Anoxia-Induced Suspended Animation in Budding Yeast as an Experimental Paradigm for Studying Oxygen-Regulated Gene Expression

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A lack of oxygen can force many organisms to enter into recoverable hypometabolic states. To better understand how organisms cope with oxygen deprivation, our laboratory previously had shown that when challenged with anoxia, both the nematode Caenorhabditis elegans and embryos of the zebrafish Danio rerio enter into suspended animation, in which all life processes that can be observed by light microscopy are halted pending the restoration of oxygen (P. A. Padilla and M. B. Roth, Proc. Natl. Acad. Sci. USA 98:7331–7335, 2001, and P. A. Padilla, T. G. Nystul, R. A. Zager, A. C. Johnson, and M. B. Roth, Mol. Biol. Cell 13:1473–1483, 2002). Here, we show that both sporulating and vegetative cells of the budding yeast Saccharomyces cerevisiae also enter into a similar state of suspended animation when made anoxic on a nonfermentable carbon source. Transcriptional profiling using cDNA microarrays and follow-on quantitative real-time PCR analysis revealed a relative derepression of aerobic metabolism genes in carbon monoxide (CO)-induced anoxia when compared to nitrogen (N2) gas-induced anoxia, which is consistent with the known oxygen-mimetic effects of CO. We also found that mutants deleted for components of the mitochondrial retrograde signaling pathway can tolerate prolonged exposure to CO but not to N2. We conclude that the cellular response to anoxia is dependent on whether the anoxic gas is an oxygen mimic and that the mitochondrial retrograde signaling pathway is functionally important for mediating this response.

Molecular oxygen (O2) can be utilized by all organisms, except obligate anaerobes, in metabolic pathways that enable the extraction of chemical energy stored in nutrient compounds. When cells reliant on aerobic respiration suffer from poor oxygen availability, the cells respond by increasing anaerobic energy production, upregulating stress genes, and in the case of mammals, stimulating angiogenesis to increase oxygen delivery (60). In many species, it is thought that the delivery of oxygen to tissues is homeostatically adjusted in order to provide adequate oxygenation for energy generation (52), consistently with the idea that organisms must compensate for decreases in environmental oxygen levels in order to thrive or to merely survive. In addition to being a topic of interest in its own right, understanding how various cell types cope with a lack of oxygen can have important implications for human health, as the progression of several pathological conditions, including heart attack, stroke, and cancer, is associated with poor oxygenation to the affected tissues (35).

Many approaches in different systems have been undertaken to better understand the mechanisms that enable cells to survive a lack of oxygen. Among eukaryotic model organisms, the growth of yeasts under depressed oxygen levels has been of great interest historically, in large part due to the role of yeasts in the baking and brewing industries (6). Although budding yeast is a facultative anaerobe, continuous culturing under anaerobic conditions requires the addition of sterols (1) and unsaturated fatty acids (2) in the medium (since molecular oxygen is required to synthesize these compounds), as well as the activation of biochemical pathways to bypass those that require molecular oxygen (54). This highlights the importance of oxygen even for organisms classified as facultatively anaerobic.

The response of many metazoan species to decreased oxygen also has been extensively studied. These include many popular model organisms, such as nematodes (53), fruit flies (20), zebrafish (26), and mice (51). In addition, much work has been done on less well-studied systems, including brine shrimp (21), turtles (55), carp (15), sharks (37), and dogs (47). These organisms all appear to manifest physiological and behavioral changes that are consistent with a decrease in metabolism when exposed to lower-than-normal oxygen concentrations. From this veritable menagerie, it is clear that many species have evolved mechanisms to cope with a lack of oxygen at various levels of severity.

Our laboratory has been interested in the response of model systems to very severe oxygen deprivation and has demonstrated that two well-studied model organisms, the nematode Caenorhabditis elegans (40) and embryos of the zebrafish Danio rerio (39), enter into a reversible state of suspended animation when exposed to anoxia (operationally defined as an atmosphere containing less than 10 Pa of O2). Similarly to results reported for Drosophila embryos (16), all life processes observable by light microscopy are halted pending the restoration of oxygen. Moreover, it was found that the san-1 gene, which encodes a component of the mitotic spindle checkpoint, is required for anoxia-induced suspended animation in C. elegans embryos, as the depletion of the SAN-1 gene product by RNA-mediated interference resulted in chromosome missegregation and death (38).

To further elucidate the molecular mechanisms that underpin the process of anoxia-induced suspended animation, we...
turned to the budding yeast *Saccharomyces cerevisiae*, a model system that we show also enters into reversible suspended animation when exposed to anoxia on a nonfermentable carbon source. We carried out transcript microarray analysis on cells that were made anoxic on a nonfermentable substrate in order to identify pathways that may be important for survival under such conditions. We used two different anoxic gases, carbon monoxide (CO) and nitrogen (N2). As CO can mimic the presence of O2 by displacing the latter in the binding sites of many heme-containing proteins (reviewed in reference 41) while N2 cannot, we hypothesized that there would be marked differences in gene expression between the transcriptomes of cells exposed to each of the two anoxic gases. Consistently with the known oxygen-mimetic properties of CO, we found that exposure to this gas caused a coordinated derepression of aerobic metabolism genes when compared to a similar exposure to N2. Moreover, we found that mutants deleted for components of the mitochondrial retrograde signaling pathway recovered normally from prolonged exposure to CO but recovered poorly after similar exposure to N2. Our findings lead us to conclude that the response of yeast to anoxia is dependent on whether the applied anoxic gas is an oxygen mimetic and that the mitochondrial retrograde signaling pathway is functionally important for mediating the proper response.

**MATERIALS AND METHODS**

**Anoxia-induced suspension of sporulation.** Diploid SK1 cells (MATa/MATα *ho::LYS2*2/ho::Y2233/2::ura3Δ3/3::ura3Δ3/3::lys2Δ2/2::sen2(HisG2/His2G;Hog1::GFP-mbp4/4-bgl his4X: LEU2::RAG3::his3/3::BRE1EU2) were grown for 24 h in 1 ml YPD (1% yeast extract, 2% peptone, 2% glucose) at 30°C. This culture then was diluted 100-fold into YPA (1% yeast extract, 2% peptone, 3% potassium acetate) presuperoxidation medium and grown for 48 h in a 1-liter baffled Erlenmeyer flask with shaking. Cells then were collected by centrifugation, washed once in sterile water, and resuspended in 100 ml sporulation medium and grown for 48 h in a 1-liter baffled Erlenmeyer flask with shaking. These plates then were sealed inside modified Pyrex crystallization dishes (Corning, Inc., Lowell, MA) at 100 cm3 per min. The test culture was bubbled with O2 up to the beginning of hour 6, with N2 (N2 was scrubbed with an Aerex CE500KFR14 inline inert gas purifier to remove trace O2 contamination) from hour 6 to the beginning of hour 18, and with O2 from hour 18 onward. Samples were collected every 3 h. Cells were collected by gentle centrifugation and fixed using 70% ethanol at −20°C. Cells then were stained with 4′,6-diamidino-2-phenylindole (DAPI) to visualize chromatin, and the percent asci formation as well as the completion of meiosis I and II were quantified.

**RNA extraction.** To obtain cells for RNA extraction, BY4741 (MATa/his3Δ1/1::LYS2/2::met15/15::ura3/3::ly2Δ2/2::sen2(HisG2/His2G;Hog1::GFP-mbp4/4-bgl his4X: LEU2::RAG3::his3/3::BRE1EU2) were grown for 24 h in 1 ml YPD (1% yeast extract, 2% peptone, 2% glucose) at 30°C. This culture then was diluted 100-fold into YPA (1% yeast extract, 2% peptone, 3% potassium acetate) presuperoxidation medium and grown for 48 h in a 1-liter baffled Erlenmeyer flask with shaking. Cells then were collected by centrifugation, washed once in sterile water, and resuspended in 100 ml sporulation medium and grown for 4 h in a 1-liter baffled Erlenmeyer flask with shaking. These plates then were sealed inside modified Pyrex crystallization dishes (Corning, Inc., Lowell, MA) at 100 cm3 per min. The test culture was bubbled with O2 up to the beginning of hour 6, with N2 (N2 was scrubbed with an Aerex CE500KFR14 inline inert gas purifier to remove trace O2 contamination) from hour 6 to the beginning of hour 18, and with O2 from hour 18 onward. Samples were collected every 3 h. Cells were collected by gentle centrifugation and fixed using 70% ethanol at −20°C. Cells then were stained with 4′,6-diamidino-2-phenylindole (DAPI) to visualize chromatin, and the percent asci formation as well as the completion of meiosis I and II were quantified.

**Microarray processing and analysis.** Microarray processing steps described here, up to and including the scanning of hybridized slides, were carried out by the DNA Array laboratory at the Fred Hutchinson Center. Four micrograms of total RNA from each sample was used as the substrate for the Ambion Amino MessageAmp protocol (Ambion Inc., Austin, TX). Dye-coupled products from the in vitro transcription step were hybridized to yeast open reading frame (ORF) microarray slides (bearing 6,229 yeast ORFs) that were printed in house. Arrays were hybridized, washed, and scanned on a GenPIX Pro 6 microarray scanner (MDS Analytical Technologies, Toronto, Canada), and images were returned to the authors for analysis.

**Quantification of percentage of budded cells.** BY4741 cells were plated onto nylon membranes on solid YPA similarly to the procedure for RNA extraction. Cells were incubated at 30°C under continuous perfusion with either CO or N2 for 2 days. Cells were washed off the membranes as described for RNA extraction and then were fixed in 4% formaldehyde in 0.1 M potassium phosphate, pH 7.5, for 15 min at room temperature with continuous tumbling. Cells were washed twice in 0.1 M potassium phosphate, pH 7.5, supplemented with 1.2 M sorbitol and resuspended in the same buffer. More than 600 cells were counted from each sample for each of three biological replicates.

**Transcript microarray analysis.** A Lowess normalization procedure was applied using GeneTraffic (Iobion Informatics, La Jolla, CA). T-profiler (10) was used to identify upstream consensus motif and gene ontology (GO) enrichment patterns within the array data. This online tool (http://www.t-profiler.org) utilizes the Student t test to derive an E value that reflects the degree of difference in the mean log-transformed expression ratio of a predefined group of genes and the mean for the rest of the genome. Student t tests are calculated for each gene group, in each data set, and at each time point in a time course. An E value of <0.05 is considered indicative of a statistically significant difference in gene expression. As this approach compares the mean expression ratios of each gene group to determine which genes are responsible for the observed expression changes, genes in each group contribute to the evaluation of statistical significance, if any, to those genes that are judged to be differentially expressed on an individual basis. MEME (http://meme.sdsc.edu/meme/meme.html) (3, 5) and MAST (http://meme.sdsc.edu/meme/mast.html) (4) were used for de novo consensus motif identification and genomewide upstream sequence enrichment searches, respectively.

**Quantitative real-time PCR (qRT-PCR).** To initiate cDNA synthesis for each sample, 5 μg total RNA was combined with 225 pmol random primers in water to a total volume of 18.5 μl and incubated at 70°C for 10 min. Samples then were immediately chilled on ice for 10 min. A cocktail of the following reagents in the appropriate multiple of these proportions was prepared: 6 μl 5× first-strand buffer, 3 μl 0.1 M dithiothreitol, 0.6 μl 25 mM each deoxynucleoside triphosphate, and 1.9 μl SuperScript II. A volume of 11.5 μl of this cocktail then was added to each RNA-primed mix and incubated for 2 h at 42°C. Reaction mixtures then were incubated at 95°C for 5 min to inactivate the reverse transcriptase. Two units of RNase H was added to each reaction, which then were incubated at 37°C for 20 min to degrade the template RNA. Finally, samples were incubated at 95°C for 5 min to inactivate the RNase H.

For qRT-PCR, each reaction mixture consisted of the following: 19.92 μl of reaction mix, 0.1 μl 10× PCR buffer, 0.9 μl 50 mM MgCl2, 1.5 μl 2.5 mM each deoxynucleoside triphosphate, 0.03 μl Sybr green, 1.5 μl cDNA reaction mix, 0.15 μl Taq, and 3 μl 30 μM each gene-specific primer. A reaction cocktail consisting of the common components sufficient for the required number of reactions was set up and then dispensed into each well of a 96-well PCR dish. All reagents for this procedure were from Invitrogen (Carlsbad, CA). qRT-PCRs were carried out on a Bio-Rad iQ5 thermocycler, with a 5-min step at 94°C.
followed by 40 repeats of the following steps: 94°C for 30 s, 55°C for 30 s, 72°C for 60 s, 78°C for 10 s, and plate read. PCR products were analyzed on a 3% agarose gel to verify the size of each product and the absence of side products. The automated detection of the qRT-PCR threshold cycle by iQ5 software was applied with reactions utilizing the same primer pairs grouped together for each of the 16 primer pairs. The manual adjustment of threshold cycle detection was necessary in a few cases in which the software failed to correctly distinguish signal from background.

Identification of mutants sensitive to prolonged anoxia. We searched the Saccharomyces Genome Database (SGD) for all genes that are annotated under the GO term signal transduction, as well as all genes annotated under subordinate terms. In total, we found 174 nonessential genes with corresponding deletion strains in the MATα deletion set. Each of these strains was inoculated into 200 μl liquid YPD and grown for 2 days at 30°C. Strains then were spotted at 1,000-fold dilution in PBS onto solid YPA medium and incubated for 4 days under continuous perfusion with hydrated CO or N₂ at 100 cm³/min in modified crystallization dishes. Control plates were maintained in room air. Candidate strains for retesting were identified by comparing plates after the formerly anoxic cells were allowed to recover in air.

Candidate strains from the initial phenotypic test were pregrown in the same manner. Tenfold serial dilutions were spotted onto solid YPA medium and subjected to the same phenotypic testing procedure as described above using three biological replicates. rtg1, rtg2, and rtg3 deletion strains were constructed de novo and verified by PCR using standard methods (http://www.fherc.org/science/labs/gottschling/yeast/). rtg1A and rtg3A also were verified by testing for previously described glutamate and aspartate auxotrophies (24). The anoxia phenotypes of each of the rtg deletion strains then were confirmed by serial dilution spot tests using four biological replicates. Pregrowth overnight culture in 5 ml YPD on a rotator drum can be substituted for 2-day growth in the 96-well dish format with similar phenotypic results.

Microarray accession number. Microarray data were deposited at the NCBI GEO, under data set accession number GSE12004.

RESULTS

Yeast cells enter suspended animation when made anoxic on a nonfermentable carbon source. In order to utilize budding yeast as a model for studying anoxia-induced suspended animation, we first had to demonstrate that it is possible to reversibly halt life processes observable via light microscopy by withdrawing oxygen. Since budding yeast can grow anaerobically on a fermentative carbon source, we used a nonfermentable carbon source in order to test whether anoxia-induced suspended animation is a conserved response to the lack of oxygen. Meiosis and sporulation comprise a well-studied developmental process in yeast that can be induced experimentally by transferring diploid cells to a defined medium that lacks nitrogen sources and contains only a nonfermentable carbon source (reviewed in reference 36). Meiosis and sporulation result in the formation of four haploid gametes (i.e., spores) enclosed in the remnant of the diploid cell, with this entire assembly being referred to as an ascus. Since meiosis and sporulation comprise a dynamic process whose progression can be easily monitored cytologically, we determined whether it was possible to reversibly halt this process by imposing anoxia.

We utilized the efficiently sporulating SK1 strain for this series of experiments. Based on previously published results (44) and our own observations, SK1 does not form ascI until more than 6 h after transfer to sporulation medium. We therefore attempted to stop sporulation by applying anoxia at the beginning of hour 6 after the transfer to sporulation medium, as the sporulation process should be well under way by that point, but hour 6 is still early enough that ascI are not yet formed. We found that it is indeed possible to reversibly halt sporulation by perfusing the culture with N₂. When cells were made anoxic at the beginning of hour 6 (after being allowed to initiate sporulation in the presence of oxygen up to that time) and maintained in anoxia up to hour 18, the majority of anoxic cells remained mononucleate and were unable to complete the sporulation process without O₂ (Fig. 1C and D). Only upon the restoration of oxygen (at hour 18 of the experiment) do the cells continue with the sporulation process, which reaches a maximum of 75.5% ascI formation by the end of the experiment (Fig. 1E). This value is 85.7% of the value observed in control cultures, which reach a maximum of 88.1% ascI formation. In contrast to the cultures that were reversibly suspended by N₂, control cultures that are allowed to sporulate normally had essentially completed sporulation by hour 18 (Fig. 1A, B, and E).

We also found that cells can be reversibly halted after having completed either of the two meiotic divisions. Figure 2A and B show that the cells are not accumulating at one particular stage when experiencing anoxia and suggest that stoppage at multiple stages within meiosis and sporulation is recoverable. Indeed, we found that the percentage of cells that had completed meiosis I but had not yet proceeded further when the sample was taken decreases over time, such that by the end of the experiment the percentage of such cells is the same for both cultures (Fig. 2C). The same is true of cells that had completed meiosis II but had not yet formed ascI (Fig. 2D). If the cells that had been arrested immediately after meiosis I or II had not resumed sporulation, then the relative percentages of such cells would be expected to increase over the course of the experiment or at least stay the same. Thus, the observed decrease (over the time course) in the percentage of cells having just completed meiosis I or II suggests that many of the cells that had been arrested immediately after meiosis I or II in anoxia resumed sporulation upon reoxygenation, albeit with less synchrony than that of the controls. In addition, spore dissections showed that spore viability was high: 82% for spores derived from cells that were made anoxic and 93% for control spores (data not shown). Taken together, these results demonstrate that anoxia-induced suspended animation is a conserved response to severe oxygen deprivation in sporulating yeast.

While it is clear from cytological evidence that sporulating yeast can undergo anoxia-induced suspended animation, we also wished to determine if the same were true of vegetative cells. If so, then any potential screening to identify genes that are required for suspended animation would be much easier to carry out using vegetative cells of any of the extant deletion sets (18), which were constructed in an S288c genetic background. Accordingly, we tested whether the haploid BY4741 strain (the S288c derivative parental strain of the MATα deletion set) also can undergo anoxia-induced suspended animation. In order to observe the growth of colonies originating from single cells, we spotted BY4741 cells at low density onto solid medium containing only a nonfermentable carbon source, acetate. When cells were deposited onto acetate medium and made anoxic for 2 days, colonies did not form as long as the cells were kept anoxic (Fig. 3). In fact, most cells did not divide over the 2 days of anoxia, and the few cells that divided did so only once (compare N₂ and CO days 0 and 2). In contrast, cells left in room air clearly had undergone multiple rounds of cell division after 2 days (compare air day 0 to day 2). When the
anoxic cells were restored to room air, they resumed cell divisions, forming colonies similar in size and appearance to those of the control cells after 2 days in air (compare N$_2$ and CO day 4 to air day 2). These results show that it is possible to reversibly stop the growth of budding yeast by exposure to anoxia on a nonfermentable carbon source. Thus, our laboratory has shown that anoxia-induced suspended animation is a response to the extreme lack of oxygen that is conserved across three well-studied model species, budding yeast (both sporulating and vegetative cells), nematodes, and zebrafish.

Vegetative cells in suspended animation can reversibly arrest while in a budded state. Yeast cells, when exposed to various conditions not conducive to continued growth, transiently arrest the cell cycle in G$_1$. These conditions include starvation for carbon, nitrogen, sulfur, phosphorus, and potassium (22); exposure to mating pheromone (11); elevated temperature (25); oxidative stress (59); and osmotic stress (7). To determine whether cells in anoxia-induced suspended animation exhibit a similar arrest in G$_1$, we quantified the percentage of budded cells from populations made anoxic with CO or N$_2$ for 2 days on acetate medium (Fig. 4). We found that 12.4% of cells treated with CO were in a budded state, while 10.4% of cells treated with N$_2$ were budded. These percentages are similar to the 13.0% budded cells observed in the overnight pregrowth cultures, with $P = 0.728$ compared to results for CO treatment and $P = 0.138$ compared to results for N$_2$ treatment (by Student $t$ test).

Note that cells growing on acetate in the presence of room air exhibited a lower percentage of budded cells (5.3%). Compared to the pregrowth, CO-, and N$_2$-treated samples, the respective $P$ values are 0.030, 0.026, and 0.056. Cells growing on nonfermentable medium require almost fourfold more time to complete a cell cycle than cells growing on fermentable medium (63). The lower percentage of budded cells likely is due to the increased time required for cells in nonfermentable medium to grow to a size that is sufficient to pass START, resulting in more cells being in an unbudded state at any particular time. The similarity in percent budded cells between pregrowth and anoxic samples, combined with the dissimilarity between aerobic and anoxic samples on acetate, suggest that budded cells made anoxic on acetate reversibly arrest as such. In contrast to cells exposed to other stresses, these budded cells are unable to complete the cell cycle in progress and, thus, do not arrest in G$_1$.

Vegetative cells retain high viability after continuous, prolonged anoxia. To assess the viability of cells in prolonged anoxia-induced suspended animation, we plated BY4741 cells at low density onto solid YPA medium and kept cells in continuous anoxia for up to 7 days (Fig. 5). Cells retained high viability (76.8% relative to room air controls) after even 1 week of continuous exposure to either CO or N$_2$, as judged by the ability to form colonies after anoxia. Thus, vegetative yeast have a robust ability to withstand prolonged arrest in a non-proliferating state while anoxic on nonfermentable medium.

Broad similarity in the transcriptional responses to both anoxic gases. To better understand the molecular underpinnings enabling anoxia-induced suspended animation, we wished to identify sets of genes that are differentially expressed as cells undergo reversible deanimation when made anoxic on a nonfermentable carbon source. These data should provide

![FIG. 1. Anoxia-induced suspension of meiosis and sporulation in SK1 cells. SK1 cells allowed to initiate sporulation (A) in the continuous presence of oxygen have completed sporulation by hour 18, forming over 80% asci (B). In contrast, cells allowed to initiate sporulation in the presence of oxygen (C) but made anoxic from hours 6 to 18 did not complete the sporulation process (D). The scale bar represents 10 μm. (E) A plot of the percent asci as a function of time shows that cells made anoxic from hour 6 to hour 18 rapidly resumed sporulation when oxygen is restored at hour 18. Error bars represent standard errors of the means for four independent trials. Three hundred cells from each culture were counted at each time point.](http://ec.asm.org/.../Fig_1.png)
insight into patterns of gene expression that define the transcriptional response of cells undergoing suspended animation, allowing us to draw comparisons to previously published microarray studies of yeast undergoing anaerobiosis (29, 30, 31, 57) and other stress conditions (12, 17). Accordingly, we carried out microarray analysis on cells collected over six time points (15, 30, 45, 60, and 120 min and 24 h) during anoxia using each of the two anoxic gases.

We elected to use T-profiler (10) to analyze the microarray data. This online tool readily identifies groups of genes, with related GO annotations, that are differentially expressed when comparing two conditions without the need to apply cutoffs that exclude a large proportion of the expression data from further consideration (see Materials and Methods). We found broad similarity in the transcriptional responses to anoxia caused by each of the two gases. Specifically, two groups of genes were upregulated at most time points in both anoxic gases: genes encoding cell wall components and genes grouped under the heading cellular component unknown (Table 1). It has been shown that yeasts upregulate many cell wall genes when undergoing anaerobiosis (29, 30, 31, 57), apparently to remodel the cell wall’s composition. Our results are consistent with these previous findings.

In the hopes of shedding light on the role(s) of the group of uncharacterized ORFs that is upregulated under both anoxic gases, consensus motif identification searches were carried out on this group of genes. Using MEME (3, 5), an online consensus motif discovery tool, we identified three consensus motifs that are enriched in the upstream regions of 55 of the most highly upregulated genes in this group, genes with a mean of at least twofold induction across the six time points. We then used MAST (4), a related online tool, to search for the enrichment of these three consensus motifs in the upstream regions of all yeast genes. This search found 122 genes with $E < 10^{-6}$. Using GO tools at the SGD, we found that 63 of these 122 genes coded for products with roles in nucleobase, nucleoside,
nucleotide, and nucleic acid metabolic processes. Forty-eight of these 63 genes are found in Ty-transposable elements. Due to the high degree of sequence similarity among transposable element genes (32), the possibility of significant cross-hybridization in microarray studies makes it difficult to derive an accurate assessment of overrepresentation relative to the genome. This difficulty notwithstanding, it is nonetheless possible that the activation of retrotransposition is a part of the transcriptional response to oxygen deprivation, as transposable elements are mobilized when cells are exposed to various stresses (32). We also note that a similar oxygen limitation-dependent upregulation of transposable elements has been reported in fission yeast (50).

FIG. 3. BY4741 cells were spotted at low density onto solid YPA medium. One group of cells was continuously perfused with an atmosphere of pure N₂ for 2 days and then was returned to room air to restart growth (top row). A second group of cells was similarly perfused with CO for 2 days and then returned to room air (middle row). A control group of cells was kept in room air for 4 days (bottom row). Cells that were made anoxic for 2 days halt their cell divisions but readily restart growth after return to room air.

The responses to both anoxic gases also were very similar among the groups of genes that become relatively less abundant in anoxia. The large majority of these genes are involved that the activation of retrotransposition is a part of the transcriptional response to oxygen deprivation, as transposable elements are mobilized when cells are exposed to various stresses (32). We also note that a similar oxygen limitation-dependent upregulation of transposable elements has been reported in fission yeast (50).

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FIG. 4. Cells in suspended animation can reversibly arrest while in a budded state. A total of 13.0% of BY4741 cells from overnight pregrowth cultures were budded. Similarly, among cells reversibly arrested in CO or nitrogen on acetate medium for 2 days, 12.4 and 10.4%, respectively, were arrested in a budded state. Note that only 5.3% of cells growing in the presence of air on acetate were budded, reflecting the slower cell cycle on nonfermentable medium. Data are from three biological replicates; error bars represent standard errors of the means.

FIG. 5. Cells retain high viability after up to 1 week of continuous anoxia. BY4741 cells were plated at low density onto solid YPA medium and continuously perfused with either CO or nitrogen for the indicated number of days and then allowed to recover in room air before colonies were counted. Data are from three biological replicates; error bars represent standard errors of the means.
in transcription or translation, along with related metabolic processes. These groups include ribosome and nucleolar components as well as ribosome biogenesis, RNA metabolism, protein metabolism, amino acid metabolism, and RNA ligase activities (Table 2). As the cells are unable to grow when made anoxic, it is not surprising to find a profound and prolonged decrease in the abundance of such transcripts. Based solely on our own data, it is unclear to what degree these genes are being repressed in the anoxic cells. However, based on previous studies (46), it is known that for cells transferred from glucose to a nonfermentable carbon source (glycerol), there is apparently little relative change in the transcript abundance of ribosome biogenesis genes for at least 60 min after the transfer. Additionally, as shown in reference 46, such transcripts actually become less abundant after prolonged growth in the nonfermentable medium. Given the findings in reference 46 and the fact that the downregulated gene groups all are of interrelated function, it is probable that the relative change in gene expression seen across these gene groups is due more to coordinate repression in anoxia than to coordinate induction in air.

**Patterns of upstream consensus motif enrichment suggest similarity to other stress-induced transcriptional responses.** In addition to identifying sets of genes that are differentially expressed in anoxia, we also used T-profiler to identify consensus motifs that are associated with genes that show differential expression. Similarly to the procedure for identifying gene group enrichment, genes are assigned to groups based upon common consensus motifs, and the gene group enrichment, genes are assigned to groups based on their similarity to other stress-induced transcriptional responses. In this manner, the results from these searches are summarized in Table 3. Among genes that are upregulated in anoxia, there is a consistent enrichment for those with MSN2/MSN4 consensus motifs (AGGGG, CCCCT, or HRC PAC (CGATGAG) and rRPE motifs (AAAATTT). The PAC motif (motif M3b in reference 56) is associated with RNA binding and processing genes, while the rRPE motif (motif mRRPE in reference 42) is found upstream of genes with roles in ribosomal biogenesis. In addition, genes with the RAPI
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<th>CO</th>
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<td><strong>15 min</strong></td>
<td>Nucleolus, nucleus, ribosome biogenesis, rRNA metabolism, RNA metabolism, cytoplasm biogenesis</td>
<td>Nucleolus, ribosome biogenesis, rRNA metabolism, nucleus, RNA metabolism, nucleic acid metabolism, cytoplasm biogenesis, cell growth and maintenance, translation</td>
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<td>RNA helicase activity, macromolecule metabolism, nucleic acid binding, RNA ligase activity, tRNA ligase activity, organic acid metabolism, translation, catalytic activity</td>
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<td><strong>60 min</strong></td>
<td>Ribosome component, nucleolus, rRNA metabolism, RNA metabolism, biosynthesis, structural molecule, nucleic acid metabolism, protein metabolism, nucleus, nucleic acid binding</td>
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<tr>
<td><strong>120 min</strong></td>
<td>Ribosome component, protein biosynthesis, biosynthesis, structural molecule activity, macromolecule biosynthesis, nucleolus, rRNA metabolism, translation, cytoplasm biogenesis, RNA metabolism</td>
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<td><strong>24 h</strong></td>
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<td>RNA helicase activity, RNA ligase activity, RNA metabolism, rRNA metabolism, cytoplasm biogenesis, protein metabolism, nucleic acid metabolism, macromolecule metabolism, RNA metabolism</td>
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* Gene groups are binned by E values, with groups having lower E values listed first within each bin at each time point.
motif (CCRTACA), which have roles in ribosome biogenesis (9), and another motif that is annotated as rRNA related by T-profiler (GCGATGAGMTGARAW), also are significantly downregulated at all time points after 30 min. These patterns of consensus motif enrichment are consistent with previously published observations of the transcriptional response to various stresses (17, 12) as well as the patterns of gene group enrichment described in this paper.

| Time point | Upregulated motifs enriched in: CO | N$_2$ | Downregulated motifs enriched in: CO | N$_2$
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<td>15 min</td>
<td>CTCC (GCR1, 0.0039), CTCCT (MSN2-4, 0.0060), CGCNNNNNNNNNNN NRCBG (unknown, 0.030)</td>
<td>AGGGG (MSN2-4, 6.8 × 10^{-5}), TCTCC (ADR1, 0.0106), TCCCGGGA (PDR3, 0.033), TCCCGGGG (unknown, 0.049)</td>
<td>CGATGAG (PAC, 0.0012)</td>
<td>AAAATT (rRPE, 3.0 × 10^{-9}), CGATGAG (PAC, 3.7 × 10^{-7}), GCGATGAGMTGARAW (RNA?, 0.0046)</td>
</tr>
<tr>
<td>30 min</td>
<td>CCCCT (MSN2-4, 2.0 × 10^{-6}), AGGGG (MSN2-4, 9.2 × 10^{-7})</td>
<td>AGGGG (MSN2-4, 2.8 × 10^{-9}), CCCCT (MSN2-4, 1.4 × 10^{-10}), TATAWAW (TPB, 2.7 × 10^{-10}), TCCCGGGG (unknown, 3.1 × 10^{-4}), TCCCGGGG (PDR3, 5.1 × 10^{-4}), TGCACCC (RCS1, 0.0085)</td>
<td>CGATGAG (PAC, 8.2 × 10^{-6}), AAAATT (rRPE, 2.6 × 10^{-4})</td>
<td></td>
</tr>
<tr>
<td>45 min</td>
<td>AGGGG (MSN2-4, 1.6 × 10^{-15}), CCCCT (MSN2-4, 0.001), TCCCGGGG (PDR3, 0.012), HRCCCCTYWD (MSN2-4, 0.025), TCCCGGGG (unknown, 0.031)</td>
<td>AGGGG (MSN2-4, &lt; 1.0 × 10^{-15}), CCCCT (MSN2-4, &lt;1.0 × 10^{-15}), TATAWAW (TPB, 2.8 × 10^{-10}), TCCCGGGG (PDR3, 5.5 × 10^{-6}), TCCCGGGG (unknown, 6.1 × 10^{-3}), HRCCCCTYWD (MSN2/4, 2.3 × 10^{-6}), CCNNNNWWRGG (MCM1, 0.018), TCTCC (ADR1, 0.029), TCCCGGGG (unknown, 0.030)</td>
<td>AAAATT (rRPE, 2.1 × 10^{-15}), CGATGAG (PAC, 2.0 × 10^{-9}), GCGATGAGMTGARAW (RNA?, 0.0030), CCRRTACA (RAP1, 0.0055)</td>
<td></td>
</tr>
<tr>
<td>60 min</td>
<td>AGGGG (MSN2-4, 2.0 × 10^{-15}), CCCCT (MSN2-4, &lt; 1.0 × 10^{-15}), TATAWAW (TPB, 0.0015), HRCCCCTYWD (MSN2/4, 0.0092), TCCCGGGG (PDR3, 0.041), CCNNNNWWRGG (MCM1, 0.044)</td>
<td>AGGGG (MSN2-4, &lt; 1.0 × 10^{-15}), CCCCT (MSN2-4, &lt;1.0 × 10^{-15}), TATAWAW (TPB, 1.8 × 10^{-9}), TCCCGGGG (PDR3, 0.0022), HRCCCCTYWD (MSN2/4, 0.020)</td>
<td>AAAATT (rRPE, &lt; 10^{-15}), CGATGAG (PAC, 1.1 × 10^{-13}), CCRRTACA (RAP1, 7.2 × 10^{-15}), GCGATGAGMTGARAW (RNA?, 7.8 × 10^{-4}), TGACTCA (GCNN4, 0.025)</td>
<td></td>
</tr>
<tr>
<td>120 min</td>
<td>AGGGG (MSN2-4, 2.4 × 10^{-10}), TCCCGGGG (PDR3, 1.2 × 10^{-8}), CCCCT (MSN2-4, 1.4 × 10^{-5}), TCCCGGGG (unknown, 1.2 × 10^{-3}), TATAWAW (TPB, 0.0045), HRCCCCTYWD (MSN2/4, 0.018)</td>
<td>CCGTTT (MSN2-4, 2.9 × 10^{-8}), AGGGG (MSN2-4, 4.2 × 10^{-7}), TCCCGGGG (PDR3, 7.0 × 10^{-6}), TCCCGGGG (unknown, 1.3 × 10^{-4}), TATAWAW (TPB, 0.0021), HRCCCCTYWD (MSN2/4, 0.027), TCCCGGGG (unknown, 0.049)</td>
<td>CCRRTACA (RAP1, &lt; 10^{-15}), AAAATT (rRPE, 6.6 × 10^{-9}), CGATGAG (PAC, 1.2 × 10^{-4})</td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>CCCCT (MSN2-4, 2.9 × 10^{-10}), AGGGG (MSN2-4, 8.2 × 10^{-10}), TCCCGGGG (PDR3, 3.8 × 10^{-9}), TCCCGGGG (unknown, 0.0011), CCNNNNNWWNNNNNCCCCCNG (GAL4, 0.003), CCNNNNNWWNNNNNCCCCCNG (PUS3, 0.0010), TCCCGGGG (unknown, 0.0018), CCNNNNNWWNNNNNCCCCCNG (GAL4, 0.0016), ATGGRAWRW (NBF, 0.035), TCCCGGGG (unknown, 0.039), CCCCT (MSN2-4, 0.041), CCGGGGTA (MIG1, 0.044)</td>
<td>TCCCGGGG (PDR3, 3.1 × 10^{-7}), AGGGG (MSN2-4, 7.8 × 10^{-4}), CGNNNNNNNNNNNCCCCCNG (GAL4, 0.0010), TCCCGGGG (unknown, 0.0018), CGNNNNNNNNNNNCCCCCNG (GAL4, 0.016), ATGGRAWRW (NBF, 0.035), TCCCGGGG (unknown, 0.039), CCCCT (MSN2-4, 0.041), CCGGGGTA (MIG1, 0.044)</td>
<td>AAAATT (rRPE, 4.9 × 10^{-11}), CGATGAG (PAC, 2.3 × 10^{-7}), CCRRTACA (RAP1, 0.0092)</td>
<td></td>
</tr>
</tbody>
</table>

* Motifs that were enriched by treatment with either gas at each time point are listed, along with the cognate transcription factor (if known) and the E value in parentheses. Besides A, C, G, and T, the other one-letter codes represent different nucleotides as follows: R for purines, Y for pyrimidines, N for any nucleotide, W for weak (A or T), S for strong (C or G), M for amino (A or C), K for keto (G or T), B for any nucleotide that is not A, H for any not G, D for any not C, and V for any not T.
Relative derepression of gene groups involved in aerobic energy generation when cells are exposed to CO but not when exposed to N2. In contrast to the broad similarity described in the preceding sections, we noted differences in gene expression between exposure to CO and exposure to N2 for the groups of genes that were upregulated early in the anoxia time course (Table 1). At 15 and 30 min of exposure to CO, many gene groups associated with aerobic energy generation were upregulated. These groups include oxidative phosphorylation (29 genes), proton-transporting ATP synthesis (16 genes), mitochondrial electron transport chain (22 genes), and aerobic respiration (61 genes). This is in marked contrast to what was observed for N2, for which, except for a significant increase in mitochondrial dysfunction (reviewed in reference 33). In contrast to the essentially normal recovery from CO, rg1, rg2, and rg3 deletion mutants tend to permanently arrest as small microcolonies when attempting to recover from N2. For each rg mutant, a small fraction of colonies manages to grow relatively large in the recovery from N2, possibly due to the accumulation of suppressing mutation(s).

Aerobic energy generation genes are relatively derepressed when retrograde signaling mutants are exposed to N2. Given the N2-specific sensitivity of the retrograde signaling mutants and the fact that, in wild-type BY4741 cells, aerobic energy generation genes show differential regulation in CO compared to that in N2, we carried out qRT-PCR to determine if the pattern of gene expression in anoxia differed between the wild type and rg mutants. We examined the strains deleted for RTG1 and RTG3, as each of these genes encodes a basic helix-loop-helix leucine zipper transcription factor. Together, Rtg1 and Rtg3 function as a heterodimer to effect nuclear gene expression as a result of signaling via the retrograde pathway (33). We found that in both rg1 and rg3 deletion mutants, the 16 aerobic energy generation genes previously described tend to be more derepressed at 30 min in N2 than in CO (compare Fig. 6 to Fig. 8). This contrast between the mutant and wild
type is particularly striking when comparing rtg3Δ (Fig. 8B) to BY4741 (Fig. 6B). We conclude that the deletion of either the RTG1 or RTG3 transcription factor gene results in gene expression patterns upon exposure to N₂ that are aberrant compared to those of the wild type.

**DISCUSSION**

**Budding yeast is a model for anoxia-induced suspended animation.** Understanding the molecular mechanisms that enable cells to cope with a lack of oxygen is of high importance to human health while also being of great interest from a basic science perspective. To better elucidate such mechanisms, our laboratory previously characterized the phenomenon of anoxia-induced suspended animation in two model organisms, the nematode *C. elegans* and the zebrafish *D. rerio*. To determine if a similar response is conserved in a model species with even more facile gene-based research tools, we turned to the budding yeast *S. cerevisiae*. We demonstrated that both sporulating and vegetative yeast on a nonfermentable carbon source undergo anoxia-induced suspended animation in response to severe oxygen deprivation. The sporulation process can be suspended even after being well under way, with high recoverability, for up to 12 h. Vegetative cells can be maintained in a suspended state for at least 7 days, also with high recoverability (more than 75%). These results, combined with the demonstration of a similar phenomenon in *Drosophila* (16),

![FIG. 7](image7.png)

FIG. 7. Retrograde signaling is required for proper recovery from prolonged exposure to N₂. Tenfold serial dilutions of each strain spotted onto solid YPA are shown after 7 days of growth in air (left column), 4 days of arrest in CO followed by 7 days of recovery in air (middle column), and 4 days of arrest in N₂ followed by 7 days of recovery in air (right column). Each of these deletion strains recovers poorly after prolonged exposure to N₂ but exhibit relatively normal recovery after similar exposure to CO. Rtg1, Rtg2, and Rtg3 are components of the so-called mitochondrial retrograde signaling pathway, which is thought to activate changes in nuclear gene expression to compensate for mitochondrial dysfunction.

![FIG. 8](image8.png)

FIG. 8. (A) Log₂-transformed change (n-fold), as determined by qRT-PCR, comparing rtg1Δ N₂-treated cells to CO-treated cells at 30 min in anoxia for the 16 transcripts depicted in Fig. 6. Many of these aerobic metabolism genes are relatively derepressed in rtg1Δ when treated with N₂. (B) A similar plot comparing rtg3Δ N₂-treated cells to CO-treated cells at 30 min in anoxia for the same 16 transcripts. Most of these aerobic metabolism genes are more markedly derepressed in rtg3Δ than in rtg1Δ when treated with N₂. Data for each panel are from two replicate qRT-PCR runs; error bars represent standard errors of the means.
show that anoxia-induced suspended animation is a response to severe oxygen deprivation that is conserved among four well-studied model organisms.

We found that vegetative yeast cells on nonfermentable medium rapidly halt their cell divisions when made anoxic. This result is reminiscent of transient cell cycle arrest under nutrient limitation (45) and prolonged arrest under starvation (14, 61, 62). However, cells that are made anoxic on nonfermentable medium do not uniformly arrest in G1. In contrast, cells exposed to various stimuli, including nutrient starvation (22), mating pheromone (11), elevated temperature (25), oxidative stress (59), and osmotic stress (7), tend to arrest in G1. We therefore conclude that the phenomenon of anoxia-induced suspended animation, which we have named in analogy to related phenomena in higher eukaryotes, is distinct from the growth arrest of wild-type yeast previously described.

In addition, note that depending on the genotype, not all types of starvation result in viable nonproliferative states. A striking example of this phenomenon was described by Botstein and colleagues, who worked with a strain that is auxotrophic for leucine and uracil. When starved for either phosphate or sulfur in liquid medium, the cells remained completely viable after 1 week, as judged by subsequent colony formation on solid YPD. In contrast, when the cells were starved for leucine for 1 week, there was a 10-fold decrease in viability. Similar starvation for uracil resulted in an even more severe viability decrease of more than 100-fold (8). Thus, reversible arrest in a viable nonproliferative state is not a default response to starvation. Instead, there are likely particular cellular mechanisms that enable cells to enter into and maintain a viable nonproliferative state, such as anoxia-induced suspended animation on nonfermentable medium.

Also, while a straightforward analogue for anoxia-induced suspended animation can be described when comparisons are made across model systems such as yeast, nematodes, fruit flies, and zebrafish, the responses to nutrient limitation or starvation are much more divergent across different species; for example, when comparing yeast to nematodes (48). This similarity in the response to oxygen deprivation among model species suggests the possibility that yeast that are made to assume an obligate aerobic lifestyle on nonfermentable medium serve as a useful model for studying conserved cellular responses to oxygen deprivation. Also, given the apparently conserved nature of this response to extremely low oxygen levels, it is curious to consider well-documented cases in the medical literature describing humans who have survived prolonged bouts of oxygen deprivation due to hypothermic circulatory arrest in various accidents with little or no adverse sequelae (58, 19).

Analysis of gene expression reveals a coordinated derepression of aerobic energy generation genes in CO but not in N2. In order to identify genes that are involved in suspended animation, we carried out transcript microarray analysis on cells that were exposed to either CO or N2. Using the T-profiler tool, we found that while the gene group and consensus motif enrichment profiles were quite similar for the two anoxic gases, there were areas of marked difference between exposure to CO and exposure to N2. Specifically, multiple gene groups whose constituent genes have roles in aerobic energy generation were significantly upregulated at 15 and 30 min in CO but not in N2. We noted 16 genes that were upregulated by at least twofold in CO at 30 min based on the microarray data. Most of these 16 genes were at least twofold more abundant in CO than in N2, again based on the microarray data. We carried out qRT-PCR to verify these results and confirmed that most of these genes were indeed relatively derepressed in CO compared to their levels in N2. The derepression of these genes is consistent with the idea that CO acts as an oxygen mimetic, presumably by binding at the heme of hemoproteins (43), which effects signal transduction events that result in the observed changes in transcription. We propose that the presence of a high concentration of CO essentially fools the cells into sensing that there is abundant O2, and thus genes that encode proteins with roles in aerobic metabolism are coordinately derepressed compared to the expression levels in the presence of N2, which is not an oxygen mimetic.

Previously, Poyton and colleagues found that the anaerobic induction of two genes (CYC7 and OLE1) in N2 can be completely blocked by treatment with CO, while the induction of a third gene (COX5B) is partially blocked by CO in cells on galactose medium (28). They found that 11 other genes, previously shown to be oxygen regulated, showed no difference in expression after treatment with either anoxic gas. To our knowledge, no one has looked for differential gene regulation in CO and compared it to that of N2 on a genomewide scale. It is worth noting that the choice of medium perhaps has a strong influence on the likelihood of observing differences in gene expression. Since a lack of oxygen brought on by exposure to pure CO or N2 would not be expected to differentially affect anaerobic metabolism per se, it is possible that cells on a fermentable medium are less likely to manifest differences in gene expression than cells on a nonfermentable medium. This is because energy generation from a nonfermentable carbon source requires O2 as well as gene expression changes associated with the need to utilize O2. If so, then the application of an O2 mimetic, namely CO, while the cells are on a nonfermentable substrate would be more likely to elicit changes in gene expression that are normally O2 dependent than a similar CO exposure on a fermentable substrate. In addition, the use of nonfermentable medium essentially converts the yeast cells into obligate aerobes, thus making the yeast model more similar to the truly obligate aerobic cells of higher eukaryotes.

An analysis of the microarray data also showed that a general stress response (previously referred to as the environmental stress response by Gasch et al. [17] and the common environmental response by Causton et al. [12]) is likely activated by yeast that were made anoxic on a nonfermentable substrate. Specifically, we found that genes with MSN2/MSN4 motifs and genes encoding cell wall proteins were significantly upregulated at almost all time points, while genes with roles in transcription, translation, and many associated processes were significantly downregulated, again at nearly all time points. These genes that are similarly regulated between the two anoxic gases appear to form the core transcriptional response to anoxia, sharing much in common with other stress responses.

The mitochondrial retrograde signaling pathway is functionally important for recovery from prolonged exposure to N2 but not to CO. Having found that aerobic energy generation genes tend to be relatively derepressed in CO, we set out to identify genes that are functionally important for enabling sur-
vival in one anoxic gas but not the other. We found that while mutants deleted for components of the mitochondrial retrograde signaling pathway were able to recover normally after prolonged exposure to CO, they recovered very poorly after a similar exposure to N₂. We then found that in both the rgt1 and the rgt3 deletion mutants a number of aerobic energy generation genes tend to be derepressed in N₂. This is in contrast to the relative repression of these same genes in wild-type cells. Thus, the disruption of mitochondrial retrograde signaling results in aberrant gene expression in an anoxic gas-dependent manner.

**CO may cause divergent signals from two distinct oxygen-sensing pathways in budding yeast.** The molecular nature of oxygen sensing in budding yeast has been an active study for a long time. Multiple molecular mechanisms for oxygen sensing in yeast have been proposed. One such mechanism proposes that cells sense the intracellular level of some compound(s) that requires O₂ for synthesis. Thus, the concentration of the compound(s) can be an effective proxy for oxygen concentration. Candidate compounds that can serve in this role are heme (64) and sterols (13). A distinct oxygen-sensing mechanism proposes that the binding of molecular oxygen to heme-containing proteins causes signal transduction events that result in changes in gene expression (43).

Based on our gene expression data, we propose a model in which both of these mechanisms are simultaneously functioning in budding yeast, such that each mechanism mediates a subset of the overall transcriptional response. First, recall that treatment with CO causes a derepression of aerobic metabolism genes relative to that seen in N₂. As noted previously, this is consistent with hemoprotein occupancy-based signaling that mimics O₂ binding. In addition, we note that other parts of the transcriptional response are very similar for both anoxic gases. Gene groups that are downregulated include ribosome biogenesis genes as well as genes involved in transcription, translation, and related biosynthetic pathways. As noted in Results, ribosome biogenesis genes are known to be downregulated only after prolonged growth on a nonfermentable medium (46). Thus, the downregulation seen in the anoxic samples relative to the reference air samples on acetate probably represents a greater degree of transcriptional repression than can be accounted for merely by the transitioning to growth on a nonfermentable carbon source. We also observed an upregulation of cell wall genes in both anoxic gases, similarly to previously published results (29, 30, 31, 57) for cells made anoxic on fermentable medium. Taking into consideration these common features of the transcriptional response to both anoxic gases, we propose that the signal that results in these similar patterns of gene expression originates from a mechanism that would be expected to respond similarly to both CO and N₂, such as the depletion of some compound(s) that requires O₂ for its synthesis.

In this model, treating cells with N₂ results in signals from both mechanisms that are convergent, i.e., both signal a lack of O₂, as N₂ is not believed to bind in hemoproteins like O₂ does, while the concentration of some compound(s) requiring O₂ for synthesis should decrease. In contrast, treating cells with CO results in divergent signals, as the oxygen-mimetic properties of CO points to the (perceived) presence of O₂, while the concentration of the compound(s) that require O₂ in order to be synthesized still would decrease, pointing to the (actual) lack of O₂. The difference in signaling between CO and N₂ ultimately results in transcriptional responses that are similar across much of the transcriptome but are markedly different for aerobic metabolism genes. Since the differences between exposure to CO and N₂ are most apparent in the aerobic metabolism genes, it is quite possible that such changes can be observed only when the cells are forced to generate energy aerobically by being put on a nonfermentable medium. As such, we propose that the experimental paradigm we arrived at to demonstrate the conservation of anoxia-induced suspended animation in budding yeast can be of considerable utility in continuing efforts to better understand the molecular mechanisms that mediate oxygen-regulated gene expression.

**ACKNOWLEDGMENTS**

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**REFERENCES**


