et al.* (2000). Our experimental design allows us to subdivide the profile by the relative contribution of growth rate differences to the observed changes in expression levels. The two most

Table 1. GO annotation of genes in each subsets

	p value	Gene hits	Term size	Term fraction (%)
S2 (237 genes)				
Process				
Trehalose biosynthetic process	3.02E-04	5	7	71.43
Fatty acid catabolic process	3.79E-04	6	12	50
Carboxylic acid catabolic process	1.89E-03	6	15	40
Glycogen biosynthetic process	3.59E-04	7	18	38.89
Glycogen metabolic process	1.65E-05	10	32	31.25
Alcohol catabolic process	5.18E-11	19	66	28.79
Glucose catabolic process	2.29E-06	13	51	25.49
Glycolysis	1.09E-03	9	38	23.68
Response to oxidative stress	1.08E-03	12	71	16.9
Hexose metabolic process	9.54E-06	18	114	15.79
Carbohydrate metabolic process	8.62E-14	42	297	14.14
Coenzyme metabolic process	2.95E-04	18	142	12.68
Response to stress	5.31E-06	46	587	7.84
Function				
Enzyme inhibitor activity	1.73E-03	7	25	28
Oxidoreductase activity	7.80E-12	41	330	12.42
Component				
Peroxisome	1.74E-03	10	59	16.95
S4 (206 genes)				
Process				
Sulfur use	1.18E-03	5	10	50
Ribosomal small subunit assembly and maintenance	1.16E-05	7	14	50
Regulation of translational fidelity	2.12E-04	6	13	46.15
Cysteine metabolic process	8.58E-03	5	14	35.71
Ribosomal subunit assembly	2.02E-11	17	55	30.91
Sulfur amino acid biosynthetic process	8.03E-06	10	35	28.57
Ribosome assembly	7.38E-10	17	67	25.37
Ribosomal large subunit assembly and maintenance	4.23E-05	10	41	24.39
Maturation of SSU-rRNA	3.10E-05	11	50	22
Sulfur compound biosynthetic process	1.36E-04	10	46	21.74
Aspartate family amino acid metabolic process	3.71E-04	11	63	17.46
Ribosome biogenesis and assembly	1.87E-32	69	432	15.97
Translation	5.01E-57	111	711	15.61
Function				
Structural constituent of ribosome	5.07E-105	104	236	44.07
snoRNA binding	1.16E-04	7	21	33.33
RNA polymerase activity	3.69E-04	8	34	23.53
rRNA binding	6.52E-07	17	107	15.89
Component				
Cytosolic ribosome	1.15E-123	104	175	59.43
Small subunit processome	4.18E-13	17	48	35.42
RNA polymerase complex	2.32E-04	8	33	24.24
Ribonucleoprotein complex	8.48E-80	126	655	19.24
Cytosol	4.04E-56	107	668	16.02
Nonmembrane-bound organelle	4.15E-69	142	1091	13.02
Nucleolus	5.48E-14	39	305	12.79
S6 (283 genes)				
Process				
Nucleoside salvage	3.13E-04	6	10	60
Purine salvage	6.67E-04	6	11	54.55
Purine nucleoside metabolic process	5.48E-04	7	16	43.75
Ribonucleoside metabolic process	9.01E-04	7	17	41.18
Histidine family amino acid metabolic process	9.79E-03	6	16	37.5
Metabolic compound salvage	7.24E-04	8	23	34.78
Nucleoside metabolic process	1.62E-04	9	26	34.62
Nucleobase metabolic process	2.49E-04	11	43	25.58
Ribosomal large subunit biogenesis and assembly	8.47E-07	16	64	25
Maturation of 5.8S rRNA	4.43E-03	9	37	24.32
tRNA metabolic process	2.30E-07	24	138	17.39
Amino acid biosynthetic process	7.06E-07	23	134	17.16
Protein–RNA complex assembly	2.17E-07	25	149	16.78
Amine biosynthetic process	2.62E-06	23	143	16.08
rRNA processing	2.78E-11	41	277	14.8
Amino acid metabolic process	1.60E-05	29	237	12.24
RNA processing	9.97E-13	62	539	11.5
Amino acid and derivative metabolic process	7.46E-05	29	254	11.42

Continued

Table 1. *Continued*

	p value	Gene hits	Term size	Term fraction (%)
Function				
rRNA primary transcript binding	6.87E-03	4	6	66.67
transferase activity, transferring pentosyl groups	4.11E-03	6	16	37.5
RNA helicase activity	5.70E-08	14	42	33.33
RNA-dependent ATPase activity	1.41E-04	9	28	32.14
RNA polymerase activity	7.75E-03	8	34	23.53
Translation factor activity, nucleic acid binding	2.46E-03	11	58	18.97
Component				
DNA-directed RNA polymerase III complex	2.46E-04	7	17	41.18
Nucleolus	1.63E-14	47	305	15.41
Nuclear lumen	1.72E-10	62	619	10.02

prominent clustered gene groups in Figure 1 show strong induction (G1) or repression (G2) in response to the heat shock. Of particular interest is the correlation with growth rate differences observed for these two groups, which we infer from differences in the level of expression during the steady state preceding the heat pulse. For group 1, the expression level during steady state before the heat stress is lower as the growth rate increases (i.e., the correlation is negative), whereas for group 2 the correlation is positive, as reported previously by Brauer *et al*. (2008) and Castrillo *et al*. (2007). Other gene clusters in Figure 1 show diverse patterns: in groups G3 and G4 the effect of growth rate on gene expression is qualitatively much smaller than that of temperature, whereas in groups G5 and G6 the opposite is observed. Thus, our experimental design seems to have succeeded in distinguishing, in at least some cases, between stress effects and growth rate effects on gene expression.

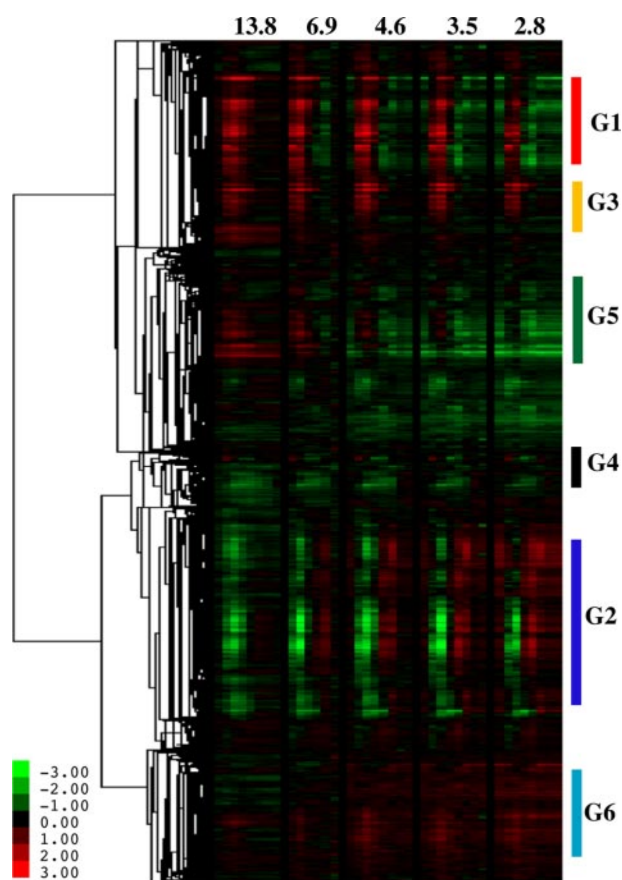

Figure 1. Hierarchical clustering of the gene expression patterns of five yeast cultures grown to steady state in phosphate-limited chemostats exposed to a mild heat pulse (28–36°C). The steady-state cultures were grown at different rates, with doubling time of 13.8, 6.9, 4.6, 3.5, and 2.8 h. With the exception of the 6.9-h doubling time culture, which did not have a 120-min postheat shock time point, samples were taken before the heat pulse (steady state at 28°C), at the maximum temperature (36°C), and after the heat pulse (5, 10, 15, 30, 60, and 120 min after reaching 36°C). Reference for the microarray is from a phosphate-limited steady-state culture with a doubling time of 6.9 h.

Brauer *et al*. (2008), in their large-scale growth rate study, constructed an empirical model that predicts the "instantaneous growth rate" based on the expression of 72 genes that were best correlated in their study with steady-state growth rate across many nutrient limitations. By applying their model to our data, we can get a sense of the instantaneous growth rate of the cells as a function time during and after the heat shock. As seen in Figure 2, in all five chemostats, the predicted instantaneous growth rate of the culture decreased remarkably rapidly in response to the heat pulse.

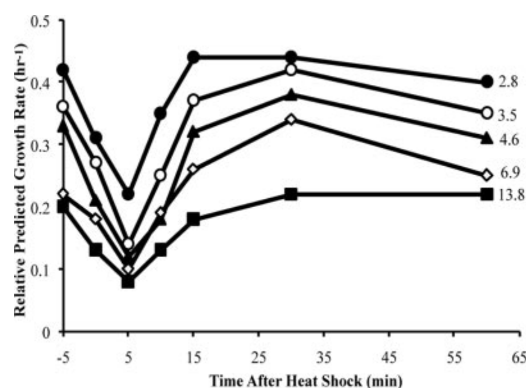

Figure 2. Negative effect of heat pulses on the growth rate of the chemostat cultures. Each line represents the predicted growth rate (Airoldi *et al*., 2008; Brauer *et al*., 2008) of a steady-state culture with a unique doubling time undergoing a heat pulse. The number next to each line corresponds to the doubling time of the culture before the heat pulse. Briefly, square, 13.8 h; white diamond, 6.9 h; triangle, 4.6 h; white circle, 3.5 h; and black circle, 2.8 h). The preheat shock time is represented as −5 min. Time zero corresponds to the moment the heat pulse reached 36°C. The conditions are described in *Materials and Methods*.

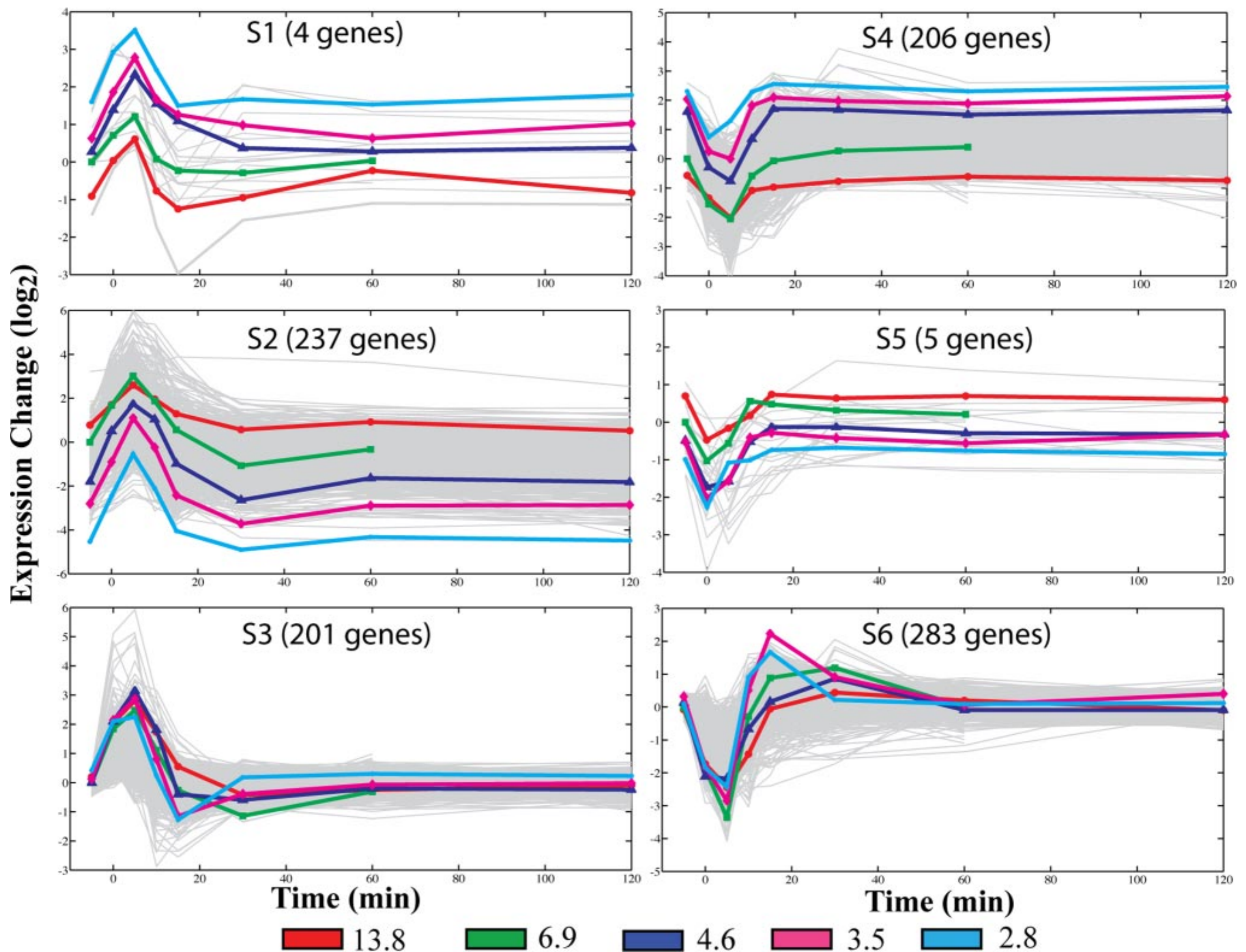

Figure 3. Genes clustered into groups based on their expression changes in response to temperature and differences in growth rate. Clusters S1–S3: genes that are induced in response to temperature; clusters S4–S6: genes that are repressed by the heat pulse. S1 and S4 contain genes that are positively correlated with growth rate. S2 and S5 contain genes that are negatively correlated with growth rate. S3 and S6 contain genes that are not affected by differences in growth rate. For each cluster, an archetype is plotted in color (in the order from slowest to fastest growth: red, green, dark blue, magenta, and light blue), whereas the rest of the genes are plotted in gray. The archetype gene for each subset is as follows: S1, *GNP1*; S2, *HXT5*; S3, *BDH1*; S4, *UTR2*; S5, *SKS1*; and S6, *AAH1*).

The nadir in the predicted growth rate occurs 5 min after the cultures achieved 36°C (or ~11 min after the heat pulse was initiated); it is at about this time that maximum expression level is observed for almost all the heat shock induced genes (Figure 3; see below).

Four Subsets of Unambiguously Heat-responsive Genes

To focus our analysis on those genes that unambiguously responded to the heat pulse, we selected the set of all the genes whose expression level changed at least twofold (up or down) in at least one chemostat (see *Materials and Methods*). Further analysis allowed us to distinguish six subsets of such genes, represented in Figure 3 as a cloud of lines overlaid by the data for a single archetype of the subset. Note that these subsets do not correspond to the clusters in Figure 1, which shows all genes and not just those that respond unambiguously to the heat pulse. Subsets S1, S2, and S3 comprise genes whose expression rises during the heat pulse and are either positively correlated (S1, containing only 4 genes), negatively correlated (S2, 237 genes), or uncorrelated (S3, 201 genes) with steady-state growth rate. Subsets S4, S5, and S6 comprise genes whose expression levels fall during the heat pulse and which are either positively correlated (S4, 206 genes), negatively correlated (S5, 5 genes), or uncorrelated (S6, 283 genes) with growth rate. The notable result here is that S1 and S5 contain very few members, but each of the other classes contains ~200 genes. Thus, there are about as many clearly heat-responsive genes whose expression is generally uncorrelated with growth rate as there are genes responsive to both temperature and growth rate changes. Clearly, the relationship between environmental stress response and growth rate does not apply to all stress-responsive genes.

It is also striking that there are virtually no heat-induced genes that show a positive correlation with growth rate (S1), and no heat-repressed genes that are negatively correlated with changes in growth rate (S5). The simplest way to understand this observation is to suppose that the heat pulse causes a reduction in growth rate, so that all genes that respond to growth rate are necessarily affected (i.e., few or none can be unaffected). This interpretation is consistent with the idea that the behavior of most or even all of S2 and

S4 genes, genes whose expression correlates with both growth rate and heat stress, arises from reduced growth rate, as postulated by Brauer *et al*. (2008) and Castrillo *et al*. (2007) and documented in Figure 2. This conclusion does not apply to the remaining genes whose expression is responsive to heat (i.e., S3 and S6) because their expression fails to correlate with the steady-state growth rate.

Functional Classifications of Heat and Growth Rate-responsive Gene Subsets

To identify any prominent themes in the gene subsets S1–S6 (Figure 3), we submitted them to Go Term Finder (Boyle *et al*., 2004) for Process, Molecular Function, and Cellular Component. For the sake of brevity, we limited the analysis to GO terms with a Bonferroni-corrected $p < 10^{-3}$ and whose term fraction defined as the number of genes assigned to a particular GO term, to be >10%. Table 1 shows the GO term enrichment for each of the categories mentioned above. Due to rather small size, no enrichment was detected for S1 and S5.

Out of 442 genes that are induced at least twofold by the heat shock, 54% (subset S2) have a negative correlation of gene expression with changes in growth rate. These genes are enriched for those with roles in energy metabolism, especially oxidative metabolism. Furthermore, genes annotated to be involved in stress response, most notably oxidative stress and trehalose biosynthesis, are also found in this cluster. Trehalose, historically regarded as a reserve carbohydrate in yeast (making up to 20% of the dry weight in stationary cells), is also known to play a vital role in promoting survival under conditions of extreme heat (Ribeiro *et al*., 1997; Singer and Lindquist, 1998; Estruch, 2000). The enrichment of oxidative stress genes is also unsurprising because it is well known that more than one type of stress can activate the same genes (Estruch, 2000).

We were hoping to identify some functional themes for genes activated by heat pulse but uncorrelated with growth rate differences. Unfortunately, when the list of S3 genes was submitted for the same analysis, no significant GO Term Enrichment was observed in all three ontologies. However, it is worthwhile to compare specific examples between the subsets that show correlation with growth rate (S2) and those that do not (S3). Virtually all the genes encoding the explicitly annotated heat shock chaperones (e.g., *SSA1*, *SSA4*, and many *HSP* genes) occur in S2 and not S3; the chaperone-encoding genes that do occur in S3 have well-characterized basic functions in diverse aspects of cell biology: *KAR2* and *MPD1* in the endoplasmic reticulum and *MDJ1* in the mitochondria. The only genes explicitly associated with thioredoxin are in S2. There are some interesting cases where one of two isoenzymes is in S2 and the other in S3: for example, superoxide dismutase (*SOD1* in S3 and *SOD2* in S2).

Subsets S4 and S6 contain genes that are repressed by at least twofold in response to heat shock. They are enriched for genes vital to growth, particularly protein synthesis. This is in agreement with previous observations (Regenberg *et al*., 2006; Castrillo *et al*., 2007; Brauer *et al*., 2008) and is a strong indication that cellular growth is negatively impacted by the heat pulse. Although the two clusters share many similarities, we also note interesting differences. For example, the S6 genes, whose expression is generally not correlated with differences in steady-state growth rate, are enriched for nucleoside metabolism, sterol metabolism, and RNA processing but not in genes that encode the ribosomal subunits. The S4 subset, whose expression is positively correlated with growth rate, contains >100 genes that are annotated to encode encoding cytoplasmic (but not mitochondrial) ribosomes, This group is also enriched for genes that participate in sulfur metabolism, notably the methionine biosynthetic pathway, the genes involved in the biosynthesis of *S*-adenosyl methionine. These differences highlight that there are differences in regulation among heat-repressed genes, just as we found for heat-induced genes.

Effect of Growth Rate on Heat-Shock Response

We next examined whether culture growth rate has any effect on the magnitude of the changes in expression of genes that respond to heat shock. Figure 4 is a comparison of the expression change for cultures with a doubling time of 14 h to one with a doubling time of 2.8 h. The magnitude of the expression change for each gene is calculated as the maximum expression difference with respect to the expression level preheat shock. From Figure 4, it is clear that the great majority of all genes are expressed to a very similar degree at both growth rates. The only exceptions comprise a relatively small group of genes that include most of those strongly induced by the heat shock; for this group, the magnitude of expression change seems to be generally higher for the culture with the faster growth rate (blue points in Figure 4).

Figure 4 is only one of many possible pairwise comparisons. To see whether this trend is general over all five growth rates, we calculated the slope of the expression change after heat shock relative to the growth rate of the culture before the heat shock. These values are plotted as a histogram in Figure 5. A positive slope indicates that the heat shock response is higher as the growth rate increases and a negative slope implies that the heat-shock response is lower as the growth rate increases. In the inset, we compare the distribution of these to a randomized distribution. The genome distribution is slightly asymmetrical, with a longer tail on the positive slope side, suggesting a very small number of genes with an unusually large positive slope. When we focused our attention on just the genes that are highly induced by heat shock (the blue points in Figure 4 and the blue bars in Figure 5), we observed a strongly positive slope as defined above, meaning that their increase in expression during the heat pulse is more drastic at higher growth rates. In contrast, genes that are also highly induced by the heat shock but show no correlation to growth rates (red points in Figure 4 and red bars in Figure 5) do not exhibit very different response at different growth rates.

Slow Steady-State Growth Confers Resistance to Heat Killing

The preceding analysis suggests that the most heat-shock–responsive genes, many of which have been previously implicated in heat resistance, are relatively highly expressed at lower growth rates. This in turn suggests that slowly growing cells might be relatively resistant to heat killing, just as cells preinduced by a short heat pulse are rendered relatively resistant to heat killing (Estruch, 2000). To test this inference directly, we subjected cultures growing at different steady-state growth rates in chemostats to 50°C, a temperature that will readily kill naive yeast cells growing in rich media. Figure 6 shows that indeed cells growing quickly at steady state (2.8-h doubling time) are very sensitive, cells growing very slowly (13.8-h doubling time) are resistant, and cells growing at an intermediate rate (4.6-h doubling time) show intermediate rates of killing at 50°C. As a control, we tested cells from another chemostat in which cells were growing at steady state at the intermediate rate, but at 36°C, with the result that these cells were as resistant as the slowly-growing cells. This increase in heat resistance with

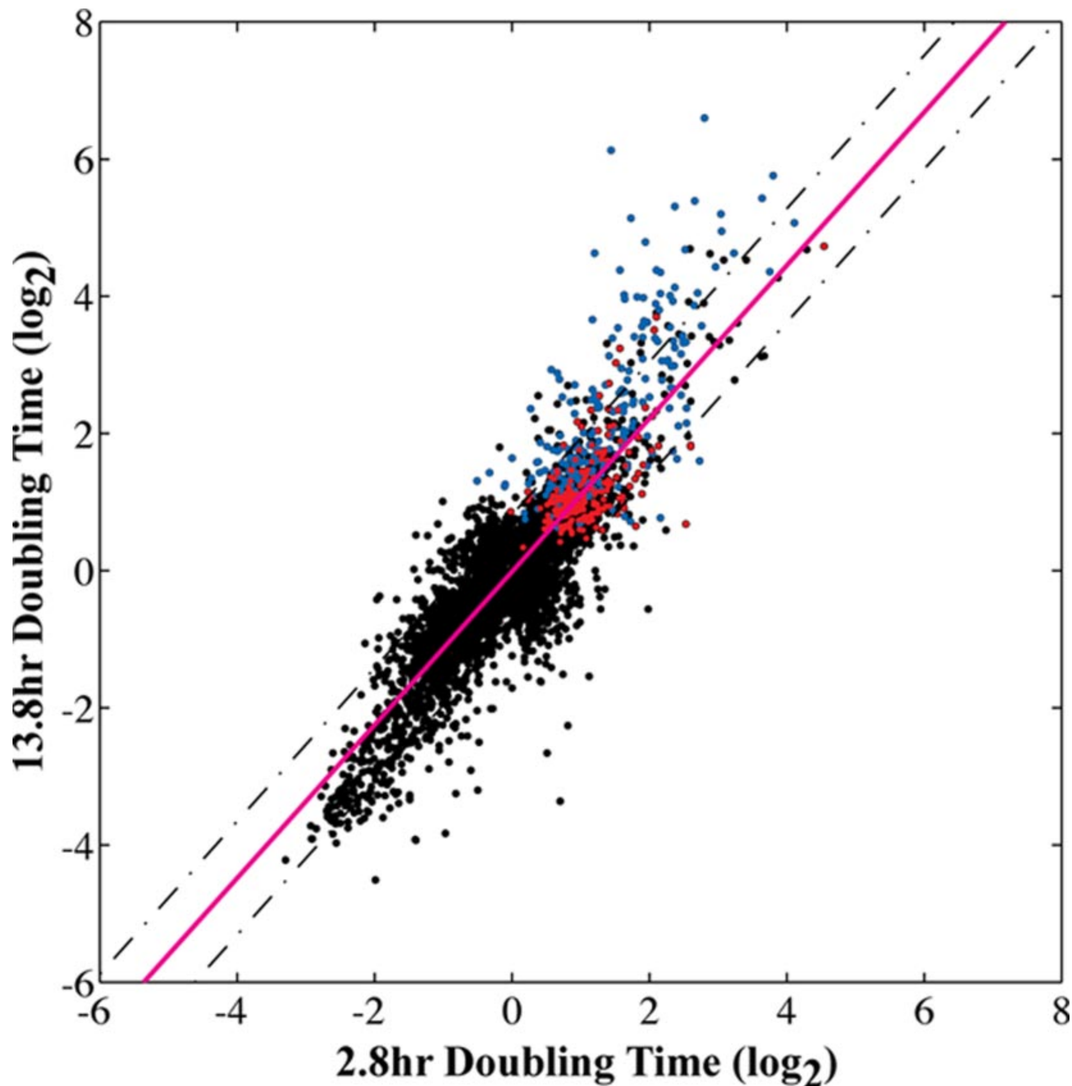

Figure 4. The magnitude of the gene expression change in response to the heat pulse between a fast growing (2.8-h doubling time) and a slow-growing cultures (13.8-h doubling time). The background (genome distribution) is shown in black. Blue and red dots depict genes from S2 and S3 cluster, respectively. Magenta line is a linear fit of the plot, with the two dashed lines representing 1.5 SDs from the linear fit.

slower growing cells coincides with the elevated expression of many known stress response genes at the low growth rate.

Thus far, in all the experiments described above we enforced the growth rate of the culture by growing cells in a phosphate-limited chemostat with different rates of media flow. To rule out the possibility that the chemostat condition, and not growth rate per se, determines survival to heat, we set out to see whether the correlation between growth rate and heat resistance can also be observed in batch culture. Previous studies have found that the exponential growth rate in batch culture can be varied by using diverse sources of nitrogen (Boer *et al.*, 2007). We chose asparagine (fastest), urea (intermediate), and proline (slowest) on this basis. When we compared the heat resistance of yeast batch cultures grown in asparagines, urea and proline (Figure 7A), we observed that the fastest growing culture with asparagine as the nitrogen source is more sensitive to heat shock than that of the proline and urea cultures. This result was also observed by Elliott and Futcher (1993). As a control, we grew the same strains in nitrogen-limited chemostats by using the same three nitrogen sources at the same dilution rate (which guarantees the same growth rate), we observed that the differences in heat sensitivity between the three chemostat cultures were minimized (Figure 7B). Thus, a major determinant of heat resistance indeed seems to be the growth rate and not the identity of the nitrogen source in each culture.

The Growth Rate Effect on Heat Resistance is Independent of Respiratory Metabolism

The increased expression of known stress genes, coupled with increased heat resistance, at low growth rate suggests that the slower the cells grow, the more stressed they become. Previous study has shown that genes that are negatively correlated with changes in growth rate are enriched for the processes of oxidative metabolism and cellular component of peroxisomes (Brauer *et al.*, 2008). Because respiratory metabolism increases the chance that cells will suffer damage from reactive oxygen species, we wanted to test the hypothesis that the heightened expression of many stress response genes at low growth rate might simply be a response to increased oxidative damage. To this end, we compared the gene expression and heat resistance phenotypes of

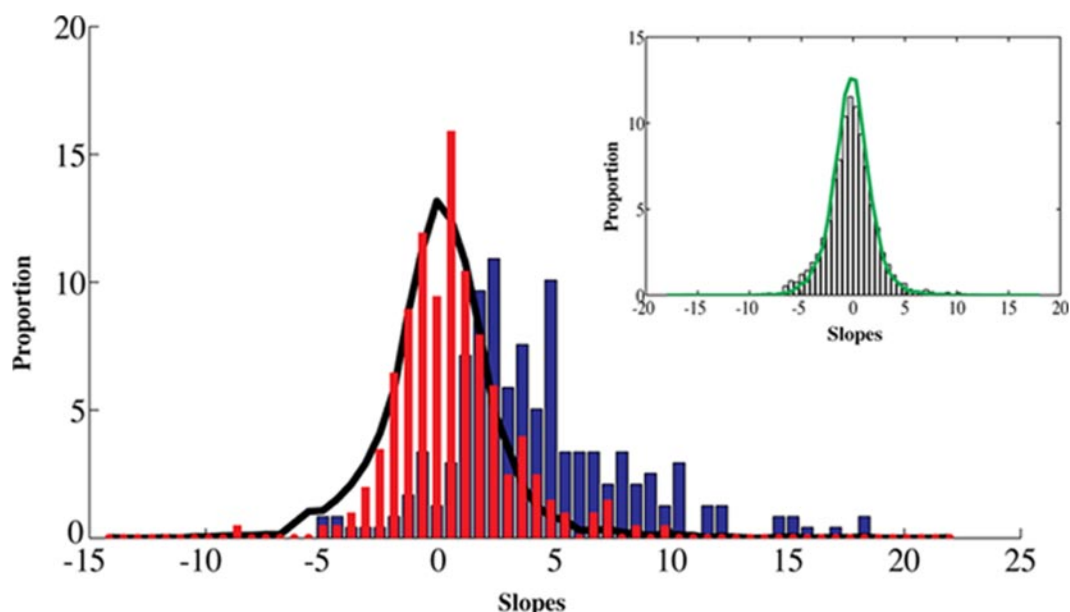

Figure 5. The distribution of the slopes of the heat-shock expression changes as a function of growth rate. Positive slopes indicate that the magnitude of the response is positively correlated with growth rate, whereas negative slopes indicate the opposite. The inset shows the distribution of the genome (black bar) compared with bootstrapped values (100,000 samples) represented as green line. In the main graph, the black line represents the distribution of the genome. The blue bar graph depicts the distribution of heat-induced genes that has a negative correlation to growth rate. The red bar graph represents the heat-induced genes that are not affected by changes in growth rate.

wild type to petite *[rho°]* mutants completely lacking mitochondrial DNA and thus respiratory deficient (Piskur, 1994). On the hypothesis that induction of stress-related genes due to increased respiratory activity (and the attendant potential for oxidative damage) at low growth rates is the cause of heat resistance, we expected that petite mutants would express stress-related genes less strongly and thereby be less resistant to heat killing.

We grew steady-state cultures of both wild type and petite strains in chemostats at the same temperature (30°C) and dilution rate (both at 0.05 and 0.1 h^{-1}), which correspond to 14- and 7-h doubling time, respectively. Figure 8 shows the distribution of the gene expression in the *[rho°]* mutant strain relative to its wild-type parent. As expected, the stress-inducible genes (as defined in Gasch *et al.*, 2000) are expressed at a much reduced level in petite steady-state cultures compared with the wild type ($p < 10^{-49}$ by a Kolmogorov–Smirnov test), suggesting that slower growth indeed has less of an inducing effect in the mutants. Similarly, the stress-repressed cluster genes (again as defined by Gasch *et al.*, 2000) tend to be expressed at a higher level relative to what is found in the wild type ($p < 10^{-34}$),

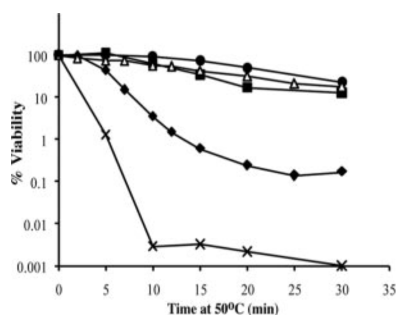

Figure 6. Survival of steady-state cultures in response to lethal heat shock (50°C) is affected by the steady-state growth rate. Each curve represents the survival curve of cultures at different steady state growth rate. Briefly, circle, 13.8 h; square, 6.9 h; diamond, 4.6 h; and X, 2.3 h., all at 30°C; and triangle, 4.6 h at 36°C. The assay is described in *Materials and Methods*.

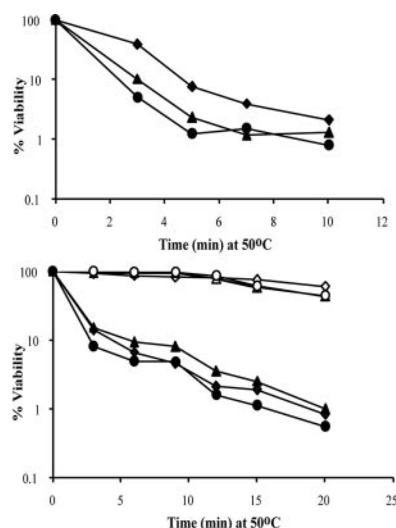

Figure 7. Comparison of the survival of cultures growing exponentially with equimolar amounts of different nitrogen sources. (A) Batch cultures growing exponentially in proline (diamond) with a doubling time of 3.5 h, asparagine (circle) with a doubling time of 1.7 h, and urea (triangle) with a doubling time of 2.1 h. (B) Chemostat cultures limited by the concentration of the same three nitrogen sources but growing at essentially the same rates. The symbol for each nitrogen source is the same as those in A. The slow growth rate (in white) has a doubling time of 13.8 h. The faster growth rates (in black) for proline, urea, and asparagine have a doubling time of 4.6, 4.9, and 4.1 h, respectively.

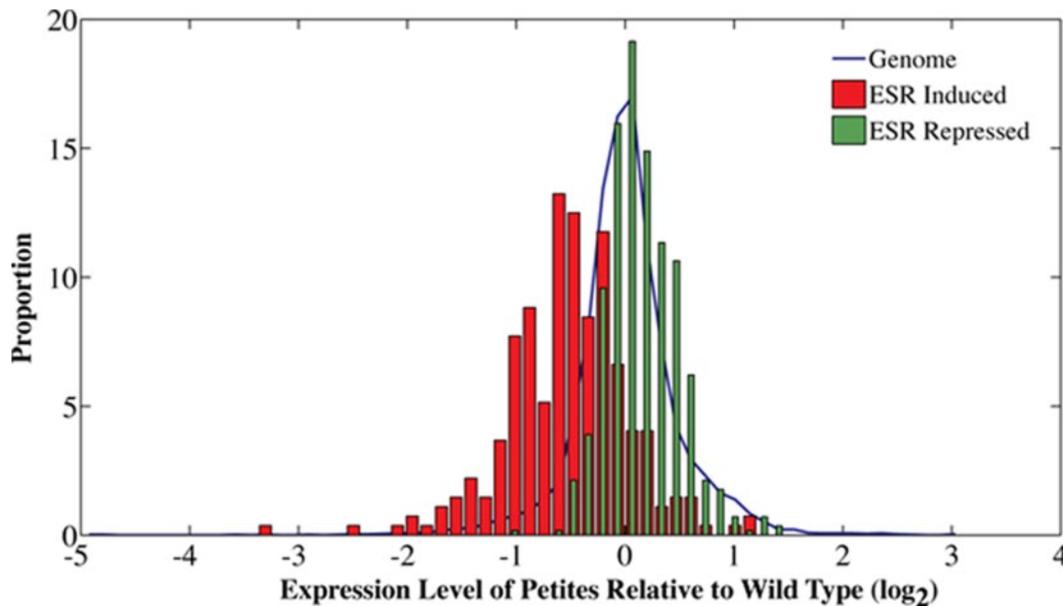

Figure 8. Reduced difference in gene expression in a petite *[rho°]* mutant compared with wild-type cultures at the same growth rate. The distribution of expression of all genes in petites (blue line) relative to the wild type and the known common environmental stress genes as defined by Gasch *et al.* (2000). Induced ESR genes, red bar; repressed ESR genes, green bar.

meaning that slower growth has less of a repressive effect on this group than observed in wild type. These data verify that slow-growing *[rho°]* mutants, as expected, seem to have less of the stress-associated gene expression patterns characteristic of wild type at the same growth rate.

The differences in gene expression between petite and wild type suggest that the parameters of the relationship between growth rate and gene expression might be altered. When we used the 72-gene predictive model of Brauer *et al.* (2008), the model predicted a higher-than-expected growth rate for the petite strains at both growth rates we tested. Thus, although the expression change of many known growth rate-responsive genes in petites is less extreme than those in the wild type at each growth rate (Figure 8), the overall relationships of expression relative to growth rate are preserved (i.e., the predictor works for both strains). This is entirely consistent with the idea that respiratory functions play a role in regulating expression of many (but not necessarily all) growth rate correlated genes.

Given this result, we might expect that these differences in gene expression would result in differential sensitivity to heat killing as a function of growth rate, as hypothesized. However, as shown in Figure 9, this is clearly not the case. At the same growth rate, there was no difference in survival between petites and wild-type cells. However, when we extended this comparison to different growth rates, we observed that the survival of petites and wild type are both higher at low growth rate, as what we would expect. This result strongly implies that the increased resistance to heat at low growth rate cannot be attributed to oxidative damage.

DISCUSSION

Previous studies exposing yeast to different stressors have shown that although the actual stress responses elicited are unique, substantial areas of overlap, especially in patterns of gene expression, also exist. These observations are in accord with the well-studied phenomenon called "cross-protection," which occurs when cells are exposed to a mild stress develop tolerance not only to the higher level of the same stress but also to those caused by other stress agents (Mitchel and Morrison, 1982, 1983; Barnes *et al.*, 1990; Jamieson, 1992; Flattery-O'Brien *et al.*, 1993; Davies *et al.*, 1995; Swan and Watson, 1999; Estruch, 2000; Pereira *et al.*, 2001; Palhano *et al.*, 2004). Overlap in the stress response at the transcript level involves about 900 genes whose expression changes across a wide range of harmful conditions (Gasch *et al.*, 2000). More recent studies have also revealed that the yeast growth rate profoundly impacts gene expression (Regenberg *et al.*, 2006; Castrillo *et al.*, 2007; Brauer *et al.*, 2008). Perhaps the most striking overlap is seen between genes that are affected by the differences in growth rate and the genes observed by Gasch *et al.* (2000) to change in most or all stresses. These observations raised the possibility that much of gene expression response might be secondary to diminished growth rate caused by stress (Castrillo *et al.*, 2007; Brauer *et al.*, 2008).

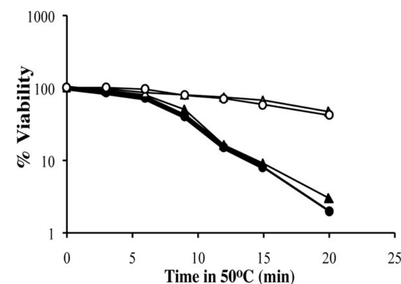

Figure 9. Comparison of heat resistance between wild type (triangle) and petites *[rho°]* (circle) at two different growth rates (13.8 h, white; 6.9 h, black). Both cultures are grown in phosphate-limited chemostat (excess glucose) at 30°C and subjected to lethal heat shock.

Four Subsets of Heat-responsive Genes

We set out to classify genes on the basis of independent experimental assessment of their response to changes in temperature and growth rate. We took advantage of the chemostat to control the growth rate of the culture. We applied a short heat pulse to steady-state yeast cultures growing with doubling times ranging from 14 to 2.8 h. On the basis of their transcriptional responses, we could subdivide heat shock-responsive genes into subsets based on their response to these two variables. Not surprisingly, many genes (∼200) known to be induced in response to heat shock were also highly expressed at *low* growth rate. Similarly, ∼200 genes that are repressed by heat shock are highly expressed at *high* growth rate. These results corroborated findings of Castrillo *et al*. (2007) and Brauer *et al*. (2008). However, in both cases, we also identified comparable numbers of genes that are heat-shock specific but were not affected by differences in growth rate. It is tempting, but not necessary, to interpret these results as dividing heat-shock–specific responses from more generic responses to environmental perturbations.

For the heat-repressed genes, we found significant Gene Ontology enrichment in both subsets S4 and S6 in Figure 3: heat-repressed genes that are not affected by growth rate (S6) and those that are positively correlated with changes in growth rate (S4). Although both subsets are enriched for genes involved in the machinery of protein synthesis, there are differences between the two subsets. Subset (S6) is enriched for nucleotide biosynthesis and genes involved in translation functions like tRNA processing, whereas subset (S4) is dominated by ribosomal protein-encoding genes. Because cellular growth rate directly depends upon the rate of production of proteins, it is to be expected that the ribosomal protein gene expression is positively correlated with changes in growth rate, particularly if one supposes that the number of ribosomes is rate-limiting. In this view, the growth rate-insensitive subset contains genes whose expression (or the gene products themselves) are not growth rate limiting. These results are consistent with the conclusion that the decrease in transcription of ribosomal genes that accompanies virtually every kind of stress (cf. Gasch *et al*., 2000) simply reflects decreased demand for ribosomes as cells divide more slowly.

Effect of Growth Rate on Heat-Shock Response

The pervasive effect of growth rate on gene expression strongly suggests that different internal conditions prevail at different steady states. We therefore compared the magnitude of the heat shock transcriptional response as a function of the growth rate to see whether growth rate has any impact. We found that the growth rate has little if any impact on the gene expression changes in response to the heat pulse. However, we did observe significant deviations for those genes that are most highly induced by the heat shock (e.g., *HXT5*, the representative gene for subset S2 in Figure 3). For these genes, the magnitude of the change in gene expression to the heat pulse is more dramatic at higher growth rate. Furthermore, we observed that these genes, for the most part, are already highly expressed at low steady-state growth rate, before the heat pulse. We therefore conclude that the magnitude of expression changes is influenced by expression levels before the heat shock.

It is worth noting that a related observation was made by Berry and Gasch (2008), who found that the gene expression response to stress is smaller if cells have been exposed previously to stress. Because a substantial fraction (∼50%) of the genes are the same in the two studies, this suggests that slow growth might be seen as a mild stress. In contrast, the mild stress might have impacted growth rate, and thus this observation, consistent between the two studies, is unhelpful in distinguishing whether the ultimate reason for the gene expression changes is "stress" or "growth rate."

The heat-resistance experiments we carried out demonstrated conclusively that slow growth confer cross protection to heat shock, and presumably other types of stress. Typically, cross-protection has been studied between oxidative, salt, osmolarity, and heat stresses. Starvation, a condition that approximates low growth rate, has also been studied. Our observation of cross-protection from heat shock suggests, at the surface, that the relevant protecting genes and functions might be found among subset S2, i.e., those genes that are induced both by heat and slow growth. Thus, one could make the argument that the similar phenotype (i.e., cross-protection) of slow growing cells with those that have been stressed by traditional means seems to suggest that the slow-growing cells behave as if they are under stress.

Role of Oxidative Metabolism in Gene Expression

This line of reasoning then leads to the question of what might be the reason that slow growth induces protective functions. One appealing possibility is that slow growth, even on a fermentable carbon source, differs from rapid growth in that it involves higher levels of respiration. With respect to the bulk of the gene expression changes associated with slow growth, on the one hand, and stress, on the other, our data strongly support this view. A respiratory-deficient (*[rho^o]*) culture shows a markedly reduced change in expression of most of the genes associated with the generic stress response (Gasch *et al*., 2000) compared with an isogenic wild-type culture growing under the identical conditions.

Additional features of our results are relevant to the role of oxidative metabolism in the influence of growth rate on gene expression. At the physiological level, we observed that at steady-state, slow growing cultures are more dense, produce less ethanol and consume more glucose. This decrease in the production of ethanol, coupled with an increase in the consumption of glucose, is consistent with the assumption that slow growing cells are shifting their carbon flow more toward respiration. At the gene expression level, we also observed an enrichment in genes that participate in the respiratory process and peroxisomal localization at the low growth rate, a result also seen by previous study (Brauer *et al*., 2008). Also, we observed in these data, as we had seen before during experiments involving the diauxic shift (Brauer *et al*., 2005), that the enzymes of the tricarboxylic acid cycle are induced in slow growth as well (Supplemental Table 2).

These observations provide indirect evidence of a metabolic shift that accompanies a reduction in growth rate. We are left with the general picture that at high growth rates and in the presence of excess glucose fermentation is the predominant mode of energy production, whereas at low growth rates the cell population shifts to increasing dependence on mitochondrial function. It is known that actively respiring cells are susceptible to oxidative damage caused by ROS produced by the electron transport chain, the principal site being close to the cytochrome *c* oxidase complex (Guidot *et al*., 1993). The increased oxidative damage experienced by cells would elicit a stress response. Not surprisingly, respiring cells grown on nonfermentable carbon source have been observed to be more resistant to oxidative

stress than those grown on glucose (Jamieson, 1992; Flattery-O'Brien *et al*., 1993). Correspondingly petite ([*rho*^{o}]) mutants, whose mitochondrial functions have been impaired, are unable to respire and thus have been observed to be more sensitive to various type of oxidative stress (Grant *et al*., 1997; Maris *et al*., 2001). Furthermore, because the stress response elicited by ROS in the respiring cells is very similar to that induced by heat shock (Godon *et al*., 1998; Lee and Park, 1998; Sugiyama *et al*., 2000), Moraitis and Curran (2004, 2007) have demonstrated that heat shock response and thermotolerance are strongly influenced by the level of ROS.

No Difference in Cross-Protection in Respiratory-deficient Cultures

With this result in hand, it is logical to expect differences in cross-protection against heat killing in respiratory-deficient and respiratory-competent strains, because most (but not all) of the genes in subsets S2 and S4 are expressed differently in the petite strain. Surprisingly, however, when we tested the cross-protection in both respiratory-deficient and respiratory-competent strains at 50°C and at two different growth rates, we found that the survival curves between the two strains with equal growth rate were virtually identical. Thus, we are forced to conclude that the genes and functions responsible for cross-protection are not being induced by some by-product of respiratory metabolism.

We can readily envision a relatively simple explanation for these apparently conflicting results. Among the many genes in subset S2, there may well be quite a few that are induced equally in wild-type and respiratory-deficient cells. The basis for their induction would have to be some direct readout of growth rate unrelated to oxidative metabolism. Alternatively, there might be a threshold of activity that is passed by even the moderate induction found in the respiratory-deficient strain.

Is Slow Growth Stressful?

The evidence presented in this paper demonstrates an inverse relationship between the rate at which cells divide and how resistant they are to heat stress. This behavior bears striking resemblance to cross-protection, in which exposure to one form of stress often confers resistance to another. This leads to the idea that cell with long doubling time seem to be stressed. Three features of slowly growing cells support this notion (Castrillo *et al*., 2007; Brauer *et al*., 2008). At the level of gene expression, most of the known stress response genes are highly induced at slow growth. At the level of cell cycle regulation, slower growing cells spend larger fractions of their cell cycle in the G0/G1 (i.e., unbudded) phase. At the physiological level, as we have shown here, slow-growing cells are more resistant to lethal heat challenge.

We have dealt with the transcriptional regulation above, by clearly demonstrating that most of the commonly known stress genes are induced during slow growth in the chemostat. Furthermore, we also observed that known stress genes are expressed at a lower level in respiratory-defective [*rho*^{o}] mutants than in wild type at equal growth rate. This implies that there may well be a role for respiratory metabolism or its consequences in activating the regulators of these genes. This might actually be oxidative damage itself, or, alternatively, it might be some other feature of oxidative metabolism such as the proton flux across the mitochondrial membrane or a metabolite associated with a peroxisomal function. In this regard, we must also consider the possibility of metabolic cycling, wherein it is thought that there might be an alternation of oxidative and fermentative metabolism in wild-type cells (Klevecz *et al*., 2004; Tu *et al*., 2005). Obviously, this explanation will not account for respiration-independent induction of cross-protection.

The cell cycle presents an attractive possibility for a respiration-independent readout of growth rate. It has been reported in the literature that thermal stress induces a transient arrest in the G1 phase of the cell cycle which causes the accumulation of unbudded cells (Johnston and Singer, 1980). Subsequent studies showed that this heat-induced arrest at the cell cycle regulatory step of START was related to the decrease in transcript abundance of G1 cyclins *CLN1* and *CLN2* (Rowley *et al*., 1993). The accumulation of cells in G1 in response to stress and slow growth suggests that cells in G1, in which no replication or division is taking place, are more stress resistant, suggesting a possible relationship between thermotolerance and cell cycle (Plesset *et al*., 1987). However, other studies have shown that thermotolerance is largely independent of cell cycle position (Barnes *et al*., 1990; Elliott and Futcher, 1993). Our results with growth rate-dependent cross protection against heat challenge in respiration-deficient cells may well motivate a reexamination of the possibility of a cell cycle-dependent readout of growth rate.

At the physiological level, a promising avenue is the link between nutrient sensing and stress response. In yeast, the cAMP-dependent protein kinase (PKA) pathway has a strong influence on stress resistance. Mutants with an elevated cAMP–PKA pathway display lower stress resistance, whereas mutants with this pathway repressed show higher stress resistance (Toda *et al*., 1987; Cameron *et al*., 1988; Park *et al*., 1997). Such mutants, although highly stress resistant, also exhibits longer lag phase and slow growth. However, a mutant *fil*1 (fermentation-induced loss of stress resistance) with a mutation in adenylate cyclase was isolated and found to have a 10-fold drop in adenylate cyclase activity (Van Dijck *et al*., 2000). What made this mutant interesting was that although it exhibited more freeze and drought resistance than that of the wild type, its fermentative growth rate with the wild type is comparable, demonstrating that high stress resistance and normal growth are not mutually exclusive. Interestingly it was also observed that the presence of *fil*1 mutation markedly increased the stress resistance of strains deleted for *MSN2*, *MSN4*, *HSP104*, and *TPS1* (Versele *et al*., 2004). This suggests that there are factors beyond trehalose, Hsp104 and the Msn2/4-controlled genes that play a role in conferring general stress resistance.

Finally, we raise the possibility that slow growth may be the default mode of growth, i.e., the environment in which the genome of yeast was forged by selection over evolutionary time. In this view, all genes that provide cross protection and are viewed by the literature as indications of unusual environmental stress are actually normally expressed, because yeast evolved in slow growth conditions. In this view, the normal scientific laboratory conditions (rich medium, excess glucose) are unusual, and yeast, normally expecting slow growth, heat pulses, radiation exposure, changes in osmolarity and salt provide themselves with the means to resist them all. Under this view, we should require an explanation of why at fast growth rates in rich media these genes are turned off. It could well be that evolution favored populations that could temporarily increase their growth rate by abandoning their defenses when conditions such as food supply and temperature are optimal. Under this view, favorable environmental conditions represent rare but evolutionarily important opportunities for rapid population ex-

pansion, and what we currently think of as "stressful" is probably the default lifestyle to which the yeast has been accustomed.

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