Global Gene Expression Analysis during Sporulation of the Aquatic Fungus Blastocladiella emersonii*†

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The Blastocladiella emersonii life cycle presents a number of drastic biochemical and morphological changes, mainly during two cell differentiation stages: germination and sporulation. To investigate the transcriptional changes taking place during the sporulation phase, which culminates with the production of the zoospores, motile cells responsible for the dispersal of the fungus, microarray experiments were performed. Among the 3,773 distinct genes investigated, a total of 1,207 were classified as differentially expressed, relative to time zero of sporulation, at least one of the time points analyzed. These results indicate that accurate transcriptional control takes place during sporulation, as well as indicating the necessity for distinct molecular functions throughout this differentiation process. The main functional categories overrepresented among upregulated genes were those involving the microtubule, the cytoskeleton, signal transduction involving Ca2+, and chromosome organization. On the other hand, protein biosynthesis, central carbon metabolism, and protein degradation were the most represented functional categories among downregulated genes. Gene expression changes were also analyzed in cells sporulating in the presence of subinhibitory concentrations of glucose or tryptophan. Data obtained revealed overexpression of microtubule and cytoskeleton transcripts in the presence of glucose, probably causing the shape and motility problems observed in the zoospores produced under this condition. In contrast, the presence of tryptophan during sporulation led to upregulation of genes involved in oxidative stress, proteolysis, and protein folding. These results indicate that distinct physiological pathways are involved in the inhibition of sporulation due to these two classes of nutrient sources.

The life cycle of Blastocladiella emersonii, an aquatic fungus of the class Blastocladiales, involves a series of complex morphological and biochemical events, which involve transcriptional, posttranscriptional, and posttranslational mechanisms of control, especially during two stages of cell differentiation, the germination and the sporulation of the fungus (21). Germination starts with the zoospore, a motile uninucleated wall-less nongrowing cell, which is responsible for the dispersal of the fungus. In the presence of appropriate stimuli, the zoospore germinates, undergoing a number of biochemical and morphological changes. The early events of germination, which include retraction of the single polar flagellum, construction of a cell wall rich in chitin, and cleavage of a giant mitochondrion into normal-size ones, do not require concomitant RNA and protein synthesis and involve posttranslational regulatory events (17, 20, 30, 31, 35, 36). Major changes in the pattern of RNA and protein synthesis are observed only at the late events of this stage, when the germ tube begins to branch, giving rise to a rhizoidal system through which nutrients are absorbed, and when the cells enter the vegetative growth phase (17, 20, 30, 31, 35, 36).

B. emersonii vegetative growth is characterized by intense nuclear division not accompanied by cell division, generating single-celled coenocytes denominated zoosporangia. At any time during vegetative growth, sporulation can be induced by subjecting vegetative cells to nutrient starvation. In the laboratory, sporulation can be triggered by washing and resuspension of the cells in a buffered solution containing 1 mM Ca2+. In fact, both calcium and the calcium-binding protein calmodulin have been shown to be necessary at the earliest stages of this process (4, 33, 34). Under starvation conditions, cells undergo an ordered and synchronized sequence of morphological and physiological changes. The series of events includes the construction of a basal septum separating the cell body from the rhizoidal system; formation of a structure in the cell wall, called the papilla, through which the zoospores exit the cell; cytoplasmic cleavage around each nucleus; and biogenesis of the flagellum. The process culminates in the production and release of a number of motile zoospores to the medium. This synchronized sequence of events can, however, be disturbed by nutritional variables such as (i) Casamino Acids, which promote the return of the cells to the growth phase when added to cultures together with the sporulation solution and before septum formation; (ii) certain amino acids that either prevent (tyrosine, phenylalanine, tryptophan, histidine, and threonine) or delay (valine, serine, arginine, and methionine) septum formation when added to cultures together with the sporulation solution and before septum formation; and (iii) sugars such as glucose, which can block zoospore biogenesis (3).

Even though high levels of proteolysis are observed, especially at the beginning of sporulation, changes in the protein profile seem to be regulated mainly at a transcriptional level throughout this developmental stage (5, 19). Although the overall RNA synthesis rate falls drastically after induction of sporulation, synthesis of new RNA molecules takes place to ensure subsequent cytodifferentiation events (26). Indeed, no
net increase in protein, RNA, or DNA levels are observed during sporulation (14, 19, 26), implying that all biochemical and morphological changes occurring during this stage are dependent on an extensive turnover of proteins and RNAs. In fact, studies on RNA metabolism revealed that mRNAs transcribed during early B. emersonii sporulation are preferentially eliminated, in contrast to those synthesized at later times, which seemed to be stored in the zoospores (14).

Recently, global gene expression changes occurring during B. emersonii germination were analyzed in cells germinating both in nutrient medium and in inorganic solution containing either potassium or adenine as inducers of this differentiation process (29). Data revealed that more than 900 genes out of 3,563 distinct genes spotted in the microarray chips were differentially expressed during germination in nutrient medium, over 500 of them being upregulated. The main biological processes upregulated were shown to be those necessary for cell growth and maintenance, including gene transcription, protein biosynthesis, energy metabolism, nutrient transport, and cell cycle control. Interestingly, most genes involved in cellular growth were not induced during germination triggered in inorganic solution, which does not advance beyond germ tube formation, indicating that the presence of nutrients controls the expression of these genes. In contrast, data revealed that most genes involved in signal transduction showed the same expression profile during the initial stages of germination with all the inducers investigated. These results indicate that the same signaling pathways are activated irrespective of the initial stimulus triggering germination (29).

In this report, we focused on global transcriptional changes occurring during sporulation of B. emersonii, as well as on the transcriptional response to the presence of subinhibitory concentrations of glucose or tryptophan in the sporulation solution. In addition, a comparative analysis of the genes differentially expressed during both germination and sporulation revealed a large number of genes inversely regulated between these two cytodifferentiation stages.

**MATERIALS AND METHODS**

**Culture conditions.** Cultures of B. emersonii were maintained in solid medium containing 0.13% peptone, 0.13% yeast extract, 0.3% glucose, and 1% agar. For the last part of the sporulation-transcribed population (21), and synchrony of sporulation were monitored by taking samples at different times and by examining the cellular phenotypes (vegetative cell, septate zoospore, papillate zoosporangium, cleavage zoosporangium, and empty zoosporangium, vitrogen), and its integrity was verified through 1% agarose–2.2 M formaldehyde extraction.

**Microarray hybridization.** For microarray hybridization, cDNA synthesis and labeling were performed using the CyScribe postlabeling kit (Amersham Biosciences). Briefly, we used 10 μg of total RNA, oligo(dT) primers, amino allyl-dUTP, 1× buffer, dithiothreitol (DTT), and the reverse transcriptase CyScribe according to the supplier’s recommendations. The reaction mixture was incubated at 42°C for 3 h following RNA degradation by addition of NaOH. The resulting first-strand cDNA was purified using a Millipore multimesure filtration plate (MAFB NOB) and dried in a speed vacuum apparatus. For cDNA labeling, the CyDyes were suspended in 100 mM sodium bicarbonate (pH 9.0) and this mixture was added to the dried cDNA. The reaction mixture was incubated in the dark for 1 h at room temperature and interrupted by addition of 4 M hydroxylamine. The labeled cDNA was purified and dried as described above. Labeled cDNA was suspended in water, 50% formamide, and 1× hybridization buffer (Amersham Biosciences). The labeled cDNA was carefully dropped onto microarray slides containing 3,773 distinct expressed sequence tag (EST) sequences, spotted at least in duplicate, as previously described (10). Arrays were hybridized at 42°C for 16 h and washed at 55°C once in 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate) and 0.2% sodium dodecyl sulfate (SDS) for 10 min, followed by two washes in 0.1× SSC and 0.2% SDS for 10 min and one final wash in 0.1× SSC for 1 min. Slides were then dried with N2 vapor, and image acquisition was carried out using a Generation III scanner (Amersham Biosciences).

**Differential expression analysis.** Differentially expressed genes representing the complete transcription profile (S0, S120, S150, and ZSP) were determined by LOWESS fitting on an intensity log scale (37) relative to the control condition (S0) (M = log2 [treatment/control]) and Z is the log mean fluorescence intensity (S = log; [treatment/2 + control]). Each condition (sporulation with and without 1% glucose) was analyzed with three independent biological experiments. Since each slide carried two replicates of the arrayed genes, a total of six intensity readings were generated for each gene in the microarray. The expression ratios shown in the Table 1 represent the median values determined for the valid replicates.

**Determination of differentially expressed genes.** We used intensity-dependent cutoff values for classifying a gene as differentially expressed based on self-self hybridization experiments, as previously described (16, 39). Briefly, the self-self approach consists of hybridizing against itself the same cDNA sample labeled separately with either Cy3 or Cy5 to estimate the experimental noise. The cDNA synthesized from RNA of cells at time zero of sporulation (S0) was chosen to perform the self-self experiment. We used a credibility interval of 0.99, a window size of 1.0, and a window step of 0.2. A gene was classified as differentially expressed at a given sporulation time point if at least 70% of its replicates were outside the intensity-dependent cutoff curves.

**Cluster analysis.** Differentially expressed genes representing the complete transcription profile (S0, S120, S150, and ZSP) were separately with either Cy3 or Cy5 to estimate the experimental noise. The cDNA synthesized from RNA of cells at time zero of sporulation (S0) was chosen to perform the self-self experiment. We used a credibility interval of 0.99, a window size of 1.0, and a window step of 0.2. A gene was classified as differentially expressed at a given sporulation time point if at least 70% of its replicates were outside the intensity-dependent cutoff curves.

**Materials and methods.** The materials and methods section describes the experimental setup for the study, including the materials used, methods employed, and data analysis techniques.
15 s and 60°C for 1 min. For each gene analyzed, two independent RNA samples were used. The gene encoding the mitochondrial RNA helicase-like protein, which was shown to be invariant in all conditions tested, was used as the calibrator gene in all experiments. The determination of the expression ratios was carried out using the 2^-ΔΔCT method as previously described (18).

**Cellular thiol determination.** The redox state of the sporulating cell was estimated by measuring the amount of reduced thiols present in the lysole obtained from cells treated with tryptophan and untreated. Quantification was performed using the dithionitrobenzoic acid (DTNB) method at 412 nm (molar extinction coefficient, 13.6 mmol^-1 cm^-1) as previously described (32). The reduced thiol concentration was expressed as the percentage of that for the control (tryptophan-untreated cells).

**Microarray data accession number.** The microarray data discussed in this work have been deposited in NCBI's Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO series accession number GSE18718 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE18718).

**RESULTS AND DISCUSSION**

**Global gene expression analysis during B. emersonii sporulation.** To determine gene expression changes throughout the sporulation phase of the *B. emersonii* life cycle, cDNA microarray experiments were performed using total RNA isolated from cells at different time points during this differentiation stage. A particular gene was classified as differentially expressed relative to time zero of sporulation if at least 70% of the replicates were outside the credibility intervals defined by self-experiments, as described in Materials and Methods.

Globally, about 32% of the 3,773 distinct *B. emersonii* genes spotted in the microarray chips were significantly upregulated or downregulated at at least one of the four time points analyzed: 615 genes were induced and 645 genes were repressed relative to time zero of sporulation at at least one of the time points analyzed. The numbers of differentially expressed genes at each of the four sporulation time points (60 min, 120 min, 150 min, and zoospores) were 48, 163, 210, and 392, respectively, for the induced genes, and 102, 327, 346, and 416, respectively, for the repressed genes. The increase in the number of genes either up- or downregulated during the sporulation phase (illustrated by the plots in Fig. S1 in the supplemental material) reflects not only the important transcriptional activities involved in the nutritional-starvation condition to which cells are subjected in order to enter the sporulation phase. However, in the latest stages of this differentiation process, *B. emersonii* cells increase the transcriptional activity of genes involved in protein biosynthesis, probably to store these mRNAs in polyribosomes in an organelle present in zoospores named the “nuclear cap,” as the earliest stages of germination are entirely preprogrammed using stored mRNAs and proteins (20, 30, 35, 36).

Many genes related to central carbon metabolism were also downregulated during *B. emersonii* sporulation, and an overview of the expression profile of these genes is depicted in Fig. 2. For instance, genes encoding glycolytic/glucogenic enzymes such as phosphoglucone isomerase (PGI), fructose-1,6-bisphosphate aldolase (FBA), glyceraldehyde 3-phosphate dehydrogenase (TDH), enolase (ENO), pyruvate dehydrogenase (PDA) and phosphoenolpyruvate carboxykinase (PCK); genes of the tricarboxylic acid/glyoxylate cycle enzymes such as citrate synthase (CIT), aconitate hydratase (ACO), isocitrate dehydrogenase (IDH), succinyl-coenzyme A (CoA) synthetase (SUC), succinate dehydrogenase (SDH), malate dehydrogenase (MDH), and malate synthase (MLS); genes of the electron transport system such as the NADH-ubiquinone oxidoreductase (NHD), succinate dehydrogenase (SDH-UQ), ubiquinol-cytochrome c reductase (OCR), cytochrome c oxidase (COX), and electron transfer flavoprotein genes; and genes related to ATP proton motive force such as the ATP synthase gene were all downregulated during the sporulation phase or were not found to be significantly altered (Fig. 2B). The decrease in transcript levels observed for genes of central carbon metabolism is probably the result of the nutritional-starvation conditions under which *B. emersonii* sporulation takes place.

**qRT-PCR validation of expression profiles.** To verify the level of reliability of the array-based data, we selected nine differentially regulated genes and analyzed their expression ratios by qRT-PCR. All time points tested revealed a coincidence in the direction of gene expression modulation determined with both methods, indicating overall consistency between microarray hybridization and qRT-PCR data. Figure 3 shows data comparing results from microarray and qRT-PCR experiments for the selected genes.

The great majority of genes differentially expressed during both sporulation and germination are inversely regulated. The expression profiles of 530 genes differentially expressed during
both germination (29) and sporulation (this work) of B. emersonii were compared through clustering analysis using the algorithm K-means. Data revealed that approximately 95% of the genes analyzed are inversely regulated between these two differentiation stages (see Fig. S2 in the supplemental material). This result indicates not only the existence of strong transcriptional control mechanisms during B. emersonii sporulation and germination but also the need of different molecular functions during these distinct developmental stages. The biological functions overrepresented among the genes induced during sporulation and repressed throughout germination were cytoskeleton biogenesis, vesicle trafficking, and chromosome organization. In the case of genes downregulated during sporulation and upregulated during germination, the overrepresented molecular functions were protein biosynthesis and energetic metabolism (see Fig. S2 in the supplemental material).

The observation that protein biosynthesis genes, including genes encoding ribosomal proteins, translational initiation and elongation factors, and aminoacyl-tRNA synthetases, are downregulated during sporulation and upregulated during germination indicates that the protein biosynthesis rate is higher in the germination stage. One of the main differences between both phases is nutrient availability in the culture media in which these developmental stages take place. Unlike germination, sporulation occurs in the absence of nutrients, and such a condition seems to be responsible for regulating the expression patterns of different groups of genes, such as those related to protein biosynthesis. As protein synthesis is an energetically expensive process, results shown here suggest that control of protein biosynthesis at the level of transcription during these two developmental stages can respond to differences in energy availability. In fact, the same analysis revealed that genes involved in energetic metabolism are preferentially upregulated during germination and downregulated during sporulation (see Fig. S2 in the supplemental material).

Genes involved in cytoskeleton activity and composition are also clearly inversely regulated between both differentiation phases. For instance, genes coding for actin, profilin, and intersectin and actin and clathrin binding proteins are strongly induced in sporulating cells and repressed in germinating cells. Indeed, intense vesicle formation and trafficking take place during sporulation to guarantee zoospore differentiation and release to the medium, explaining the greater requirement for such functions during sporulation than during germination.

Genes encoding proteins involved in chromosome organization, such as histones and histone acetyltransferase, are also inversely regulated between sporulation and germination. Levels of these gene transcripts increase during sporulation, reaching maximum amounts in the zoospores. These transcripts and...
the corresponding proteins will probably be needed early during zoospore germination, possibly allowing for the increment of the transcriptional rate observed during this developmental stage (40). Throughout germination, levels of these gene transcripts decrease drastically, probably to reach a steady-state level necessary during vegetative growth. It is worth mentioning that mRNA levels for the gene encoding a putative nucleoside-diphosphate kinase decrease during sporulation and increase strongly during the germination phase. As this enzyme is involved in the synthesis of nucleoside triphosphates, such an expression profile probably reflects the distinct transcriptional rates during these two \textit{B. emersonii} differentiation stages.

A large number of genes without putative identification were found to be not only differentially expressed throughout both the germination and sporulation of \textit{B. emersonii} but also inversely regulated between these two stages. These data suggest that most of these genes, whose roles are still unknown, could exert important functions in the \textit{B. emersonii} life cycle. The complete list of differentially expressed genes common to both \textit{B. emersonii} developmental stages is shown in Table S2 in the supplemental material.

**Effect of glucose during \textit{B. emersonii} sporulation.** In the presence of high glucose concentrations (≥2%), \textit{B. emersonii} cells induced to sporulate fail to progress to the empty-zoo-

sporangium stage even 320 min after induction (the half-life [$T_{50}$] of normal sporulation control for empty zoosporangia is 210 min) (3). In the presence of 2% glucose, significant delays in basal septum formation and especially in the appearance of papillated zoosporangia are observed (3). To investigate the effect of glucose on global gene expression during sporulation of \textit{B. emersonii}, cells were induced to sporulate in the presence of a subinhibitory concentration of glucose (1%) and microarray experiments were carried out. The functional category most affected by 1% glucose was microtubule and cytoskeleton composition. As depicted in Table 1, genes associated with this functional category, such as the myosin, dynein, actin, tubulin, and cofilin genes, were highly overexpressed around 120 to 150 min after sporulation induction in the presence of glucose, compared to normal sporulation conditions.

The effect of different glucose concentrations (1% and 2%) on the expression of genes involved in cytoskeleton composition and activity in late sporulation cells (150 min of induction) was evaluated using qRT-PCR in order to confirm data obtained from microarray experiments (Fig. 4). Genes encoding small GTPases were also analyzed due to their regulatory role in cellular processes such as cell differentiation and division, vesicle transport, nuclear assembly, and control of the cytoskeleton (22). All genes analyzed showed increased expression...
when cells were exposed to a high glucose concentration, suggesting that this carbohydrate induces the overexpression of cytoskeleton genes through an unknown mechanism in *B. emersonii*.

Large increases in the rate of synthesis of cytoskeleton and microtubule proteins have been associated with several abnormalities in the cell cycle in different biological systems. For instance, increased synthesis of beta-tubulin has been shown to inhibit vegetative growth in *Aspergillus nidulans* (42). Overexpression of a class V mouse beta-tubulin in mammalian cells was reported to produce a strong, dose-dependent disruption of microtubule organization, increased microtubule fragment-

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**TABLE 1. Gene expression profile comparison between standard sporulation and sporulation in the presence of 1% glucose**

| Clone ID | Annotation | Standard sporulation at: | Glucose sporulation at: |
|----------|------------|--------------------------|--------------------------|
|          |            | S0 | S60 | S120 | S150 | ZSP | S0 | S60 | S120 | S150 | ZSP |
| BeE60N09D06 | Vesicle-soluble NSF attachment protein | 1.00 | 1.13 | 2.73 | 1.50 | 1.44 | 1.00 | 1.08 | 2.69 | 4.20 | 1.30 |
| BeE90D04C07 | Cofilin | 1.00 | 1.77 | 2.27 | 1.61 | 0.99 | 1.00 | 4.50 | 4.72 | 2.64 | 1.00 |
| BeE60H28B01 | Actin | 1.00 | 0.93 | 2.71 | 1.64 | 0.28 | 1.00 | 1.13 | 3.03 | 6.54 | 0.20 |
| BeE120N35H01 | Vesicle-mediated transport-related protein | 1.00 | 2.45 | 4.26 | 2.14 | 2.20 | 1.00 | 3.16 | 17.27 | 6.87 | 1.98 |
| BeE120N07A12 | Roadblock-related dynein light chain | 1.00 | 1.31 | 2.46 | 1.62 | 0.39 | 1.00 | 1.19 | 3.66 | 2.89 | 0.53 |
| BeE60N07E05 | Myosin regulatory light chain 2 | 1.00 | 1.83 | 4.20 | 4.06 | 0.78 | 1.00 | 2.16 | 8.22 | 11.71 | 0.84 |
| BeE60N12E01 | Myosin regulatory light chain cde4 | 1.00 | 1.09 | 2.66 | 1.94 | 0.78 | 1.00 | 1.34 | 8.94 | 2.45 | 0.99 |
| BeE60H23E05 | Muscle-specific protein 20 (calponin) | 1.00 | 0.84 | 2.16 | 2.75 | 0.78 | 1.00 | 0.78 | 1.62 | 4.53 | 1.00 |
| BeE90N15E03 | Dynein light polypeptide 4 | 1.00 | 1.18 | 2.58 | 2.50 | 2.03 | 1.00 | 1.44 | 2.97 | 12.04 | 1.63 |
| BeE90N17C06 | Dynein 1 alpha heavy chain | 1.00 | 1.96 | 2.23 | 1.73 | 1.06 | 1.00 | 1.42 | 3.97 | 3.34 | 1.00 |
| BeE90H10E11 | Cyttoplasmic dynein light chain | 1.00 | 1.67 | 1.63 | 1.41 | 2.22 | 1.00 | 1.65 | 2.83 | 2.68 | 1.14 |
| BeE120N30D02 | Myh11 protein | 1.00 | 1.03 | 3.29 | 2.17 | 0.77 | 1.00 | 0.80 | 5.62 | 6.87 | 0.80 |
| BeE90D13B04 | Tubulin alpha-6 chain | 1.00 | 2.93 | 4.89 | 4.20 | 0.34 | 1.00 | 2.45 | 8.63 | 8.94 | 0.57 |
| BeE90D18C07 | Tubulin, alpha 2 | 1.00 | 3.20 | 3.63 | 4.11 | 0.36 | 1.00 | 2.33 | 7.41 | 11.88 | 0.36 |
| BeE30N08A11 | Alpha-tubulin | 1.00 | 2.77 | 4.14 | 2.99 | 0.38 | 1.00 | 2.11 | 7.26 | 10.34 | 0.48 |

* a The Gene Ontology functional category for each clone was MC (microtubule and cytoskeleton).
* b ID, identification.
* c S0, S60, S120, and S150 correspond to cells at 0, 60, 120, and 150 min of sporulation, respectively. ZSP, zoospores.
tation, and a concomitant reduction in cellular microtubule polymer levels. These changes were shown to disrupt mitotic spindle assembly and block cell proliferation (2). Thus, overexpression of several genes encoding different tubulin chains during \textit{B. emersonii} sporulation in the presence of 1\% glucose (Table 1) might contribute to the inhibition of zoospore biogenesis observed at high glucose concentrations.

Profilin and cofilin are small actin monomer-biding proteins that regulate the size, localization, and dynamics of the large pool of unpolymerized actin in cells. The most important physiological function of cofilin is to increase actin dynamics by depolymerizing the filaments from their pointed ends, while profilin promotes the assembly of actin monomers (27). Transcriptional levels of cofilin and profilin genes increase about 3- and 4-fold, respectively, in the presence of 1\% glucose, and this increase reaches about 8-fold at 2\% glucose for both genes (Fig. 4). In \textit{Saccharomyces cerevisiae}, it was reported that cells overexpressing the cofilin gene were unable to survive, indicating that expression of cofilin should be appropriately regulated for normal cell growth (13). Since the actin cytoskeleton is involved in various cellular processes such as cell motility and division and since cofilin is the unique actin-regulatory protein essential for cell viability in \textit{S. cerevisiae} (12, 25), \textit{Drosophila melanogaster} (11), and \textit{Caenorhabditis elegans} (24), it is possible that the transcriptional rise observed for cofilin and profilin genes during \textit{B. emersonii} sporulation in the presence of glucose could explain the block in sporulation that was shown to occur at higher concentrations of this carbohydrate (3).

Molecular motors such as the kinesin, dynein, and myosin isoforms are also involved in a wide range of cellular processes, many of which require the transport or movement of cargo along cytoskeletal tracks. Generally, small GTPases such as Rho, cdc42, Rab, Sar, and others play an essential role in those processes (22). Additionally, the processivity of such motor molecules can be increased by some activators, as observed for the dynein motor, whose processivity is increased by dynactin (15). As zoospore differentiation is undoubtedly a complex process that involves an intense traffic of vesicles and organelles, requiring motor proteins and cytoskeletal tracks among other molecules, it is possible that overproduction of such proteins could cause cytoskeletal disturbances, explaining the failure of correct zoospore biogenesis observed at high glucose concentrations (≥2\%), as reported by Correa and Lodi (3).

FIG. 4. Overexpression of genes involved in cytoskeleton composition and activity in \textit{B. emersonii} cells sporulating in the presence of glucose. Gene expression ratios were evaluated by qRT-PCR, and results are median values from two independent biological experiments.
the caspase family found in plants, fungi, and protozoans and are involved in apoptosis (37). Recent results from our laboratory concerning the effect of cadmium stress on *B. emersonii* gene expression revealed that many genes involved in the oxidative stress response are induced in both developmental phases (germination and sporulation) but that metacaspase genes were induced only during sporulation (10). The induction of genes that code for molecular chaperones, such as HSP10, HSP90, T-complex subunits, prefoldin, and different peptidyl-prolyl cis-trans isomerases also suggests the existence of stress conditions when tryptophan is added to the sporulation solution.

In brain cortices of rats, tryptophan seems to be involved in reactive oxygen species formation. In animals and patients with hypertryptophanemia and other neurodegenerative diseases in which tryptophan accumulation is observed, increases in lipid peroxidation have been detected, indicating the existence of oxidative stress, probably due to higher intracellular concentrations of some compounds originating from tryptophan oxidation through the kynurenine pathway, such as quinolinic acid, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid, all of which are able to generate ROS (6–9). The existence of such tryptophan metabolites in fungi has been supported by some findings, such as the purification of 3-hydroxykynurenine from cell extracts of *B. emersonii*, the sequencing of cDNAs encoding enzymes of the kynurenine pathway, such as kynurenine 3-monooxygenase, in *B. emersonii* expression libraries (http://blasto.iq.usp.br), and the finding of kynureninase and kynurenine 3-monooxygenase genes in the *Batrachochytrium dendrobatidis* genome (http://www.broad.mit.edu).

Altogether, data shown here suggest that the presence of tryptophan during sporulation of *B. emersonii* could generate ROS, and such a stress condition could explain the block of zoospore differentiation reported by Correa and Lodi (3) at high tryptophan concentrations (≥ 100 μM).

**Final remarks.** The sporulation phase of *B. emersonii* is marked by several important morphological alterations, which culminate in the formation of highly differentiated cells, the zoospores. The success of such structural transformations is guaranteed by accurate biochemical changes. In the present work, large-scale gene expression analyses were carried out during the sporulation phase in order to determine the changes in gene expression pattern during zoospore biogenesis. Data presented show that the complexity of the *B. emersonii* sporulation process is associated with the induction of several genes involved in distinct signaling pathways, including those involving in cyclic GMP (cGMP) synthesis and degradation, which do not seem to be commonly found in fungi (41). Intense cytoskeleton activity is also required, particularly during late sporulation events, as suggested by the upregulation of genes related to this functional category. On the other hand, genes belonging to functional categories such as protein biosynthesis and energetic metabolism are downregulated in the sporulation phase, probably reflecting the low energy availability at this stage of the life cycle.

Analysis of the genes differentially expressed during both sporulation and germination revealed that the vast majority of the genes found in this situation (in the midst of which one finds a large number of genes without putative identification) are inversely regulated in these two developmental stages (see Fig. S2 in the supplemental material). Such an expression pattern seems to reflect not only the morphological differences between sporulation and germination but also the existence of an accurate mechanism of gene expression control, which guarantees the correct series of events during these differentiation processes in *B. emersonii*. The effect of subinhibitory concentrations of glucose and tryptophan on gene expression during the sporulation phase was also analyzed, and data obtained suggest that these nutrients affect the sporulation process differently, even though both nutrients can block zoospore biogenesis when added in large amounts (3). The presence of 1% glucose in the sporulation solution led to the overexpression of genes involved in cytoskeleton composition and function. As the cytoskeleton apparatus is involved in several cellular processes such as differentiation, cell division, and organelle trafficking, appropriate expression levels of this class of genes seem to be required to guarantee the correct synchrony of these events. On the other hand, treatment with tryptophan (50 μM) during sporulation of *B. emersonii* appears to cause oxidative stress, as suggested by the genes found to be induced under this condition. It is possible that such a stress condition is responsible for the impairment of the sporulation process observed in the presence of higher concentrations of this amino acid.

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**REFERENCES**

1. Aizawa, H., K. Sutoh, S. Tsubuki, S. Kawashima, A. Ishii, and I. Yahara. 1995. Identification, characterization, and intracellular distribution of cofillin in Dictostelium discoideum. J. Biol. Chem. 270:10923–10932.

2. Bhattacharya, R., and F. Cabral. 2004. A ubiquitous beta-tubulin disrupts microtubule assembly and inhibits cell proliferation. Mol. Biol. Cell 15:3123–3131.

3. Correa, L. C., and W. R. Lodi. 1986. Induction od sporulation in Blastocladiella emersonii: influence of nutritional variables. Exp. Mycol. 10:270–280.

4. Correa, L. C., and W. R. Lodi. 1999. The induction of sporulation in the aquatic fungus blastocladiella emersonii is dependent on extracellular calcium. FEMS Microbiol. Lett. 179:353–359.

5. da Silva, A. M., J. C. da Costa Maia, and M. H. Juliani. 1986. Developmental changes in translatable RNA species and protein synthesis during sporulation in the aquatic fungus Blastocladiella emersonii. Cell Differ. 15:263–274.

6. Feksa, L. R., A. Latini, V. C. Rech, P. B. Feksa, G. D. Koch, M. F. Amaral, G. Leipnitz, C. S. Dutra-Filho, M. Wajner, and C. M. Wannmacher. 2008. Tryptophan administration induces oxidative stress in brain cortex of rats. Metab. Brain Dis. 23:221–233.

7. Feksa, L. R., A. Latini, V. C. Rech, M. Wajner, C. S. Dutra-Filho, A. T. de Souza Wyse, and C. M. Wannmacher. 2006. Promotion of oxidative stress by L-tryptophan in cerebral cortex of rats. Neurochem. Int. 49:87–93.

8. Forrest, C. M., G. M. Mackay, L. Oxford, N. Stoy, T. W. Stone, and L. G. Darlington. 2006. Kynurenine pathway metabolism in patients with oseoporosis after 2 years of drug treatment. Clin. Exp. Pharmacol. Physiol. 33:1078–1087.

9. Forrest, C. M., G. M. Mackay, N. Stoy, M. Egerton, J. Christofides, T. W. Stone, and L. G. Darlington. 2004. Tryptophan loading induces oxidative stress. Free Radic. Res. 38:1167–1171.

10. Georg, R. C., and S. L. Gomes. 2007. Transcriptome analysis in response to heat shock and cadmium in the aquatic fungus Blastocladiella emersonii. Eukaryot. Cell 6:1053–1062.

11. Gonzalvez, K. C., S. Bonaccorsi, E. Williams, F. Verni, M. Gatti, and M. L. Goldberg. 1995. Mutations in twistar, a Drosophila gene encoding a coflin/ADF homologue, result in defects in centrosome migration and cytokinesis. J. Cell Biol. 131:1243–1259.
26. Murphy, M. N., and J. S. Lovett. 1966. DNA and protein synthesis during zoospore differentiation in synchronized cultures of Blastocladiella emersonii. Dev. Biol. 46:58–95.

27. Paavilainen, V. O., E. Bertling, S. Falck, and P. Lappalainen. 2004. Regulation of cytoskeletal dynamics by actin-monomer-binding proteins. Trends Cell Biol. 14:386–394.

28. Peralta, R. M., and W. R. Lodi. 1988. An analysis of developmental timing in Blastocladiella emersonii sporulation. Dev. Biol. 128:78–85.

29. Salem-Izacc, S. M., T. Koide, R. Z. Vencio, and S. L. Gomes. 2009. Global gene expression analysis during germination in the chytridiomycete Blastocladiella emersonii. Eukaryot. Cell 8:170–180.

30. Silva, A. M., J. C. Maia, and M. H. Juliani. 1987. Changes in the pattern of protein synthesis during zoospore germination in Blastocladiella emersonii. J. Bacteriol. 169:2069–2078.

31. Silverman, P. M., M. M. Huh, and L. Sun. 1974. Protein synthesis during zoospore germination in the aquatic phycomycete Blastocladiella emersonii. Dev. Biol. 40:59–70.

32. Silverstein, R. M. 1975. The determination of the molar extinction coefficient of reduced DTNB. Anal. Biochem. 63:281–282.

33. Simao, R. C., and S. L. Gomes. 2001. Structure, expression, and functional analysis of the gene coding for calmodulin in the chytridiomycete Blastocladiella emersonii. J. Bacteriol. 183:2280–2288.

34. Soll, D. R., and D. R. Sonneborn. 1969. Zoospore germination in the water mold Blastocladiella emersonii. II. Influence of cellular and environmental variables on germination. Dev. Biol. 20:218–235.

35. Soll, D. R., and D. R. Sonneborn. 1971. Zoospore germination in Blastocladiella emersonii. 3. Structural changes in relation to protein and RNA synthesis. J. Cell Sci. 9:679–699.

36. Soll, D. R., and D. R. Sonneborn. 2004. DNA microarray-based analysis of microarray data by principal component discriminant analysis: prioritizing relevant transcripts linked to the degradation of different carbohydrates in Pseudomonas putida S12. Microbiology 150:257–272.

37. Vencio, R. Z., and T. Koide. 2005. HTself: self-self based statistical test for low replication microarray studies. DNA Res. 12:211–214.

38. Vencio, R. Z., T. Koide, S. L. Gomes, and C. A. Pereira. 2006. BayGO: Bayesian analysis of ontology term enrichment in microarray data. BMC Bioinformatics 7:86.

39. Vieira, A. L., E. Linares, O. Augusto, and S. L. Gomes. 2009. Evidence of a Ca2+·NO-cGMP signaling pathway controlling zoospore biogenesis in the aquatic fungus Blastocladiella emersonii. Fungal Genet Biol. 46:575–584.

40. Waring, R. B., G. S. May, and N. R. Morris. 1989. Characterization of an inducible expression system in Aspergillus nidulans using aCa and tubulin-coding genes. Gene 79:119–130.