Transcriptional Response of the Obligatory Aerobe *Trichoderma reesei* to Hypoxia and Transient Anoxia: Implications for Energy Production and Survival in the Absence of Oxygen†

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ABSTRACT: Oxygen is essential for the survival of obligatorily aerobic eukaryotic microorganisms, such as the multicellular fungus *Trichoderma reesei*. However, the molecular basis for the inability of such cells to survive for extended periods under anoxic conditions is not fully understood. Using cDNA microarray analysis, we show that changes in oxygen availability have a drastic effect on gene expression in *T. reesei*. The expression levels of 392 (19.6%) out of 2000 genes examined changed significantly in response to hypoxia, transient anoxia, and reoxygenation. In addition to modulating many genes with no previously assigned function, cells respond to hypoxia by readjusting the balance of expression between genes required for energy production and consumption, and altering the expression of genes involved in protective mechanisms and signaling pathways. Moreover, we show that transient anoxia strongly represses genes for enzymes that are critical for glycolysis, and are essential for energy production under anaerobic conditions. Our study thus reveals crucial differences between the facultative anaerobe *Saccharomyces cerevisiae* and *T. reesei* with regard to the oxygen-dependent transcriptional control of the glycolytic pathway, which can account for the differential survival of the two species in the absence of oxygen.

Most eukaryotic cells require a continuous supply of oxygen in order to carry out many biochemical and physiological processes, and also to obtain energy (in the form of ATP) from glucose by aerobic metabolism (respiration). Cells can sense critical reductions in oxygen tension (hypoxia), and respond by differentially transcribing genes required to trigger adaptive responses that lead to changes in the physiological and the metabolic status of the cell, which ultimately allow it to cope with the stresses associated with oxygen limitation. Well-known examples of such changes in mammalian cells have been described in muscle cells at high altitude or during exercise (*1*, *2*), and in tumor cells (*3*, *4*). Because human skeletal muscle tissue at high altitude or during exercise (*1*), and cells in solid tumors (*5*), are subjected to hypoxic conditions, they are unable to obtain all the required energy from glucose by oxidative metabolism—a process that produces ATP with a high yield but at a low rate. Instead, they generate part of their ATP by anaerobic metabolism, reducing pyruvate, the end product of the glycolytic pathway, to lactate. This process operates at a higher rate but yields less ATP from an equivalent amount of substrate. However, because brain tissue must obtain its energy by respiration of glucose, most vertebrates, with the exception of anoxia-tolerant freshwater turtles (*6*), die within minutes when deprived of oxygen.

The unicellular eukaryote *Saccharomyces cerevisiae* can grow in the absence of oxygen (anoxia). A genomewide transcriptional analysis showed that, in this yeast, nearly all genes for respiratory complexes and enzymes of the tricarboxylic acid (TCA[1]) cycle were down-regulated under anaerobic growth conditions, while genes involved in glycolysis and glycerol synthesis and genes for the cytoplasmic isoforms of alcohol dehydrogenase were up-regulated (*7*). Measurement of metabolic fluxes showed that anoxia increased glycolytic flux toward ethanol and glycerol production (*8*), indicating that the redirection of metabolites from respiratory to anaerobic (fermentative) metabolism is mainly controlled at the transcriptional level.

Although *S. cerevisiae* has been used as a model system to understand many basic cellular functions and biochemical processes in eukaryotic systems, the fact that it is a facultative anaerobe means that it throws little light on the responses of obligatory aerobes to extreme hypoxia and anoxia, and limits its usefulness as a model system for mammalian cells.

In order to gain insight into the transcriptional programs that direct the physiological and metabolic alterations which occur in obligatorily aerobic microorganisms under hypoxic and anoxic conditions, and also to elucidate why such

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[1] Abbreviations: DO, dissolved oxygen; EST, expressed sequence tag; PCR, polymerase chain reaction; TCA cycle, tricarboxylic acid cycle.

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organisms are unable to survive indefinitely in the absence of oxygen, we chose the multicellular fungus *Trichoderma reesei*. Using cDNA microarrays, we have analyzed the behavior of around 2000 transcripts in *T. reesei* cells that were exposed to different concentrations of dissolved oxygen. The results reveal how a multicellular organism responds to changes in oxygen availability by balancing the expression of genes required for energy-generating and energy-consuming processes, and modulating those required for cell defense and signaling pathways so it can cope with the negative effects of hypoxia and anoxia. The gene expression profiles observed in *T. reesei* cells placed under transient anoxia show that energy production by anaerobic metabolism is blocked, owing to strong repression of the majority of genes required for the operation of ATP-producing pathways. The work thus provides an answer to the basic question of why obligatorily aerobic eukaryotic cells are unable to survive in the absence of oxygen.

EXPERIMENTAL PROCEDURES

Microorganism and Culture Conditions. T. reesei QM 9414 was obtained from the American Type Culture Collection (ATCC 26921). *T. reesei* was grown at 28 °C in chemostats (2-L working volume) using a Bioflo III bioreactor (New Brunswick). Cultures were fed with complete medium (*9*) supplemented with 0.1% yeast extract and 100 mM glucose. The dilution rate was set at 0.1 h^{-1}, the pH was measured online and kept constant at 6.0 by the automatic addition of 2 M KOH, and the stirrer speed was set at 900 rpm. Dissolved oxygen (DO) was monitored with an InPro6100 oxygen sensor (Ingold). A blend of air and nitrogen was sparged into the bioreactor at a fixed total flow rate. To control the DO value, two mass flow controllers (5850E, Brooks Instruments) were used to adjust the flow rate of each gas according to an algorithm written in LabView (National Instruments). To calculate respiration activity, the exhaust gases—O_2 and CO_2—were monitored with a paramagnetic oxygen sensor (Beckman) and an infrared CO_2 monitor (Fuji). The reactor was inoculated with 1×10^6 spores/mL. After the culture had reached steady state, at which time the concentration of glucose was 100 μM and that of DO was 5 mg/L, the concentration of oxygen in the feed line was decreased from its initial value to 0% in 1-h steps. The culture was sparged with pure nitrogen for 30 min, and then the oxygen concentration was restored to the initial value (5 mg/L). At different DO levels aliquots were withdrawn from the culture vessel for determination of cell dry weights and extracellular glucose concentrations, and for the isolation of total RNA.

Mycelia were immediately removed by vacuum filtration, frozen in liquid nitrogen, and stored at −70 °C. The glucose concentration in the filtrate was measured with a SERA-PAK Glucose kit (Bayer).

cDNA Microarray Analysis. We have established an EST database for *T. reesei* (http://trichoderma.iq.usp.br) representing 2000 unique transcripts (*10*). *T. reesei* DNA probes were amplified by PCR using M13 reverse and M13 (−20) primers (Stratagene), and products were purified using the Millipore MultiScreen Assay System. The purified PCR products (each of which yielded a single band upon agarose gel electrophoresis) were spotted in triplicate on glass slides (Telechem, Inc.). Total RNAs were isolated using the TRizol reagent (Invitrogen). Labeled Cy3 and Cy5 cDNAs were prepared from the isolated RNA samples using the 3DNA Array 50 (Version 2) kit (Genisphere). Hybridization was performed as described previously (*10*).

Images of the microarrays were extracted using a GenePix 4000A microarray scanner (Axon Instruments), and quantification was performed using GenePixPro 3.0 software.

After elimination of spots flagged (weak signals, lack of reproducibility) by the scanning software, inspection of $M = \log_2(\text{Cy5/Cy3})$ vs $A = (1/2)\log_2(\text{Cy5})(\text{Cy3})$ plots indicated heterocedasticity. Thereafter, data from each hybridization were subjected to local normalization using the *lowess* procedure (http://www.stat.berkeley.edu/users/terry/zarray/html/matt.html). The normalized average of triplicate Cy5/Cy3 log-ratios was obtained for each probe, and the corresponding coefficient of variation (CV = standard deviation divided by the mean) was calculated to monitor reproducibility and data quality (*11*). This process eliminated around 10% of the spotted genes and reduced the averaged CV values for each array (mean CV = 3−7%). This mean value of CV indicates that the observed changes of gene expression under different concentration of dissolved oxygen are significant.

A second *lowess* normalization was then applied to the remaining data, and 392 genes were identified as having valid log-ratios for all arrays, including the self-self-reference hybridization. Data for these genes were subjected to cluster analysis using self-organizing maps (SOM) as implemented in GeneCluster 1.0 software (*12*). The color scheme and cluster profiles of Figure 2 were generated from the clustered data using a visualization program developed utilizing basic language implemented by Metal l.7.1 software available at http://www.iit.edu/~sarimar/GDS/metal.html.

Validation of the microarray data was performed by Northern analysis and quantitative Real-Time PCR.

RESULTS

Effect of Oxygen Availability on Glucose Utilization. In obligatorily aerobic microorganisms oxygen is required for oxidative metabolism of glucose. To evaluate glucose utilization and determine the patterns of transcriptional change in *T. reesei* kept under different degrees of hypoxia and transient anoxia, a chemostat culture was first grown to steady state on a nonrepressing concentration of glucose under normoxic conditions.

At steady state the concentration of glucose was 100 μM, and that of dissolved oxygen (DO) was 5 mg/L. The oxygen concentration in the supply line was reduced stepwise and maintained for 1 h at each of the following levels: 1.0, 0.5, 0.2, and 0.1 mg/L oxygen. The duration of each step was sufficient to allow the DO concentration in the culture to attain the new equilibrium before the transcriptional profile was analyzed. The rate of glucose utilization began to fall at DO levels below 0.2 mg/L (Figure 1A). The culture was kept under anoxic conditions for 2 h and thereafter returned to the normoxic state (5 mg/L O_2). This period of anoxia was long enough to cause detectable alterations in gene expression without compromising the viability of the cells (see below).

Although glucose consumption and cell density were not significantly affected by reduction of the oxygen concentra-

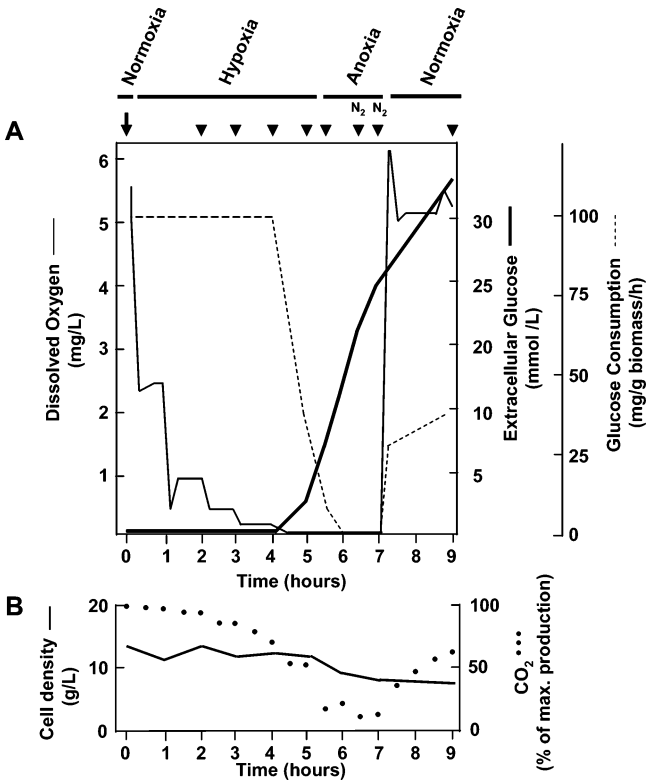

FIGURE 1: Effect of oxygen availability on glucose consumption (A) and cell density and CO_2 production (B). *T. reesei* cells were grown as described in Experimental Procedures. After the culture had reached steady state (arrow), at which the concentrations of glucose and oxygen were 100 μM and 5 mg/L, respectively, the level of oxygen supplied to the culture was reduced in 1-h steps (to 1%, 0.5%, 0.2%, 0.1%, and 0%). The vessel was purged with pure nitrogen for 1 h, and then the level of oxygen supplied was restored to the initial value. Aliquots were withdrawn from the culture medium at different time points, as indicated by the arrowheads for determination of extracellular glucose and cell dry weight and for RNA analyses (for details see Experimental Procedures).

tion from 5 mg/L to 0.2 mg/L, CO_2 production slowly declined, falling by 28% at an oxygen concentration of 0.2 mg/L compared to the value observed under normoxic conditions (Figure 1A,B).

At 0.1 mg/L oxygen, cells showed a sharp drop in glucose utilization and CO_2 production. Furthermore, the subsequent shift from hypoxia to anoxia resulted in complete cessation of glucose utilization. Concomitantly, glucose accumulated in the culture medium, and CO_2 production and cell density decreased to around 10% and 80%, respectively, of the levels observed under normoxic conditions (Figure 1A,B).

The 20% decrease in cell density during the 2 h of anoxia is most probably not due to cell death, but rather to growth arrest and continuous washing of the cells out of the culture vessel. Upon reoxygenation, the cells were able to resume consumption of glucose (Figure 1A) and production of CO_2 (Figure 2B), indicating that they had remained viable during the period of anaerobiosis.

An Overview of the Transcriptional Profiles under Different Oxygen Concentrations. The expression levels of 2000 transcripts in *T. reesei* cells cultured in the presence of different concentrations of dissolved oxygen were assessed using cDNA microarrays. The sequences of these transcripts are available at http://trichoderma.iq.usp.br. Most of these ESTs were obtained from clones randomly selected from a unidirectional cDNA library constructed using mRNA isolated from mycelia of *T. reesei* grown on glycerol as the sole carbon source (*10*). Analysis of the sequenced DNAs against the GenBank database showed that sufficient coverage had been achieved to allow a comprehensive study of the transcriptional response during hypoxia and transit anoxia.

We compared the transcript population from cells withdrawn at 5 mg/L oxygen (arrow in Figure 1A, reference sample, normoxic condition) to those expressed in the presence of various lower levels of dissolved oxygen, under anoxic conditions, and after reoxygenation (arrowheads in Figure 1A). The cDNA samples were fluorescently labeled and hybridized to the microarrays as described in the Experimental Procedures section.

In all, the expression levels of 392 (19.6%) of the 2000 genes tested were significantly altered by hypoxia, anoxia, or reoxygenation. Cluster analysis was used to group genes that showed similar expression profiles over the course of the experiment. Profiles of those genes that are significantly induced or repressed by hypoxia/anoxia, compared to the reference sample, are presented in Figure 2A,B. Significant and gradual change in gene expression was apparent as oxygen concentrations were decreased from 5.0 to 0.1 mg/L. However, a drastic change in gene expression was observed when cells were maintained under anoxic conditions (Figure 2A,2B). Upon restoration of oxygen to the normal concentration (5 mg/L), the transcriptional profiles of the majority of the genes that were altered by hypoxia or anoxia returned to their original expression state (compare lanes 1 and 9 in Figure 2A), at which stage the cells started to consume glucose and produce CO_2 once again (Figure 1A,B).

A functional classification of the 392 genes based on the EGAD database developed at the Institute for Genomic Research (TIGR, Rockville, MD) (*13*) is presented in Figure 3. We have assigned functional roles to 123 genes, 34 of which are induced (Table 1) and 89 repressed (Table 2). Interestingly, more than 68% of the genes that are affected by oxygen availability have not yet been assigned a functional role in specific biological processes. On the basis of our findings, these genes may be involved in, or control, biological processes that are affected by oxygen.

Cellular Responses to Hypoxia and Anoxia. Because the rate of utilization of glucose falls significantly at oxygen levels below 0.2 mg/L, and essentially ceases under anoxic conditions, the hexose begins to accumulate in the culture medium. Thus, at this point, two different physiological effectors that alter gene expression—oxygen and glucose—are present in suboptimal levels. To distinguish between the effects of oxygen and glucose on gene expression, we analyzed the transcriptional profile obtained after oxygen had been restored to the normoxic condition. Although cells resumed glucose consumption and CO_2 production at this point, the concentration of glucose in the medium was still high enough (30 mM) to cause glucose repression (lane 9, Figure 2A). Therefore, unless otherwise stated, those genes that show altered expression under hypoxia or anoxia (lanes 2 to 8, Figure 2A), but returned to the level observed under normoxia (lane 1, Figure 2A) even in the presence of 30 mM glucose (lane 9, Figure 2A), will be considered as genes that respond only to reduced oxygen availability.

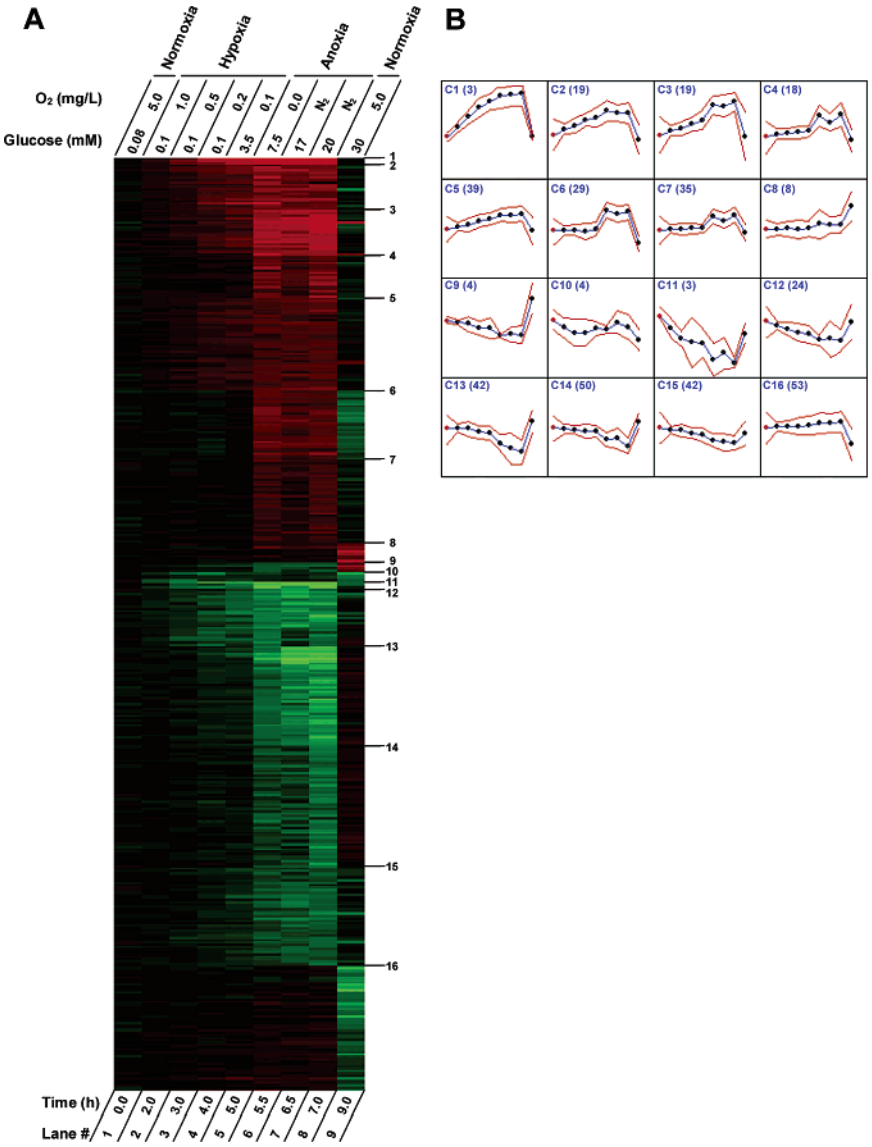

FIGURE 2: Temporal transcription patterns of differentially expressed genes during the period of hypoxia, anoxia, and reoxygenation. Data were analyzed using GeneCluster software v.1.0 (*12*). (A) The color of each element represents the expression level of each gene: red = induced; green = repressed; black = unchanged. Clusters are numbered on the right margin. (B) Plots representing the 16 clustered groups shown in A. The red and blue lines represent, respectively, the range and average expression levels of groups of genes that behave similarly. The clusters are numbered as in A, and the number of genes in each group is given in parentheses

The 123 genes that showed alteration in their gene expression and had assigned biological functions are listed in Table 1 and Table 2. In the following we consider these genes according to their functional classification.

Cell Defense. Several genes that show sequence similarities to genes involved in cell defense were induced during exposure of *T. reesei* cells to oxygen limitation. Thus, three heat shock proteins (A3340, A0004, and A0074) and a monooxygenase (A0803) were up-regulated under hypoxic conditions, and strongly induced by anoxia. It is important to note that, in fission yeast (*Schizosaccharomyces pombe*), the heat shock proteins have been shown to be induced in response not only to heat but also to other stresses such as heavy metal stress caused by cadmium (*14*).

Hypoxic and anoxic stress triggered down-regulation of two genes which display similarities to superoxide dismutase (A3343) and a putative thiol-specific antioxidant (A2590), respectively. Superoxide dismutase is an enzyme that catalyzes the disproportionation of the reactive oxygen species (ROS) O_2^-, which is formed during cellular respiration, to H_2O_2. As in *S. cerevisiae* (*15*), this gene is repressed by oxygen limitation in *T. reesei*. Superoxide dismutase and the putative thiol-specific antioxidant are required for protection from ROS that are generated during respiratory metabolism, and, therefore, their repression under hypoxic and anoxic conditions is not unexpected. In agreement with this predicted function, the superoxide dismutase gene is strongly induced after reoxygenation (see Figure 2A,B, cluster 9).

Cell Division. In this category we observed three repressed genes (A1043, A2012, and A1561) showing similarities to septin 2, SIS1, and histone H3. They are down-regulated by hypoxia and strongly repressed under anoxic conditions. The septins are a family of cytoskeletal GTPases that play an essential role in cytokinesis in yeast and mammalian cells (*16*, *17*). Evidence has been presented suggesting a functional interaction of NEDD5, a mammalian septin, with the actin-based cytoskeletal system, and inhibition of *NEDD5* expression in astrocytoma cells by antisense RNA interferes with

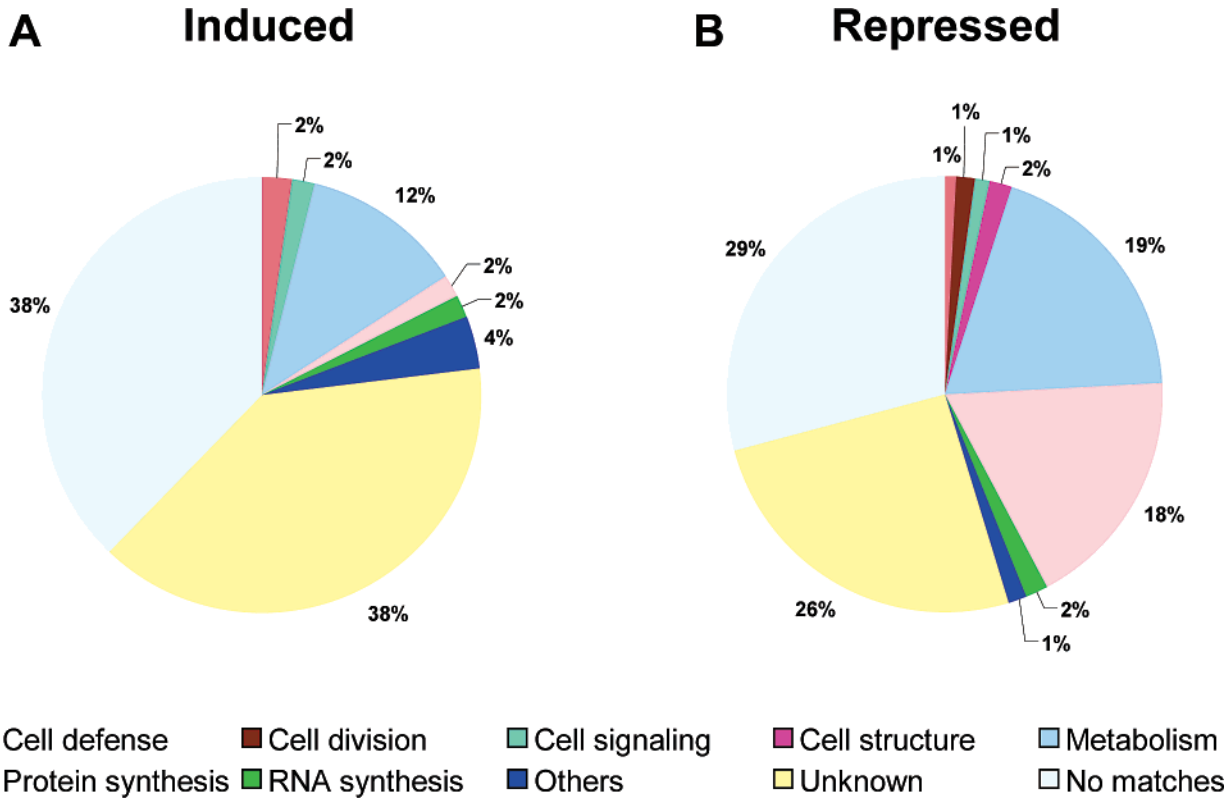

FIGURE 3: Classification of differentially expressed genes. Genes represented in Figure 2 (see also Table 1 and Table 2) were classified according to the EGAD database developed at the Institute for Genomic Research (TIGR, Rockville, MD) (*13*). Genes that show similarity to protein or DNA sequences to which no cellular role has yet been assigned are referred to as "Unknown". New sequences with no significant similarity to protein or DNA sequences in the public database are grouped in the category "No matches".

the process of cytokinesis during cell division (*18*). The second gene, A2012, is similar to *SIS1* of *S. cerevisiae*. In *S. cerevisiae* Sis1 is a chaperone which is essential for viability, and is required for initiation of translation and for nuclear migration during mitosis (*19*, *20*). The last gene is histone H3, one of the basic proteins that package and order the DNA into nucleosomes.

The down-regulation of these genes is consistent with growth arrest and the consequent drop in cell density observed under anoxic conditions (Figure 1B).

Cell Signaling. We identified 7 genes whose expression was altered in response to oxygen limitation and which belong to the signaling category. Four of them (A0880, A2346, A2774, A4057) were up-regulated under anoxia, and these encode proteins similar to Snf1, a Ran1-like protein, a MAPKK kinase (mitogen-activated protein kinase-kinase), and SCAP, respectively. In *S. cerevisiae*, Snf1 is a serine/threonine protein kinase that is considered to be a central component of the signaling pathway for glucose repression (*21*, *22*), and it has also been shown to be required for the response to heat stress (*23*). *SNF1* is required for the expression of glucose-repressed genes following withdrawal of glucose from the culture medium. Interestingly, the expression of the transcript of the putative *T. reesei SNF1* (A0880) was not altered in the hypoxic state, but was strongly induced under anoxia (Figure 2A,B, cluster 7). Therefore, its up-regulation in the presence of the high concentrations of glucose observed in the medium during incubation of *T. reesei* under anoxic conditions most probably does not reflect a metabolic response to high glucose concentration, but rather is a consequence of anoxic stress. The fact that this transcript returned to the normal level even in the presence of a repressing concentration of glucose (30 mM) after restoration of oxygen to the original state (see Figure 2A,B, cluster 7) supports this conclusion. The second gene in this category (A2346) shows similarity to a gene for a RAN1-like protein kinase of *Nectria haematococca* and to the *ran1* gene of the fission yeast *S. pombe*. Inactivation of this protein kinase in *S. pombe* is necessary to divert a vegetatively growing cell from mitotic division to meiotic differentiation (*24*). Like *SNF1*, this gene was a member of cluster 7 (Figure 2A,B). Its expression was not altered by hypoxia but strongly induced under anoxic conditions, indicating a possible role in maintaining *T. reesei* cells in the vegetative form. The A2774 gene is very similar to MAPKK genes from *Ustilago maydis* (*25*), *S. cerevisiae* (*26*), and human lung endothelial cells (*27*). MAPKKs regulate the activity of mitogen-activated protein kinases (MAPKs) by phosphorylation. Interestingly, in human lung epithelial cells, the MAPK cascade was shown to be involved in protection from oxidant injury during reoxygenation (*27*). Note that the putative MAPKK of *T. reesei* was found to belong to cluster 8 (Figure 2A,B). It is slightly up-regulated during anoxia and, interestingly, strongly induced after reoxygenation. This expression profile indicates a possible role for this MAPKK of *T. reesei* in protecting against damage due to the ROS that are generated after sudden reoxygenation. The potential activities of the protein kinases described above strongly indicate the involvement of these signaling enzymes in activating various cellular processes to protect the *T. reesei* cell under anoxic conditions and prevent oxidative damage upon reoxygenation.

Table 1: Induced Genes

clone ID	annotation	score	cluster
	Cell Defense		
TrEST-A0803	monooxygenase [*Penicillium paxilli*]; AAK11530	89	4
TrEST-A3340	30 kDa heat shock protein [*N. crassa*]; P19752	163	4
TrEST-A0004	heat shock protein HSP98 [*N. crassa*]; P31540	179	6
TrEST-A0074	heat shock protein HSP98 [*N. crassa*]; P31540	318	6
	Cell Signaling		
TrEST-A0880	serine threonine protein kinase SNF1 [*Hypocrea jecorina*]; AAK69560	384	7
TrEST-A2346	Ran1-like protein kinase [*S. pombe*]; AAA96531	285	7
TrEST-A2774	mitogen-activated protein kinase kinase [*S. cerevisiae*]; P32490	238	8
	Metabolism		
TrEST-A2977	pyruvate decarboxylase [*Aspergillus nidulans* FGSC A4]; XP_409025 (PDC)	107	2
TrEST-A3734	flavohemoglobin [*F. oxysporum*]; BAA33011	88	2
TrEST-A3912	glycerol-3-phosphate dehydrogenase [*Aspergillus fumigatus*]; EAL90210	292	3
TrEST-A0216	glutamine synthetase (Glutamate−ammonia ligase) [*Fusarium phaseoli*]; Q9UUN6	247	5
TrEST-A1365	cytochrome *c* [*N. crassa*]; P00048	186	5
TrEST-A2513	squalene epoxidase 1 (ERG1) [*A. fumigatus*]; AAS60234	95	5
TrEST-A4093	transaldolase TAL1 [*S. pombe*]; O42700	185	5
TrEST-A1726	glyceraldehyde 3-phosphate dehydrogenase 2 (GAPDH2) (TDH); P17730	301	5
TrEST-A4280	sterol delta 5,6-desaturase ERG3 [*Leptosphaeria maculans*]; AAN27998	347	5
TrEST-A3326	phosphoglycerate kinase (PGK) [*H. jecorina*]; P31865311	171	5
TrPDA[a]	pyruvate dehydrogenase [*H. jecorina*]		5
TrEST-A0111	L-xylulose reductase [*H. jecorina*]; AAM20896	342	6
TrEST-A2324	acetate kinase (acetokinase) [*Borrelia burgdorferi*]; O51567	168	6
TrEST-A0674	phosphoglycerate dehydrogenase [*Pseudomonas syringae*]; ZP_00127772	96	7
TrEST-A1813	PalI related protein (imported) [*N. crassa*]; T48810	139	7
	Protein Synthesis		
TrEST-A3417	protein translation initiation factor SUI1 [*S. cerevisiae*]; P32911	129	5
TrEST-A3737	eukaryotic translation initiation factor 6 (eIF-6); O94476	207	7
TrEST-A0377	cathepsin C isoform-a preproprotein; dipeptidyl-peptidase I [Homo sapiens]; NP_001805	409	8
	RNA Synthesis		
TrEST-A4276	ATP-dependent RNA helicase CA3 [*S. cerevisiae*]; NP_011437	122	3
TrEST-A1086	DNA-directed RNA polymerase II 14.2 kDa polypeptide [*S. cerevisiae*]; P27999	96	5
TrEST-A4080	transcription factor PACC [*Acremonium chrysogenum*]; CAC38840	179	8
	Others		
TrEST-A1116	nonribosomal peptide synthetase; NRPS [*Hypocrea virens*]; AAM78457	126	2
TrEST-A1935	NADP(+)-dependent glycerol dehydrogenase [*A. nidulans*]; CAD42649	135	3
TrEST-A3492	putative oxidoreductase [*Streptomyces avermitilis* MA-4680]; NP_822375	140	3
TrEST-A1902	aldo/keto reductase family oxidoreductase [*S. pombe*]; NP_594384	100	4
TrEST-A2502	CAP20 [*Glomerella cingulata*]; AAA77678	152	5
TrEST-A3994	hypothetical nitrilase-like protein [*Aspergillus oryzae*]; BAC55941	163	5
TrEST-A1628	probable hydroxyquinol-1, 2-dioxygenase [*N. crassa*]; CAE76372	90	6

[a] Gene probes amplified from *T. reesei* genome using gene-specific oligonucleotide primers.

The last induced gene in this category, A4057, codes for a product that is similar to the human endoplasmic reticulum membrane-bound SCAP (SREBP cleavage-activating protein) (*28*). SREBP (sterol regulatory element binding protein) is also anchored in the membrane of the endoplasmic reticulum and is transported by SCAP to the Golgi apparatus, where it is cleaved to release an active transcription factor that activates more than 30 genes needed for the uptake and synthesis of cholesterol, fatty acids, triglycerides, and phospholipids (*29*). The SREBP pathway is activated in response to lack of sterols (*30*), and it was recently shown to function as an oxygen sensor in fission yeast (*31*). Interestingly, two genes involved in sterol synthesis, squalene epoxidase (A2513) and sterol 5,6-desaturase (A4280), were also found to be induced under hypoxic and anoxic conditions (see Table 1, under Metabolism), indicating a potential role for SCAP in controlling the induction of enzymes of lipid metabolism to maintain membrane integrity under oxygen limitation.

Three genes belonging to the cell signaling category were found to be down-regulated by hypoxia and strongly repressed by anoxia. Their products display similarities to calnexin from *Aspergillus niger*, and RHOA, a GTP-binding protein from *A. nidulans* and *S. pombe*, and RIM15, a putative serine/threonine kinase from *Gibberella moniliformis*. Calnexin is a membrane-bound lectin and a molecular chaperone that binds newly synthesized glycoproteins in the endoplasmic reticulum (ER) (*32*). Overexpression of calnexin increases manganese peroxidase production in *A. niger* (*33*) and has also been reported to be involved in the folding of human thyroperoxidase (*34*). These results suggest a functional role for calnexin in the incorporation of the prosthetic group into hemoproteins. The fact that heme synthesis requires oxygen, together with our finding that a calnexin gene homologue (A1058) is down-regulated by oxygen limitation in *T. reesei*, further strengthens the notion that calnexin participates in the process of hemoprotein folding.

The second gene (A2439) encodes a predicted RHOA homologue. RHOA is a GTP-binding protein that plays a role in the regulation of glucan synthesis. The *S. pombe* Rho1 protein is a member of the glucan synthase complex and has a regulatory function in stimulating $\beta(1\rightarrow3)$ glucan synthesis (*35*). In addition, it was suggested that RHOA plays a role in cell polarity by controlling hyphal branching patterns and cell wall deposition in *A. nidulans* (*36*). The activities

Table 2: Repressed Genes

clone ID	annotation	score	cluster
	Cell Defense		
TrEST-A3343	superoxide dismutase [Mn], mitochondrial precursor [*N. crassa*]; Q9Y783	301	9
TrEST-A2590	thiol-specific antioxidant protein 3 [*Cryptococcus neoformans* var. *grubii*]; AAP68995	232	14
	Cell Division		
TrEST-A1043	septin 2 [*Coccidioides immitis*]; AAN63564	85	15
TrEST-A2012	SIS1 protein [*S. cerevisiae*]; P25294	100	15
TrEST-A1561	Histone H3 [*N. crassa*]; P07041	251	12
	Cell Signaling		
TrEST-A1058	calnexin [*Aspergillus niger*]; CAC82717	184	15
TrEST-A2439	RhoA protein (Rho1 protein homologue) [*Emericella nidulans*]; Q9C3Y4	208	13
TrEST-A0194	response regulator receiver RIM15p [*Gibberella moniliformis*]; AAR30133	305	14
	Cell Structure		
TrEST-B16G09	extracellular matrix protein precursor [*F. oxysporum* f. sp. *lycopersici*]; AAL47843	84	12
TrEST-A0714	HEX1 [*H. jecorina*]; AAQ56234	148	13
TrEST-B33C03	chitin synthase 3 (chitin-UDP acetyl-glucosaminyl transferase 3) [*N. crassa*]; P29070	247	13
TrEST-A0147	T-complex protein 1, zeta subunit (TCP-1-zeta) (CCT-zeta) [*S. pombe*]; O94515	260	14
	Metabolism		
TrEST-A4104	NAD-specific glutamate dehydrogenase (NAD-GDH) [*N. crassa*]; P00365	250	9
TrPGM[a]	phosphoglucomutase [*H. jecorina*]		9
TrPFK1[a]	phosphofructokinase [*H. jecorina*]		9
TrGPM[a]	phosphoglycerate mutase [*H. jecorina*]		9
TrEST-B08D01	pyruvate kinase (PYK) [*H. jecorina*]; P31865	171	9
TrYGR[a]	succinyl-CoA synthetase [*H. jecorina*]		9
TrFUM[a]	fumarase [*H. jecorina*]		9
TrHXt1	glucose transporter; TrHXT1 [*H. jecorina*]; AAR23147	211	10
TrEST-B06E06	monosaccharide transporter MSTA protein [*A. niger*]; AAL89822	229	11
TrEST-A1305	fructose-bisphosphate aldolase (FBA) [*A. fumigatus*]; EAL92414	368	11
TrEST-A2339	aldehyde dehydrogenase 2 (ALD2) [*S. pombe*]; CAB65612	249	11
TrEST-A2353	acetyl-CoA synthetase (ACS) [*N. crassa*]; P16929	248	11
TrICL[a]	isocitrate lyase (ICL) [*H. jecorina*]		11
TrMLS[a]	malate synthase (MLS) [*H. jecorina*]		11
TrEST-A1190	malate dehydrogenase (MDH) [A. fumigatus]; EAL86898	220	11
TrEST-A3061	phosphoenolpyruvate carboxykinase (PCK) [*N. crassa*]; Q7RVS9	216	11
TrEST-A0231	pyridoxine biosynthesis protein PDX1 [*Cercospora nicotianae*]; O59905	241	12
TrEST-A0793	peptide transporter [*A. fumigatus*]; CAE47951	198	12
TrEST-B35A11	mannitol-1-phosphate dehydrogenase [*A. niger*]; AAL89587	134	12
TrHXK[a]	hexokinase [*H. jecorina*]		13
TrGLK[a]	glucokinase [*H. jecorina*]		13
TrEST-A4206	triosephosphate isomerase (TPI) [*N. crassa*]; Q7S2Z9	188	13
TrPYC[a]	pyruvate carboxylase [*H. jecorina*]		13
TrEST-A0127	S-adenosylmethionine synthetase [*N. crassa*]; P48466	196	13
TrEST-B08D10	plasma membrane ATPase (proton pump) [*N. crassa*]; P07038	283	13
TrEST-B16G08	ATP citrate lyase, subunit 2 [*Sordaria macrospora*]; CAB76164	252	13
TrEST-A0205	nuclear transport factor 2 [*A. nidulans*]; AAK71467	101	14
TrEST-A1859	C-1-tetrahydrofolate synthase, mitochondrial precursor; P09440	185	14
TrEST-A3972	adenylosuccinate synthetase [*E. nidulans*]; AAL56637	189	14
TrEST-B21C05	cobalamin-independent methionine synthase [*Epichloe festucae*]; AAQ73630	246	14
TrEST-B26D02	3-isopropylmalate dehydrogenase A (3-IPM-DH A) [*A. niger*]; P87256	91	14
TrEST-B42H12	D-3-phosphoglycerate dehydrogenase 2 (3-PGDH 2) [*S. cerevisiae*]; P40510	192	14
TrEST-B06H05	citrate synthase (CIT) [*N. crassa*]; P34085	309	14
TrEST-A3335	isocitrate dehydrogenase (IDH) [*A. fumigatus*]; EAL90612	307	14
TrEST-A0599	oxoglutarate dehydrogenase precursor [MIPS] [*N. crassa*]; XP_325280	219	14
TrCOX2[a]	cytochrome *c* oxidase polypeptide II [*H. jecorina*]; NP_570154	728	14
TrEST-A2312	mannose-1-phosphate guanyltransferase [*S. pombe*]; NP_596551	120	15
TrEST-A2463	outer mitochondrial membrane protein porin [*N. crassa*]; P07144	169	15
TrEST-A3037	high affinity copper transporter [*Podospora anserina*]; CAC83163	124	15
TrEST-A3671	nucleoside diphosphate kinase (NDK) [*N. crassa*]; Q9UUY8	249	15
TrEST-B14A01	ATP synthase beta chain, mitochondrial precursor [*N. crassa*]; *P*23704	172	15
TrGPH[a]	glycogen phosphorylase [*H. jecorina*]		15
TrGND[a]	6-phosphogluconate dehydrogenase [*H. jecorina*]		15
TrEST-B34G06	aconitase (ACO) [*E. nidulans*]; AAN61439	154	15
TrEST-A0307	cytochrome P450 monooxygenase [*A. parasiticus*]; AAS66021	92	16
TrEST-A0386	high affinity potassium transporter, TRK-1 [*N. crassa*]; CAA08813	238	16
TrEST-A0713	pyridoxamine 5′-phosphate oxidase [*S. pombe*]; Q9UTQ1	93	16
TrEST-B19C03	cytochrome *c* oxidase polypeptide V, mitochondrial precursor [*N. crassa*]; P06810	137	16

Table 2 (Continued)

clone ID	annotation	score	cluster
	Protein Synthesis		
TrEST-A0041	vacuolar protease A (aspartic proteinase) [*N. crassa*]; XP_331049	211	12
TrEST-A2457	peptidylprolyl isomerase A precursor [*Cordyceps subsessilis*]; S71849	232	12
TrEST-A3560	related to aspartyl proteinase SAP3 precursor [*N. crassa*]; CAE76540	140	12
TrEST-A4013	carboxypeptidase [*A. nidulans*]; JC7666.	324	12
TrEST-B07H02	subtilase [*Ophiostoma piliferum*]; AAL08510	155	12
TrEST-A0265	lysyl-tRNA synthetase [*N. crassa*]; CAD79693	139	14
TrEST-A2625	ubiquitin/S27a fusion protein [*N. crassa*]; T46664	238	14
TrEST-B15E06	glutamyl-tRNA synthetase [*S. pombe*]; NP_593483	232	14
TrEST-A0595	subtilisin-like serine protease PR1H [*Metarhizium anisopliae* var. *acridum*]; CAC95047	89	15
TrEST-B23E11	26S ATP/ubiquitin-dependent proteinase chain S4 [*N. crassa*]; XP_326717	264	15
TrEST-B42E12	related to SCJ1 protein [*N. crassa*]; CAD70988	179	15
TrEST-B20A07	guanosine-diphosphatase [*N. crassa*].; CAC28854	115	16
ribosomal proteins	31 *T. reesei* ESTs match different ribossomal proteins	89−333	9; 12−15
	RNA Synthesis		
TrEST-A1624	rRNA intron-encoded homing endonuclease [*Oryza sativa*]; AAK13589	80	12
TrEST-A0146	U2 small nuclear ribonucleoprotein A′ (U2 snRNP-A′) [*S. pombe*]; Q9USX8	115	15
TrEST-A0314	related to cyclic-amp-dependent transcription factor atf-2 [*N. crassa*]; CAD70949	99	16
TrEST-A0331	cell cycle control protein cwf16 [*S. pombe*]; Q9P7C5	153	16
	Others		
TrEST-B09B08	putative coatmer delta subunit [*S. pombe*]; NP_588336	157	14
TrEST-A3183	probable beta (1−3) glucanosyltransferase gel3p [*N. crassa*]; CAD70754	158	15
TrEST-A0174	probable phosphoketolase 1 [*Nostoc* sp. PCC 7120]; Q8YWW1	330	16

[a] Gene probes amplified from *T. reesei* genome using gene-specific oligonucleotide primers.

suggested for this gene product indicate that oxygen limitation down-regulates cell wall biosynthesis, and this is compatible with growth arrest and the decrease in cell density observed under anoxic conditions.

The last gene in this category, A0194, encodes a homologue of the *Gibberella moniliformis* protein RIM15 (*37*), a putative serine/threonine protein kinase. Mutations in the *S. cerevisiae* homologue reduce the ability to undergo meiosis (*38*), and cause defects in the capacity to induce thermotolerance and starvation resistance (*39*). It is important to note that consumption of glucose in *T. reesei* ceases under anoxic conditions. The cells are therefore most probably subject to indirect glucose starvation. Repression of this gene, therefore, may represent a response to nutrient depletion.

Cell Structure. The genes identified in this category encode Hex1 of *T. reesei* (*40*), a predicted protein similar to an extracellular matrix protein precursor from *Fusarium oxysporum* (*41*), a protein similar to chitin synthase from *Neurospora crassa* (*42*), and a member of the T-complex, a cytoplasmic chaperonin.

The HEX1 protein is the major component of the Woronin body, a membrane-bound organelle that plays a role in the maintenance of cell integrity following localized damage to hyphae. In filamentous fungi, cellular compartments are interconnected through septal pores that allow transport of cytoplasm, organelles, and nuclei between cells. When hyphae are damaged, Woronin bodies rapidly plug the septal pores, thus preventing the death of cells adjacent to the damaged zone (*43*, *44*). Deletion of hex1 in *N. crassa* eliminates Woronin bodies and results in cytoplasmic bleeding from hyphae in response to cell lysis (*45*). We have not observed any lysis of *T. reesei* cells under hypoxic or anoxic conditions, and, therefore, the strong repression of the *hex1* gene expression in the absence of oxygen is most probably an indirect consequence of the down-regulation of cell division and growth.

The cytoplasmic chaperonin is a component of the T-complex, which facilitates the folding of tubulins and actin under normal growth conditions (*46*, *47*). Interestingly, and in accordance with the physiological function of the T-complex described above, we observed strong repression of actin gene transcription under anoxia (Figure 4).

Down-regulation by oxygen limitation of genes involved in cell structure indicates a general repression of cell division, which would explain the reduction in cell density observed in our cultures under conditions of extreme hypoxia and anoxia (Figure 1B).

Protein Synthesis. In this category, we identified 46 genes that responded to oxgen limitation or reoxygenation. Only three of these were up-regulated; all the others were found to be down-regulated.

The three genes that are up-regulated (A3417, A3737, and A0377) encode proteins similar to the translation initiation factors elF1 (SUI1) and eIF6, and to human cathepsin C, respectively. The *T. reesei* genes for the elF1 and eIF6 homologues are slightly induced by hypoxia and strongly up-regulated under anoxia, whereas the transcript that has similarity to cathepsin C is strongly induced upon reoxygenation (cluster 8, see Figure 2A,B). eIF1 and 6 bind to the 60S ribosomal subunit and prevent it from associating with the 40S ribosomal subunit to form the 80S initiation complex (*48*). The strong induction of these factors under anoxic conditions is therefore expected to contribute to down-regulation of protein synthesis.

Cathepsin C, also known as dipeptidyl peptidase I, is a lysosomal cysteine proteinase that appears to play a central role in the activation of many serine proteinases in immune/inflammatory cells (*49*). Although no role for this enzyme in *T. reesei* has yet been established, the strong induction of this protein after reoxygenation may reflect a critical function

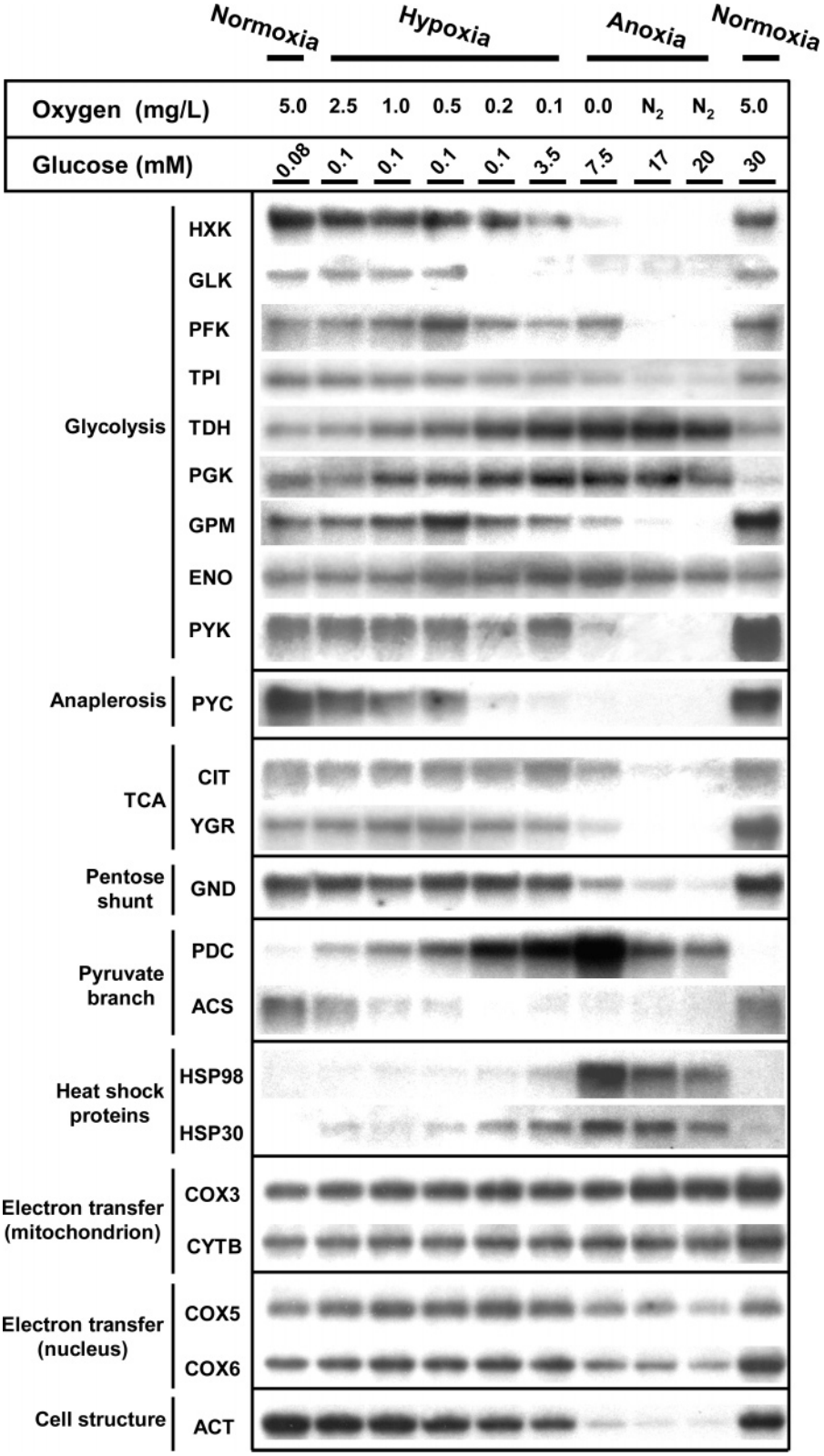

FIGURE 4: Effect of oxygen availability on the expression of selected genes involved in glucose metabolism and cell defense. Aliquots (10 μg) of total RNA, obtained from the samples used in the microarray experiment presented in Figure 2, were analyzed by Northern hybridization, using gene-specific probes amplified from the corresponding TrEST.

of this protein in processing various proenzymes to their active forms to enable resumption of growth after exposure to anoxia.

Of the 43 genes repressed by oxygen limitation, 31 were found to encode ribosomal proteins. These genes were slightly down-regulated by hypoxia and strongly repressed by anoxia (see Figure 2A,B, clusters 9, 12−15). The other repressed genes in this category show similarities to various proteases, peptidylprolyl isomerase, tRNA synthases, and a ubiquitin-dependent proteinase. Because protein synthesis consumes much of the energy generated in cells, its down-regulation should help to maintain the supply of ATP

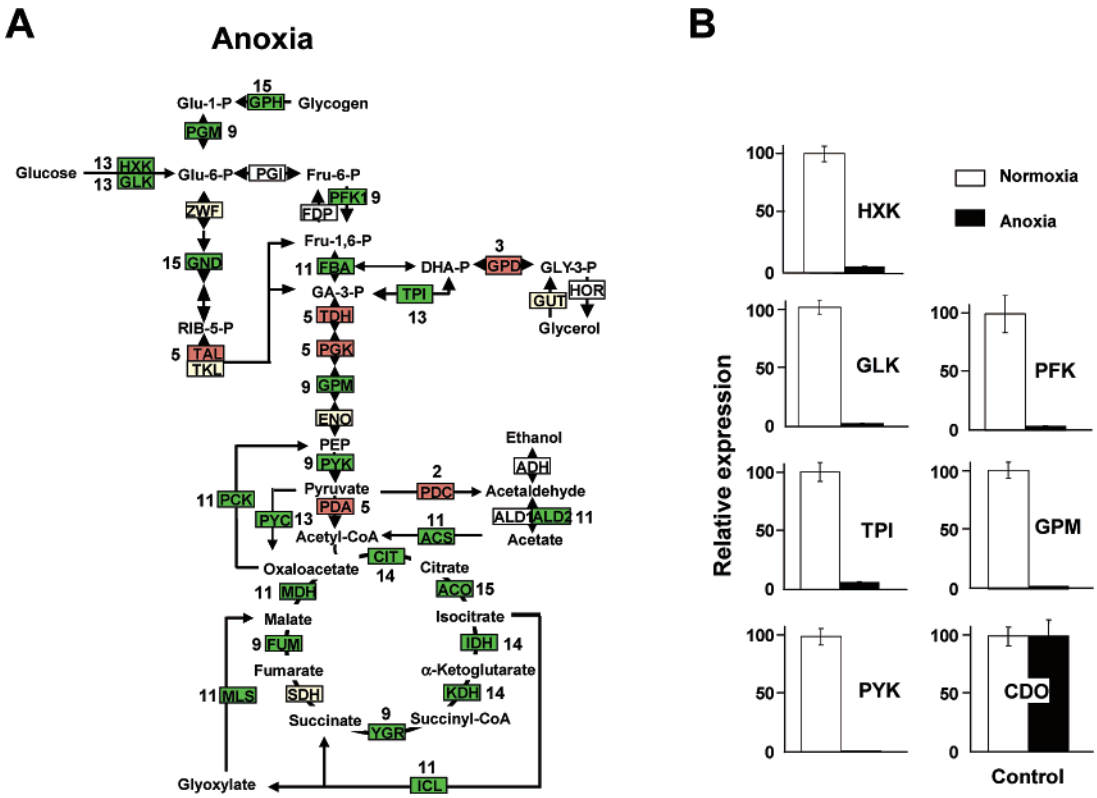

FIGURE 5: (A) Expression patterns of genes for enzymes that participate in central carbon metabolism under anoxic conditions (assayed after exposure to N_2 for 1 h). Red, green, and yellow boxes indicate those genes whose expression levels increased, decreased, or were unaffected, respectively, by the imposition of anoxia. White boxes represent genes that have yet not been isolated from *T. reesei*. The number next to each box refers to the expression cluster to which each gene belongs (see Figure 2B). (B) Effects of transient anoxia on the expression of critical genes involved in energy production under anaerobiosis, as revealed by quantitative Real-Time PCR. Real-time PCR analysis was performed using GeneAmp 5700 (Applied Biosystems) and the SYBR GREEN PCR Master Mix (Applied Biosystems). The experiment was repeated four times, and the data were normalized according to the $2^{-(\Delta\Delta C_T)}$ method (*56*). CDO is cysteine dioxygenase.

required for the physiological adaptation of the cell to hypoxia, and for survival during transient anoxia.

Central Carbon Metabolism. Under hypoxic conditions at DO concentrations >0.2 mg/L, the genes encoding enolase (ENO), glyceraldehyde-3-phosphate dehydrogenase (TDH), phosphoglycerokinase (PGK), and pyruvate decarboxylase (PDC) were found to be significantly induced. In contrast, the gene encoding fructose-bisphosphate aldolase (FBA) was down-regulated, while transcription of the genes involved in the glyoxylate cycle [isocitrate lyase (ICL) and malate synthase (MLS)], anaplerotic reactions [puruvate carboxylase, (PYC)], and gluconeogenesis (phosphoenolpyruvate carboxykinase,PCK) were strongly repressed (see Figure 2B and Figure 4). The expression of transcripts required for the function of the TCA cycle was not found to be significantly altered.

It is worthwhile mentioning here that, although CO_2 production steadily decreases as the level of DO falls, at DO = 0.2 mg/L—when CO_2 production has fallen by 28% from that observed under normoxic conditions—glucose consumption is still unaffected. This observation indicates that some of the glucose metabolized at 0.2 mg/L oxygen is most probably diverted from the TCA cycle into a different pathway. We analyzed the culture medium for the presence of glycerol, acetate, and ethanol, but none of these metabolites were detected.

Assuming that most of the relevant genes are controlled mainly at the transcriptional level, this overall pattern of gene expression indicates that *T. reesei* is able to metabolize the major fraction of glucose consumed at oxygen concentrations of >0.2 mg/L by oxidative processes.

The most pronounced transcriptional change that affects the metabolic state of *T. reesei* cells was observed under anoxic conditions. The production of CO_2 reached its minimum level (10% of that observed at normoxic condition), and glucose consumption fell almost to zero, resulting in accumulation of glucose in the culture medium (Figure 1A,B). Under anoxic conditions, six genes for enzymes of the glycolytic pathway—hexokinase (HXK), glucokinase (GLK), phosphofructokinase (PFK1), triosephosphate isomerase (TPI), phosphoglycero mutase (GPM), pyruvate kinase (PYK)—were strongly repressed (Figure 4). Profiles of gene expression of the members of the glycolytic pathway, the TCA cycle, the pentose phosphate pathway, and the glyoxylate cycle are presented in Figure 5 A (see also Figure 2B).

In light of the broad implications of the repression of glycolysis for energy production by anaerobic metabolism, and in order to validate the microarray data using an independent and quantitative technique, we examined the expression of these six genes by quantitative Real-Time-PCR.

The results of this analysis are shown in Figure 5B: they confirm the data obtained by microarray and Northern analysis. The calculated degree of repression of each gene is as follows: *HXK*, 95%; *GLK*, 97%; *PFK1*, 96%; *TPI*, 94%; *GPM*, 98%; *PYK*, 99%. This transcriptional profile is incompatible with a level of metabolite flow through the

glycolytic pathway that would permit the cells to produce enough ATP and regenerate sufficient NAD+ through anaerobic metabolism to remain viable for long. In addition, genes of the TCA cycle, and genes for two components of ATP synthase and for cytochrome *c* oxidase polypeptide II, are strongly repressed under anoxic conditions (see Table 2). Although this profile implies potentially fatal consequences for the energy budget of the cell, *T. reesei* was nevertheless able to withstand this anoxic situation for 2 h, judging by its response to reoxygenation: consumption of the accumulated glucose begins again, CO_2 production rises, and transcriptional reprogramming permits resumption of growth and respiration (Figure 1A,B).

The transcriptional profile of the genes involved in central carbon metabolism in *T. reesei* reveals a basic difference between this obligate aerobe and the facultative anaerobe *S. cerevisiae* with respect to energy production under anaerobiosis. While the genes for anaerobic energy production in *S. cerevisiae* are strongly induced in the absence of oxygen (*7*), in *T. reesei* most of the genes for components of the glycolytic pathway are strongly repressed, thus effectively preventing energy production under anaerobic conditions.

DISCUSSION

Using the obligatorily aerobic fungus *T. reesei* as a model system, we have investigated the shifts that occur in the pattern of gene expression in response to hypoxia, transient anoxia, and reoxygenation. Of the 2000 genes analyzed, 392 genes (19.6%) showed significant alteration in their level of expression—42% were induced, whereas 57% were repressed—indicating that oxygen availability has a marked effect on gene expression.

Analyses of transcripts in each of three distinct states—hypoxia, anoxia, and reoxygenation—shed light on how a multicellular organism responds to shifts in oxygen availability. Genes involved in cell protection, ergosterol synthesis, glycolysis, and the pentose phosphate pathway were up-regulated, whereas genes involved in protein synthesis (including many for ribosomal proteins), maintenance of cell structure, and cell division were down-regulated by hypoxia. The response to oxygen limitation is thus characterized by a strong tendency to cut down the flux of materials into energy-consuming processes, such as protein synthesis.

Under anoxic conditions, this tendency becomes even more pronounced, and genes involved in protein synthesis, cell structure, and cell division are strongly repressed. Moreover, and perhaps most strikingly, transcription of the majority of the genes for enzymes of the glycolytic pathway and the TCA cycle is inhibited. This pattern of gene expression implies significant impairment of major cellular functions. Indeed, cells essentially cease to produce CO_2 at this stage and become unable to utilize glucose. Upon reoxygenation after 2 h of anoxia, genes needed for the consumption of glucose by oxidative metabolism are activated and the cells begin to metabolize glucose and produce CO_2 again. In addition, genes involved in protection against oxidative injury are up-regulated.

Despite substantial advances in our understanding of the transcriptional regulation of glucose utilization for energy production in the facultatively anaerobic yeast *S. cerevisiae* (*7*, *50*) and in obligatorily aerobic multicellular fungi (*10*, *51*, *52*), we still do not know precisely why obligately aerobic eukaryotic cells are unable to obtain energy from glucose in the absence of oxygen. The transcriptional profile of the glycolytic genes, under transient anoxia, described in this work, provides some intriguing clues. Even though some genes of the glycolytic pathway were up-regulated under hypoxic conditions, a drastic change was observed in the complete absence of oxygen. The genes coding for glyceraldehyde-phosphate dehydrogenase and phosphoglycerate kinase are induced, but hexokinase, glucokinase, phosphofructokinase, triosephosphate isomerase, phosphoglyceromutase, and pyruvate kinase are all repressed by over 90%. This profile of gene expression should result in interruption of the flow of metabolites into the glycolytic pathway and consequently to impairment of ATP production. It is interesting to note that in *S. cerevisiae*, unlike *T. reesei*, all the genes for glycolytic enzymes are induced by anoxia (*7*), thus allowing production of ATP via anaerobic metabolism and permitting the cell to survive in the absence of oxygen. Whether the pattern of transcription of the glycolytic genes under anoxic conditions is unique to *T. reesei*, or is a general feature of other obligately aerobic microorganisms and mammalian cells, has still to be established.

Although it is generally thought that insertion of regulatory elements and factors, and rearrangement of primitive prokaryotic glycolytic operons into unlinked genes on the eukaryotic chromosomes, proceeded in such a way as to allow the coordinate function of the pathway (*53*), the profile of gene expression under anoxia presented in this work indicates that evolution of the glycolytic genes in *T. reesei* did not occur in this way. It appears, therefore, that evolution has produced two diametrically opposed systems of oxygen-dependent transcriptional control of glycolytic genes in eukaryotic microorganisms: in one set of organisms, lack of oxygen induces these genes; in the other it represses them.

The ability of eukaryotes to use oxygen to obtain energy by aerobic metabolism (*54*), the selection pressure imposed by energy limitation, and the high yield of ATP obtained by respiration have been implicated as key factors in the rise of complex multicellular life (*55*). Therefore, it is tempting to speculate that the strong repression by anoxia of genes for the enzymes of glycolysis reflects evolutionary adaptation of the regulatory process to select for obligatorily aerobic lifestyles.

The transcriptional profile of the genes involved in production of ATP in *T. reesei* under anoxic conditions presented in this work reveals a key reason why this obligate aerobe is unable to survive for long in the absence of oxygen. In addition, it provides important information regarding the expression of genes required for the utilization of glucose in the presence of different levels of oxygen. This basic transcriptional information is essential for the metabolic engineering of *T. reesei*—the most efficient eukaryotic producer of glucose from cellulose yet found—for the production of useful compounds and fuels, such as alcohol, from biomass.

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