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## Proteomic Analysis of the Secretions of *Pseudallescheria boydii*, a Human Fungal Pathogen with Unknown Genome

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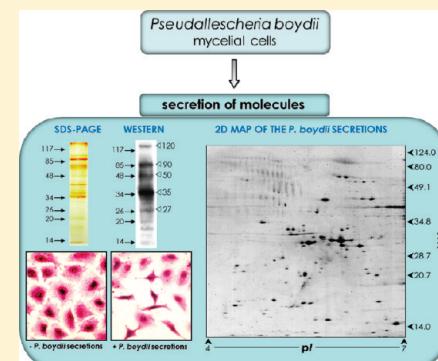
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**ABSTRACT:** *Pseudallescheria boydii* is a filamentous fungus that causes a wide array of infections that can affect practically all the organs of the human body. The treatment of pseudallescheriosis is difficult since *P. boydii* exhibits intrinsic resistance to the majority of antifungal drugs used in the clinic and the virulence attributes expressed by this fungus are unknown. The study of the secretion of molecules is an important approach for understanding the pathogenicity of fungi. With this task in mind, we have shown that mycelial cells of *P. boydii* were able to actively secrete proteins into the extracellular environment; some of them were recognized by antibodies present in the serum of a patient with pseudallescheriosis. Additionally, molecules secreted by *P. boydii* induced *in vitro* irreversible damage in pulmonary epithelial cells. Subsequently, two-dimensional gel electrophoresis combined with mass spectrometry was carried out in order to start the construction of a map of secreted proteins from *P. boydii* mycelial cells. The two-dimensional map showed that most of the proteins (around 100 spots) were focused at pH ranging from 4 to 7 with molecular masses ranging from 14 to >117 kDa. Fifty spots were randomly selected, of which 30 (60%) were consistently identified, while 20 (40%) spots generated peptides that showed no resemblance to any known protein from other fungi and/or MS with low quality. Notably, we identified proteins involved in metabolic pathways (energy/carbohydrate, nucleotide, and fatty acid), cell wall remodeling, RNA processing, signaling, protein degradation/nutrition, translation machinery, drug elimination and/or detoxification, protection against environmental stress, cytoskeleton/movement proteins, and immunogenic molecules. Since the genome of this fungus is not sequenced, we performed enzymatic and immunodetection assays in order to corroborate the presence of some released proteins. The identification of proteins actively secreted by *P. boydii* provides important new information for understanding immune modulation and provides important new perspectives on the biology of this intriguing fungus.



**KEYWORDS:** *Pseudallescheria boydii*, secreted proteins, secretome, virulence attributes

### 1. INTRODUCTION

*Pseudallescheria boydii* is a ubiquitous filamentous fungus present in soil, sewage, and polluted waters.<sup>1</sup> Pseudallescheriosis/scedosporiosis represents a broad spectrum of clinical diseases caused by etiologic agents belonging to the *Pseudallescheria/Scedosporium* complex, affecting both immunocompetent and immunocompromised individuals.<sup>1</sup> These infections present a large spectrum of manifestations varying from localized mycetomas, sinusitis, and pulmonary infections to disseminated infections, especially in

immunodeficient patients. *P. boydii* is a therapy refractory pathogen, since it is resistant or poorly susceptible to almost all current antifungal agents.<sup>1</sup> As a consequence of the treatment failure, a high mortality rate is associated with the *Pseudallescheria/Scedosporium* infections.<sup>2</sup>

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Despite the growing importance of *P. boydii* infections in the last decades, very little is known about the physiology, biochemistry, and genetics of this human fungal pathogen.<sup>3</sup> Further hindering the evolution of knowledge about the cell biology of this fungus is the absence of the sequencing of its genome. Therefore, proteomic approaches should help in elucidating some of the fungal cell life processes, revealing the presence of proteins associated with events such as nutrition, proliferation, growth, differentiation, and interaction with host structures. Secretome-related studies are particularly relevant in understanding the physiology of filamentous fungi because many of them secrete a vast number of proteins to accommodate their saprophytic lifestyle. Many of these proteins are of special interest in the study of pathogens.<sup>4</sup> For instance, pathogenic filamentous fungi could use extracellular enzymes to degrade the structural barriers of the host to facilitate this tissue invasion, and they could also participate in the infection by eliminating some mechanisms of the immune defense and/or helping in the obtaining of nutrients.<sup>3</sup>

Experimental evidence about the expression of secreted molecules by fungi belonging to the *Pseudallescheria/Scodosporium* complex was first reported by Larcher and co-workers, who had purified an extracellular serine protease able to degrade human fibrinogen, suggesting an action as a mediator of the severe chronic bronchopulmonary inflammation from which cystic fibrosis patients suffer.<sup>5</sup> Our group described the secretion of two acidic metallo-type proteases of 28 and 35 kDa capable of hydrolyzing various substrates, including serum human proteins (e.g., albumin, hemoglobin, and immunoglobulin G), extracellular matrix components (e.g., laminin and fibronectin), and sialylated proteins (e.g., fetuin and mucin). Those results demonstrated that extracellular metalloproteases produced by *P. boydii* could help the fungus to escape from natural human barriers and defenses.<sup>6</sup> Bertrand and co-workers reported the secretion of two siderophores of hydroxamates class, dimerumic acid and N<sup>α</sup>-methyl coprogen B, by *S. apiospermum*, and comparison of various strains suggested a higher production of N<sup>α</sup>-methyl coprogen B by clinical isolates of respiratory origin.<sup>7,8</sup> Furthermore, different species of *Pseudallescheria* are able to release antibiotic substances, including the following: (i) tyroscherin, an antitumor antibiotic that selectively inhibited the growth of MCF-7 human breast cancer cells;<sup>9</sup> (ii) YM-193221 (2-dimethylamino-1-(4-hydroxyphenyl)-8,10-dimethyl-6-dodecene-3-one), which exhibited potent antifungal activity against *Candida albicans*;<sup>10</sup> (iii) a new antibacterial dioxopiperazine (dehydroxybisdethiobis(methylthio)gliotoxin) and the previously described bisdethiobis(methylthio)gliotoxin and gliotoxin, which exhibited potent antibacterial activity against the methicillin-resistant and multidrug-resistant *Staphylococcus aureus*;<sup>11</sup> and (iv) a fungistatic substance capable of reducing the disease incidence of black leaf spot of spoon cabbage caused by *Alternaria brassicicola* and inhibiting the germination of *A. brassicicola* conidia.<sup>12</sup> *P. boydii* spores also produce cyclic nonribosomal peptides with a unique structure, named pseudacyclins, which can be used as early indicators of fungal infection caused by *Pseudallescheria* species.<sup>13</sup> Therefore, the identification of proteins actively secreted by *P. boydii* provides important new information for understanding immune modulation and provides important new perspectives on the biology of this fungal pathogen.

In the present work, we have initially reported that mycelial cells of *P. boydii* were able to release proteins to the extracellular

environment, some of them recognized by antibodies presented in the serum obtained from a patient with pseudallescheriosis, showing the antigenic nature of these extracellular proteins. Also, molecules secreted by *P. boydii* induced injury in pulmonary epithelial cells. Two-dimensional gel electrophoresis combined with mass spectrometry was carried out in order to start the construction of a map of secreted proteins from *P. boydii* mycelia. Proteomics analysis revealed a variety of proteins associated with fungal survival and host pathogenicity.

## 2. MATERIALS AND METHODS

### Microorganism and Growth Condition

*Pseudallescheria boydii* (RKI07\_0416) belongs to the clade 4 to the *Pseudallescheria boydii/Scodosporium apiospermum* complex, as previously proposed by Gilgado and co-workers.<sup>14</sup> The fungus was grown on Sabouraud-dextrose broth (containing 2% glucose, 1% peptone, and 0.5% yeast extract) at 25 °C for 7 days with constant shaking (200 rpm). Then, mycelial cells were filtered with filter paper and washed twice with sterile phosphate-buffered saline (PBS; 150 mM NaCl, 20 mM phosphate buffer, pH 7.2).<sup>6</sup>

### Secretion Assay

The *P. boydii* mycelia (approximately 10 g) were resuspended in 10 mL of sterile isotonic PBS supplemented with 2% glucose and incubated for 20 h at 25 °C with constant agitation (200 rpm).<sup>6</sup> After this interval, the cells were removed by centrifugation (2500g/20 min/4 °C) and the supernatant was passed over a 0.22-μm membrane (Millipore, São Paulo, Brazil). The cell-free PBS–glucose supernatant was concentrated in a 10,000 molecular weight cutoff AMICON micropartition system (AMICON, Beverly, MA, USA), obtaining a final concentration of 100-fold, and kept at –20 °C until use. Proteins in these extracts were then quantified by the method of Lowry and co-workers,<sup>15</sup> using bovine serum albumin (BSA) as the protein standard.

### Fungal Viability

The viability of the mycelia during the incubation period in the isotonic phosphate buffer was assessed by fluorescence microscopy observation. Mycelial cells were incubated for 30 min at room temperature with calcofluor white (Sigma). Subsequently, the systems were washed with PBS and incubated with propidium iodide at 10 μg/mL (Sigma) for 10 min. After, the systems were washed with PBS and taken immediately for viewing on a Zeiss epifluorescence microscope (Axioplan). Cells killed by autoclaving (121 °C/30 min) were used as positive controls for staining with propidium iodide.

### Transmission Electron Microscopy (TEM)

After secretion experiments, mycelial cells were washed in PBS and then fixed with 2.5% glutaraldehyde and 4% paraformaldehyde with 10 mM calcium chloride added in 100 mM cacodylate buffer, pH 7.4, for 1 h at 25 °C, washed in cacodylate buffer, and then postfixed with 1% OsO<sub>4</sub> and 0.8% K<sub>4</sub>Fe(CN)<sub>6</sub>·3H<sub>2</sub>O in 100 mM cacodylate buffer for 2 h. Samples were then washed in cacodylate buffer, dehydrated in graded series of acetone, and embedded in Spurr resin. Ultrathin sections were routinely stained with aqueous uranyl acetate and alkaline lead citrate and examined in a TEM type JEOL 1200 EX, operating at 80 kV.

### Secretory Protein Profile

PBS-glucose supernatant of *P. boydii* mycelial form (equivalent to 5 µg of protein) was treated with an equal volume of sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (125 mM Tris, pH 6.8; 4% SDS; 20% glycerol; 0.002% bromophenol blue) supplemented with 10% β-mercaptoethanol, followed by heating at 100 °C for 5 min. Polypeptides were analyzed on 10% SDS-PAGE by the method described by Laemmli.<sup>16</sup> Electrophoresis was carried out at 100 V for 90 min at 4 °C, and the gel was silver stained. Prior to electrophoresis, Gibco BRL (Grand Island, NY, USA) molecular mass standards were boiled in SDS-PAGE sample buffer and then applied to the same gel.

### Detection of Antigenic Proteins Secreted by Mycelial Cells

Extracellular protein extracts from *P. boydii* mycelial cells were separated by SDS-PAGE and silver-stained or, alternatively, transferred at 4 °C at 100 V/300 mA for 2 h to a nitrocellulose membrane. Blotted proteins were blocked in PBS containing 0.1% Tween 20 (PBS-T) and 5% low fat dried milk. After blocking, the membrane was incubated for 1 h at 37 °C in the presence of a serum from a human patient with pseudallescheriosis (kindly provided by Dr. Bodo Wanke, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil) or with a healthy serum both at 1:1000 dilution and washed three times in PBS-T, followed by incubation for 1 h at room temperature with a peroxidase-labeled anti-human antibody at 1:2500 dilution. Immunoblot was exposed to an X-ray film after chemiluminescence reaction using the ECL system (Pierce). Alternatively, membranes containing the secreted proteins of *P. boydii* were individually incubated for 1 h at 37 °C with the anti-actin (Sigma) and anti-tubulin monoclonal antibody (Sigma) and then revealed as described above.

### Cytotoxicity Assay

In order to determine the cytotoxicity of the molecules released by *P. boydii* mycelial cells, different supernatant concentrations (ranging from 0.1 to 5 µg/µL of protein) were placed in contact with monolayers of A549 cells (that is an established lineage from human Caucasian lung carcinoma) in RPMI medium and then incubated at 37 °C in a 5% CO<sub>2</sub> atmosphere for 24 h. In the control system, the cell lineages were incubated in RPMI medium supplemented only with PBS. After that, cellular viability was evaluated by the neutral red dye-uptake method.<sup>17</sup> Briefly, mammalian cells were incubated in the presence of 0.01% neutral red solution for 3 h at 37 °C in a 5% CO<sub>2</sub> atmosphere. Then, the medium was removed and the cells were fixed with 4% formalin in PBS. The dye incorporated by the viable cells was eluted using a mixture of methanol/acetic acid/water (50:1:49), and the dye uptake was determined by measuring the optical density of the eluate at 490 nm in an automatic spectrophotometer (ELx800TM, Bio-TeK Instruments, Inc.). The 50% cytotoxic concentration (CC<sub>50</sub>) was defined as the compound concentration which caused a 50% reduction in the number of viable cells.

### Two Dimensional Gel Electrophoresis

Proteins from the supernatant containing a protease inhibitor cocktail were precipitated by 17% trichloroacetic acid (TCA) and then solubilized in rehydration solution (2% CHAPS, 20 mM dithiothreitol (DTT), and 8 M urea) and stored at -70 °C. Protein concentration was determined by the RC DC method (Bio-Rad, Hercules, CA, USA), using BSA as standard protein. This assay is based on the Lowry assay but has been modified to

be reducing agent compatible (RC) as well as detergent compatible (DC).<sup>15</sup> For the first dimension, aliquots of 700 µg of protein were applied to 17 cm immobilized pH gradient (IPG) gel strips (Bio-Rad, Hercules, CA, USA) with a linear separation pH range by in-gel sample rehydration. After 11 h of active rehydration at 50 V at 20 °C using a Protean isoelectric focusing system (IEF) (Bio-Rad, Hercules, CA, USA), proteins were separated using the following protocol: 250 V, 20 min; 10,000 V, 2 h 30 min; 10,000 V, 90,000 VH. Before the second dimension, proteins were reduced (10 mg/mL DTT) and alkylated (25 mg/mL iodoacetamide) in equilibration buffer (6 M urea, 2% SDS, 300 mM Tris-HCl, pH 8.8, and 20% glycerol) and then separated on 12% SDS-PAGE gels and stained with Coomassie Brilliant Blue G-250. Gel images were captured with a densitometer (GS-800, Bio-Rad, Hercules, CA, USA), digitalized, and analyzed by PDQuestTM software (Bio-Rad, Hercules, CA, USA).

### Protein Digestion and Sample Preparation

Following 2D SDS-PAGE, several spots were manually excised from the gel in order to perform trypsin digestion as described previously.<sup>18</sup> The selected spots were destained with 100 µL of 25 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 8.0, in 50% acetonitrile for 15 min. After three washings, gel pieces were dehydrated with 100% acetonitrile and then dried using a Speed Vac evaporator (Savant, Farmingdale, NY, USA). Proteolytic in-gel digestion was performed using 10 µL of ice-cold sequence grade modified trypsin (Promega, Madison, WI, USA) solution (20 ng/µL in 25 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 8.0) at 37 °C, overnight, followed by peptide extraction from the gel with 50% acetonitrile in 5% trifluoroacetic acid (TFA) in a sonicator for 30 min. The extracts were dried under vacuum and then solubilized in 50% acetonitrile in 0.1% TFA. Contaminants from the peptide mixture were removed using C18 ZipTip micropipet tips (Millipore, Bedford, MA). Before loading the samples, the C18 media was first activated with 10 µL of wetting solution (100% acetonitrile), followed by equilibration in 10 µL of 0.1% TFA. The samples were loaded onto the ZipTip by 8 to 10 repeated aspiration and dispensing cycles, followed by a washing step with 0.1% TFA. The bound peptides were eluted by carefully aspirating and dispensing 8–10 times 5 µL of 70% acetonitrile in 0.1% TFA. Afterward, approximately 0.3 µL of the peptide samples were cocrystallized with an equal volume of matrix solution (10 mg/mL α-cyano-4-hydroxycinnamic acid solution (Sigma) in 0.1% TFA in 50% acetonitrile) directly onto a MALDI target plate.

In a few samples, 4-sulfophenyl-isothiocyanate (SPITC) derivatization was carried out according to Wang and co-workers, in order to improve the *de novo* sequencing of the peptides.<sup>19</sup> Approximately 5–10 µL of the peptide mixture from the in-gel digestion was dried down in a vacuum centrifuge and concentrated to 1 µL. This peptide solution was derivatized by mixing with 10 µL of SPITC (10 mg/mL in 20 mM NaHCO<sub>3</sub>, pH 9.0) and incubating for 30 min at 55 °C. The reaction was terminated by adding 1 µL of 1% TFA, and the samples were loaded onto a C18 ZipTip micropipet tip to remove contaminants prior to analysis by mass spectrometry.

### MALDI-TOF/TOF Analysis

The tryptic peptides were analyzed on a 4700-Proteomics Analyzer MALDI-TOF/TOF (Applied Biosystems, Foster City, CA). All mass spectra were acquired in positive ion reflector mode with 2000 shots per spot and externally mass calibrated with a peptide mixture. The mass spectra were recorded in a mass

range from 900 to 3,000 or 3,500 Da. The 10 or 15 most intense ion peaks from the peptide mass fingerprinting (or MS run) were further submitted to fragmentation using PSD mode with CID gas off and 1 keV collision energy. Subsequent to MS acquisition, the peak list files ppw (Protein-Peptide by Well), pps (Protein-Peptide by Spot Set), and pmf (Peptide Mass Fingerprinting) were generated from the raw mass spectrum data using the “peak to mascot” script of the 4000 Series Explorer Software according to the settings: for pmf, mass range from 500 to 4000 Da, peak density of 15 per 200 Da, signal-to-noise of 10, minimal area value of 100, and maximal 60 peaks per spot. For pps and ppw files, mass range from 60 to precursor –20 Da, peak density of 55 peaks per 200 Da, signal-to-noise of 2, minimal area value of 10, and maximal 200 peaks per precursor.

### Bioinformatics Analysis and Identification of Proteins

Following MS acquisition, each mass spectrum was submitted to a search using Mascot version 2.2 online (Matrix Science, [http://www.matrixscience.com/cgi/search\\_form.pl?FORMVER=2&SEARCH=MIS](http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=MIS)). For the identification of proteins, the search was performed against the NCBI nonredundant database (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/>) with no taxonomy restriction. When necessary, further searches were performed against the Fungi database. The parameters used were trypsin as the enzyme of choice and two missed cleavage,  $\pm 1$  Da for the precursor mass, and  $\pm 0.5$  Da for the fragment ion mass. Oxidation of methionine and tryptophan along with N-terminal acetylation of proteins, cyclization of glutamine and glutamate (pyro-glutamate), and alkylation of cysteine (carbamidomethylcysteine) were allowed as variable modifications. In order to validate a protein identification, the Mascot ion score was considered greater than 40 and the significance threshold  $P < 0.05$ . If a protein hit was identified by only one peptide, the MS/MS data should exhibit a clear spectrum with sequence tags that matched at least 3 consecutive y, b, or even a fragment ion series. The MS/MS spectra that did not automatically match a peptide sequence were manually interpreted and blast searched against the NCBI protein or nucleotide Fungi database (taxid 4751). The proteins identified with a blast E value lower than  $1e^{-1}$  were considered as positive matches. One should bear in mind that the *P. boydii* genome is not sequenced and identification hits were especially based on orthologous proteins found in other fungus species whose genome has already been sequenced.

### Measurement of Enzymatic Activity

**Malate Dehydrogenase.** Assays were conducted as described by Steffan and McAlister-Henn in a reaction medium containing 50 mM Tris-HCl (pH 7.4), 5 mM MgCl<sub>2</sub>, 2 mM sodium malate, and 2 mM NAD<sup>+</sup>.<sup>20</sup> Reactions were initiated by addition of PBS-conditioned supernatant to a final concentration of 20  $\mu$ g/mL, and the time course of NADH formation was evaluated spectrophotometrically at 340 nm. One unit of malate dehydrogenase was attributed to the formation of 1  $\mu$ mol of NADH per minute of reaction.

**Triosephosphate Isomerase.** Triosephosphate isomerase activity was evaluated from conversion of glyceraldehyde-3-phosphate into dihydroxyacetone phosphate followed by reduction of this product into  $\alpha$ -glycerophosphate catalyzed by  $\alpha$ -glycerophosphate dehydrogenase. Assays were conducted as described previously by Sola-Penna and co-workers in a reaction medium containing 50 mM Tris-HCl (pH 7.4), 5 mM MgCl<sub>2</sub>, 2 mM glyceraldehyde-3-phosphate, 0.2 mM NADH, and 3 U/mL

$\alpha$ -glycerophosphate dehydrogenase.<sup>21</sup> Reaction was initiated by addition of PBS-conditioned supernatant to a final concentration of 20  $\mu$ g/mL. Oxidation of NADH was evaluated spectrophotometrically at 340 nm, and one unit of triosephosphate isomerase was attributed to the oxidation of 1  $\mu$ mol of NADH per minute of reaction.

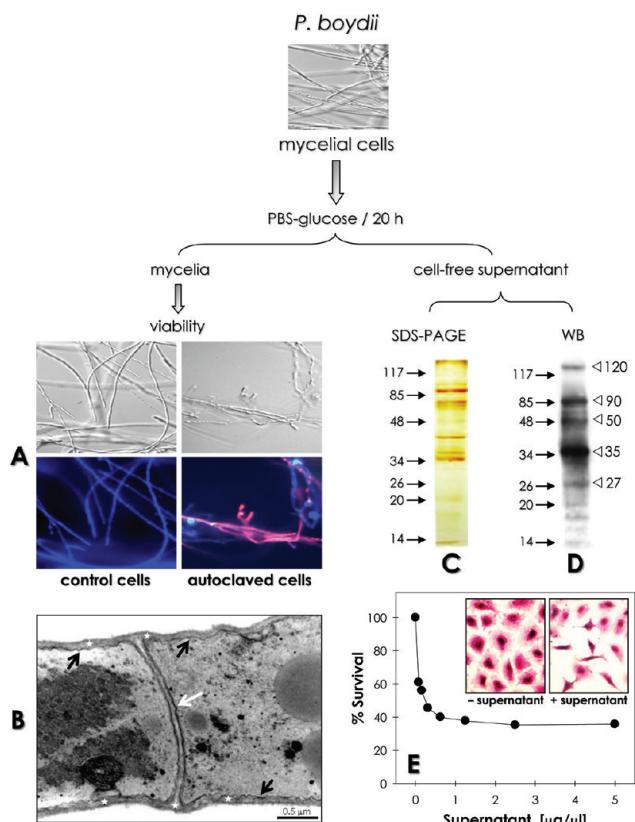
**Aldolase.** Aldolase activity was evaluated as described previously by Meira and co-workers.<sup>22</sup> Reaction medium contained 50 mM Tris-HCl (pH 7.4), 5 mM MgCl<sub>2</sub>, 1 mM fructose-1,6-bisphosphate, 0.2 mM NADH, 3 U/mL triosephosphate isomerase, and 3 U/mL  $\beta$ -glycerophosphate dehydrogenase. Reaction was initiated by addition of PBS-conditioned supernatant to a final concentration of 20  $\mu$ g/mL. Oxidation of NADH was evaluated spectrophotometrically at 340 nm, and one unit of aldolase was attributed to the oxidation of 1  $\mu$ mol NADH per minute of reaction.

**Mannitol-1-phosphate-5-dehydrogenase.** Assays were conducted as described by Iwamoto and co-workers in a reaction medium containing 50 mM Tris-HCl (pH 7.4), 5 mM MgCl<sub>2</sub>, 1 mM fructose-6-phosphate, and 0.2 mM NADH. Reaction was initiated by addition of PBS-conditioned supernatant to a final concentration of 20  $\mu$ g/mL, and the time course of NADH oxidation was evaluated spectrophotometrically at 340 nm.<sup>23</sup> One unit of mannitol-1-phosphate-5-dehydrogenase was attributed to the oxidation of 1  $\mu$ mol of NADH per minute of reaction.

**Aspartyl Proteinase.** The enzymatic activity over the cathepsin D substrate (7-methoxycoumarin-4-acetyl-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(DNP)-D-Arg-amide) was determined in the absence or in the presence of 1  $\mu$ M pepstatin A, a powerful aspartic protease inhibitor. Cleavage of cathepsin D substrate was monitored continuously in a spectrofluorometer (SpectraMax Gemini XPS, Molecular Devices, CA, USA) using an excitation wavelength of 340 nm and an emission wavelength of 490 nm. A 500  $\mu$ M stock solution of the fluorogenic substrate sample was prepared in dimethylsulfoxide (DMSO). The reaction was started by the addition of the substrate at 2  $\mu$ M to the PBS-conditioned supernatant (10  $\mu$ g protein) in a total volume of 60  $\mu$ L of 100 mM sodium acetate, pH 4.0. The reaction mixture was incubated at 37 °C for 20 min. The assays were controlled for self-liberation of the fluorophore over the same time interval.

**Phosphatase.** The phosphatase activity was determined using a microtiter assay plate by measuring the rate of *p*-nitrophenol (*p*-NP) production from the hydrolysis of *p*-nitrophenyl phosphate (*p*-NPP). PBS-conditioned supernatant (equivalent to 10  $\mu$ g/mL of protein) was mixed with 20 mM Tris-HCl buffer at pH 7.0 and 5 mM *p*-NPP as substrate. The preparation was incubated for 60 min at 26 °C, in the absence or presence of 15 mM sodium tartrate, a secreted phosphatase inhibitor. Reactions were terminated by adding 50  $\mu$ L of 2 M NaOH to each well, and the mixture (100  $\mu$ L) obtained was measured with an ELISA microplate reader (415 nm), using *p*-NP as standard.<sup>24</sup> The phosphatase activity was calculated by subtracting the nonspecific *p*-NPP hydrolysis measured in the absence of PBS-conditioned supernatant and expressed per milligram of protein.

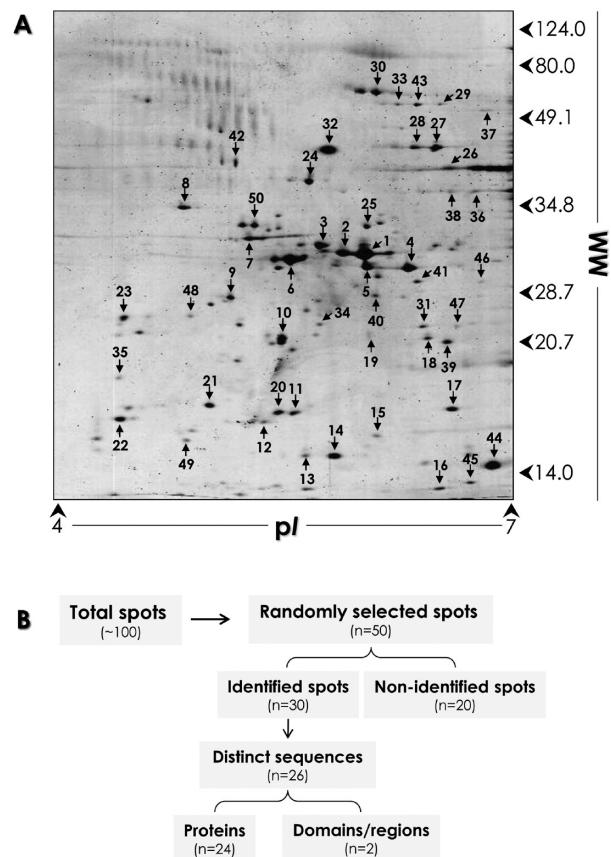
**Superoxide Dismutase.** Superoxide dismutase activity was determined using a method that involves generation of superoxide and reduction of the tetrazolium dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to its formazan, which is measured at 570 nm, as described by Madesh and Balasubramanian.<sup>25</sup>



**Figure 1.** Secretion of proteins by *Pseudallescheria boydii*. After growth of mycelial cells for 7 days in Sabouraud medium, the culture was harvested by centrifugation and mycelia were washed in PBS and incubated for 20 h in PBS-glucose. Then, fungal cells were separated from the supernatant by centrifugation. (A) The fungal viability was assessed by incubation of mycelial cells sequentially with calcofluor white (blue staining) and propidium iodide (red staining) and then observed by both optical (upper panels) and fluorescence (lower panels) microscopy. In this set of experiments, mycelia submitted to autoclavage were used as nonviable cells. (B) Transmission electron microscopy showing the preserved ultrastructural architecture of mycelial cells after incubation for 20 h in PBS-glucose. The white asterisks show the cell wall, black arrows indicate the cytoplasmic membrane, and the white arrow evidences the septum. (C) SDS-PAGE revealing the protein profile of the concentrated PBS-conditioned supernatant. The gel was silver-stained. (D) Secreted proteins were separated by SDS-PAGE, blotted to a nitrocellulose membrane, and then incubated with the serum of a pseudallescheriosis patient. The numbers on the right indicate the major reactive bands. In parts C and D, the numbers on the left indicate the relative molecular mass markers expressed in kilodaltons. (E) Different concentrations of secreted proteins were added to a confluent monolayer of A549 epithelial cells, and the viability was evaluated by the neutral red dye-uptake method. The inset represents the monolayer of A549 after incubation in the absence (− supernatant) or in the presence (+ supernatant) of PBS-conditioned supernatant at the CC<sub>50</sub> value.

### 3. RESULTS

The survivability of the mycelia along the incubation period (20 h) in PBS-glucose medium was assessed by light and fluorescence microscopy observations after double labeling of cells with calcofluor white (a nonspecific fluorochrome stain that binds to chitin in the cell wall of fungi) and propidium iodide (a DNA intercalate agent that is excluded by viable cells but can penetrate cell membranes of dying or dead cells), in order to ensure noncontamination of the secretome-containing medium



**Figure 2.** Secretome map of *Pseudallescheria boydii*. (A) An aliquot of 700 µg of proteins was applied to 17 cm immobilized pH range 4–7 IPG strips. The 2-DE gel was stained with Coomassie Brilliant Blue G-250. Protein spots indicated by numbers were randomly selected for subsequent identification by mass spectrometry (Table 1). Molecular mass markers (MM) expressed in kilodalton. (B) Rational sequence used for the analysis of the spots obtained from the secretome map of *P. boydii* mycelial cells.

by components released by dying or damaged fungal cells. By means of optical microscopic inspection, mycelial cells maintained their morphological architecture with a preserved cell wall, as evidenced by calcofluor white staining, and no propidium iodide staining was observed (Figure 1A). Conversely, mycelia killed by heat (autoclaving) showed intense incorporation of propidium iodide (Figure 1A). In parallel, TEM images evidenced the normal ultrastructural architecture of mycelia after secreting in PBS-glucose, presenting typical electron-dense cytoplasm, a distinct cell wall, a well-delineated cytoplasmic membrane, and an intact septum (Figure 1B). Taken together, these results discharged the possibility of cell membrane and/or cell wall injuries, testifying that extracellular proteins were not artifacts and were in fact present due to *bona fide* secretion of living mycelial cells.

The proteins secreted by *P. boydii* mycelia were concentrated and then separated by 1D SDS-PAGE, revealing the presence of at least 20 polypeptides with molecular masses ranging from 14 to >117 kDa (Figure 1C). In order to assess whether the secretions of mycelial cells have a biological effect on the host, we tested the immunoreactivity of these proteins with a serum obtained from a patient with pseudallescheriosis. The recognition of diverse proteins by hyperimmune patient serum indicates that these secreted proteins could be important in the pathogenesis of

Table 1. Identification of Selected Spots from *Pseudallescheria boydii* Secretions<sup>f</sup>

Spot num	Access number	Protein name	Orthologous to	Peptide sequence (Mr)	Theoretical pI	Mr (Da)	Score <sup>d</sup> / E value <sup>e</sup>
1	EGU87110.1	hypothetical protein FOXB_02370	<i>Fusarium oxysporum</i> Fo5176	LPPGTDHVHYVLQTR (1733.01 Da) <sup>a</sup>	6.20	21599.39	n.d./9e-3
1	XP_002557051.1	Pc12g01560 (contains a Forkhead associated domain (FHA), a kinesin domain (KISc) and a pleckstrin homology domain (PH))	<i>Penicillium chrysogenum</i> Wisconsin 54-1255	DQDQTTEVQGTYVR (1837.78 Da) <sup>a</sup>	6.06	182403.63	n.d./3e-3
1	XP_002569230.1	Pc21g22620	<i>Penicillium chrysogenum</i> Wisconsin 54-1255	I(338.19 Da)EHVHYVLQTR (1947.93 Da) <sup>a</sup>	9.09	82944.96	n.d./1e-3
1	XP_002557051.1	Pc12g01560 (contains a Forkhead associated domain (FHA), a kinesin domain (KISc) and a pleckstrin homology domain (PH))	<i>Penicillium chrysogenum</i> Wisconsin 54-1255	DRDQDQTTEVQGTYVR (2124.93 Da) <sup>a</sup>	6.06	182403.63	n.d./3e-3
1	EFX02309.1	hypothetical protein CMQ_2358	<i>Grosmannia clavigera</i> kw1407	WEFGEDVVVHAVSSTGENFNLA VR (2662.37 Da) <sup>a</sup>	5.17	25081.42	n.d./4e-11
2	XP_002557051.1	Pc12g01560 (contains a Forkhead associated domain (FHA), a kinesin domain (KISc) and a pleckstrin homology domain (PH))	<i>Penicillium chrysogenum</i> Wisconsin 54-1255	DQDQTTEVQGTYVR (1638.61 Da) <sup>a</sup>	6.06	182403.63	n.d./3e-3
3	XP_963786.1	phosphomannomutase	<i>Neurospora crassa</i> OR74A	NNDYEILYEDSR (1416.48 Da) <sup>a</sup>	5.38	30423.57	n.d./4e-5
3	EGC42776.1	phosphomannomutase	<i>Ajellomyces capsulatus</i> H88 (and several others)	TIHFFGDK (963.55 Da) <sup>b</sup>	9.50	76627.44	92/1e-3
3	EFY98547.1	phosphomannomutase	<i>Metarhizium anisopliae</i> ARSEF 23 (and several others)	DTICLFDVDGTLTPAR (1792.72 Da) <sup>b</sup>	5.38	30423.00	92/1e-11
4	CCA39192.1	triosephosphate isomerase	<i>Pichia pastoris</i> CBS 7435 (and several others)	FFVGGNFK (914.44 Da) <sup>b</sup>	6.61	31015.11	177/3e-3
4	EFQ24919.1 / XP_003003266.1	triosephosphate isomerase	<i>Glomerella graminicola</i> M1.001 / <i>Verticillium albo- atrum</i> VaMs.102	VVSSQLAALK (1343.70 Da) <sup>a</sup>	5.60 / 6.26	26710.00 / 27103.97	n.d./2e-2
4	XP_002584263.1 / XP_001936088.1	triosephosphate isomerase	<i>Uncinocarpus reesii</i> 1704 / <i>Pyrenopora triticirepentis</i> Pt-1C-BFP	VATTEQAQEVAHLR (1622.75 Da) <sup>b</sup>	5.49 / 5.62	26710.00 / 27034.87	177/1e-9
5	CCA39192.1	triosephosphate isomerase	<i>Pichia pastoris</i> CBS 7435 (and several others)	FFVGGNFK (914.44 Da) <sup>b</sup>	6.61	31015.11	239/8e-3
5	XP_571026.1	triosephosphate isomerase	<i>Cryptococcus neoformans</i> var. <i>neoformans</i> JEC21	KFFVGGNFK (1042.49 Da) <sup>b</sup>	6.34	26710.00	239/1e-3
5	EGR48570.1	triosephosphate isomerase	<i>Trichoderma reesei</i> QM6a	(329.18 Da)VVSSQLAALK (1343.70 Da) <sup>a</sup>	5.43	27189.08	n.d./5e-2
5	EGR48570.1	triosephosphate isomerase	<i>Trichoderma reesei</i> QM6a	DAGINWILGHSER (1567.69 Da) <sup>a</sup>	5.43	27189.08	n.d./6e-7
5	XP_753309.1	triosephosphate isomerase	<i>Aspergillus fumigatus</i> Af293 (and others)	VATTEQAQEVAHLR (1622.75 Da) <sup>b</sup>	5.87	28068.18	239/2e-9
5	EGS20729.1	triosephosphate isomerase	<i>Chaetomium thermophilum</i> var. <i>thermophilum</i> DSM 1495	(318.95 Da)DGGISVIWCCVSSLAER (2226.88 Da) <sup>a</sup>	5.23	27073.76	n.d./3e-6

Table 1. Continued

Spot num	Access number	Protein name	Orthologous to	Peptide sequence (Mr)	Theoretical pI	Mr (Da)	Score <sup>d</sup> / E value <sup>e</sup>
5	EGD97445.1	triosephosphate isomerase	<i>Trichophyton tonsurans</i> CBS 112818 (and others)	(544.04 Da)WSNIVIAYPEIWI (2561.17 Da) <sup>a</sup>	5.58	27009.66	n.d./1e-10
5	EFY93910.1 / EFY98912.1	triosephosphate isomerase	<i>Metarhizium acridum</i> CQMa 102 / <i>Metarhizium anisopliae</i> ARSEF 23	(356.98 Da)VAAQNAYDQPCGAYTWI(661.41 Da) (2926.29 Da) <sup>a</sup>	5.42 / 5.64	27203.13 / 27084.99	n.d./9e-5
6	XP_003008525.1	haloacid dehalogenase (HAD)-superfamily hydrolase	<i>Verticillium albo-atrum</i> VaMs.102	VIGYTGPYPAAER (1321.63 Da) <sup>a,c</sup>	5.03	26547.46	n.d./6e-5
6	EFY84137.1 / EFZ04426.1	HAD-superfamily hydrolase	<i>Metarhizium acridum</i> CQMa 102 / <i>Metarhizium anisopliae</i> ARSEF 2	EFTGESLISEFVGQNFR (1958.88 Da) <sup>a</sup>	5.15 / 5.25	27175.19 / 27122.21	n.d./6e-10
6	EFQ27958.1	HAD-like hydrolase	<i>Glomerella graminicola</i> M1.001	(630.38 Da)YFIDIVFSAATSL (2170.02 Da) <sup>a</sup>	4.96	27481.62	n.d./2e-3
6	EFQ27958.1	HAD-like hydrolase	<i>Glomerella graminicola</i> M1.001	(211.96 Da)TDAGAVIVIDDWSEF(628.41 Da) (2458.19 Da) <sup>a</sup>	5.00	26842.91	n.d./1e-5
6	XP_003008525.1	HAD-superfamily hydrolase	<i>Verticillium albo-atrum</i> VaMs.102	(185.09 Da)EVDQEFTGESLISEFVGQNFR (2615.18 Da) <sup>a</sup>	5.03	26547.46	n.d./2e-10
6	EGC48949.1 / XP_002625690.1	HAD-superfamily hydrolase	<i>Ajellomyces capsulatus</i> H88 / <i>Ajellomyces dermatitidis</i> SLH14081	(863.28 Da)FSAATSLPVPTSQ(2151.88 Da) (3403.7 Da) <sup>a</sup>	5.49 / 5.49	27113.13 / 27113.13	n.d./9e-5
7	XP_001906095.1	hypothetical protein (contains a GH16 fungal CRH1 transglycosylase region)	<i>Podospora anserina</i> S mat+	QAPTISSPGYIF(350.26 Da) (1594.75 Da) <sup>a</sup>	4.80	36972.05	n.d./1e-2
7	EGE03803.1 / EGD95117.1	cell wall glucanase / glycosyl hydrolase	<i>Trichophyton equinum</i> CBS 127.97 / <i>Trichophyton tonsurans</i> CBS 112818	DCPADPALGGDF(709.41 Da) (1924.79 Da) <sup>a</sup>	5.27 / 5.27	47729.56 / 47756.59	n.d./1e-4
7	EGA62177.1 / EDZ71957.1 / EDV10067.1	Crh1p / YGR189Cp-like protein / cell wall protein (all these proteins contain a GH16 fungal CRH1 transglycosylase region)	<i>Saccharomyces cerevisiae</i> FostersO / <i>Saccharomyces cerevisiae</i> AWRI1631 / <i>Saccharomyces cerevisiae</i> RM11-1a	(756.23 Da)QWAGGNANFNDG (2851.24 Da) <sup>a</sup>	4.46 / 4.43 / 4.46	53390.14 / 51282.51 / 51268.53	n.d./6e-2
8	EFY84533.1	Major Facilitator Superfamily (MFS) multidrug transporter, putative	<i>Metarhizium acridum</i> CQMa 102	LVPLGFGIR (970.59 Da) <sup>a</sup>	8.30	19440.86	n.d./2e-2
8	XP_001229897.1	hypothetical protein CHGG_03381 (belongs to the N-acetyltransferase superfamily)	<i>Chaetomium globosum</i> CBS 148.51	RGEILGFAFLSVWQPGLGGTALGSSR (2676.39 Da) <sup>b</sup>	8.10	77609.08	33/2e-20
9	XP_003349182.1	hypothetical protein SMAC_08885 (belongs to the translationally controlled tumor protein [TCP] superfamily)	<i>Sordaria macrospora</i> k-hell	VNNIIHSFR (1098.59 Da) <sup>b</sup>	4.57	19080.56	67/2e-4
9	ABB20806.1	translationally controlled tumor protein-like variant I	<i>Madurella mycetomatis</i> (and others)	DIISGDEIIIDS(537.35 Da) (1781.84 Da) <sup>a</sup>	4.55	19112.00	n.d./1e-6
10	EFY91531.1	tropomyosin-1	<i>Metarhizium acridum</i> CQMa 102 (and others)	LQLLEEEAEEADKTLR (1086.01 Da) <sup>b</sup>	4.92	17847.77	83/1e-12
10	EFY91531.1	tropomyosin-1	<i>Metarhizium acridum</i> CQMa 102 (and others)	RLQLLEEEAEEADKTLR (2042.12 Da) <sup>b</sup>	4.92	17847.77	83/1e-12

Table 1. Continued

Spot num ber	Access number	Protein name	Orthologous to	Peptide sequence (Mr)	Theoretical pI	Mr (Da)	Score <sup>d</sup> / E value <sup>e</sup>
11	XP_360621.1	cofilin, putative hypothetical protein FG06245.1 (actin depolymerization factor/cofilin-like domain)	<i>Magnaporthe oryzae</i> 70-15 (and others)	ITFIAWSPPDDA(545.37 Da) (1761.86 Da) <sup>a</sup>	5.48	16629.66	n.d./8e-7
11	XP_386421.1		<i>Gibberella zeae</i> PH-1	YAVYDFEYQLASGDGIR (1965.89 Da) <sup>a</sup>	5.25	16123.19	n.d./3e-11
11	EFQ30012.1	cofilin/tropomyosin-type actin-binding protein	<i>Glomerella graminicola</i> M1.001	TRSGAAVAQECITAYNDLK (2066.93 Da) <sup>a</sup>	5.26	17006.11	n.d./7e-9
12	AAQ87930.1	Asp f 13-like protein (contains a cerato-platanin domain)	<i>Cochliobolus lunatus</i>	FPTQGNLPK (1000.53 Da) <sup>a</sup>	5.47	14248.00	n.d./1e-3
12	AAQ87930.1	Asp f 13-like protein (contains a cerato-platanin domain)	<i>Cochliobolus lunatus</i>	(158.02 Da)VGYDQGFDDASR (1486.62 Da) <sup>a</sup>	5.47	14248.00	n.d./2e-4
13	AAQ87930.1	Asp f 13-like protein (contains a cerato-platanin domain)	<i>Cochliobolus lunatus</i>	FPTQGNLPK (1000.58 Da) <sup>a</sup>	5.47	14248.00	n.d./1e-3
13	XP_001803929.1	hypothetical protein SNOG_13722 (contains a cerato-platanin domain)	<i>Phaeosphaeria nodorum</i> SN15	(314.18 Da)YDQGFDDASR (1486.69 Da) <sup>a</sup>	4.88	13996.00	n.d./3e-3
14	XP_001818414.1 / XP_002373640.1	transporter (belongs to the Major Facilitator Superfamily)	<i>Aspergillus oryzae</i> RIB40 / <i>Aspergillus flavus</i> NRRL3357	(337.13 Da)ISNNIYR (1215.58 Da) <sup>a</sup>	7.57 / 7.56	63054.00 / 62573.34	n.d./3e-3
14	XP_001396492.1	multidrug resistance protein CDR1	<i>Aspergillus niger</i> CBS 513.88	LLVAVTCANYLASR (1549.83 Da) <sup>a</sup>	6.65	164922.77	n.d./5e-3
18	XP_001906882.1 and XP_366634.1 / XP_003007875.1	hypothetical protein and hypothetical protein MGG_02710 (both belong to the peroxiredoxin family) / peroxiredoxin type-2	<i>Podospora anserina</i> S mat+ and <i>Magnaporthe oryzae</i> 70-15 / <i>Verticillium albo-atrum</i> VaMs.102	YAIVVVDHGK (1000.57 Da) <sup>a</sup>	5.53 and 5.62 / 4.94	17647.06 and 18004.37 / 16026.22	n.d./2e-4
18	EGN94556.1	hypothetical protein SERLA73DRAFT_62273 (belongs to the peroxiredoxin family)	<i>Serpula lacrymans</i> var. <i>lacrymans</i> S7.3	EVDPGIAITGAEAVLAR (1680.98 Da) <sup>a</sup>	5.62	18004.00	n.d./3e-4
24	EGR48231.1	aldose-1-epimerase	<i>Trichoderma reesei</i> QM6a	LDFGLSSGTGLGEDSAR (1623.85 Da) <sup>a</sup>	4.99	33988.21	n.d./4e-4
25	XP_369490.1	hypothetical protein MGG_05974 (contains the ABC-type nitrate/sulfonate/bicarbonate transport systems)	<i>Magnaporthe oryzae</i> 70-15 (and several others)	VGEIYTPWSSWK (1451.77 Da) <sup>b</sup>	6.01	32289.00	52/2e-9
26	EFZ03442.1	malate dehydrogenase	<i>Metarhizium anisopliae</i> ARSEF 23 (and several others)	LFGVTTLIDIVR (1232.78) <sup>b</sup>	8.84	44630.36	373/7e-6
26	EFZ03442.1	malate dehydrogenase	<i>Metarhizium anisopliae</i> ARSEF 23 (and several others)	VQFGGDEVVKAK (1275.70 Da) <sup>b</sup>	8.84	44630.36	373/1e-6
26	EFZ03442.1	malate dehydrogenase	<i>Metarhizium anisopliae</i> ARSEF 23 (and several others)	DDLFNINAGIVK (1317.75) <sup>b</sup>	8.84	44630.36	373/4e-7
26	EFZ03442.1	malate dehydrogenase	<i>Metarhizium anisopliae</i> ARSEF 23 (and several others)	DADIIVIPAGIPR (1348.83) <sup>b</sup>	8.84	44630.36	373/4e-8
26	EFQ28643.1	malate dehydrogenase	<i>Glomerella graminicola</i> M1.001 (and several others)	AVVAGASGGIGQQPLSLKK (1750.09) <sup>b</sup>	6.47	34401.00	373/4e-12

Table 1. Continued

Spot num ber	Access number	Protein name	Orthologous to	Peptide sequence (Mr)	Theoretical pI	Mr (Da)	Score <sup>d</sup> / E value <sup>e</sup>
27	XP_002796107.1	fructose-bisphosphate aldolase	<i>Paracoccidioides brasiliensis</i> Pb01 (and several others)	EASVAGAIAAAHFIR (1482.89) <sup>a</sup>	6.09	39632.00	n.d./3e-8
27	XP_754452.1	fructose-bisphosphate aldolase, class II	<i>Aspergillus fumigatus</i> Af293 (and several others)	ILPWLDGMLDEDER (1700.87) <sup>b</sup>	5.55	39766.00	93/1e-9
27	XP_754452.1	fructose-bisphosphate aldolase, class II	<i>Aspergillus fumigatus</i> Af293 (and several others)	KLLPWLDGMLDEDER (1844.96 Da) <sup>b</sup>	5.55	39766.00	93/1e-11
27	XP_002489713.1	fructose 1,6-bisphosphate aldolase	<i>Pichia pastoris</i> GS115	AVAPLYGIPVVLHTDHCAK (2060.16 Da) <sup>b</sup>	6.02	40045.00	71/5e-13
28	XP_754452.1	fructose-bisphosphate aldolase, class II	<i>Aspergillus fumigatus</i> Af293 (and several others)	ILPWLDGMLDEDER (1700.87) <sup>b</sup>	5.55	39766.00	42/1e-9
28	XP_002489713.1	fructose 1,6-bisphosphate aldolase	<i>Pichia pastoris</i> GS115	AVAPLYGIPVVLHTDHCAK (2060.16 Da) <sup>b</sup>	6.02	40045.00	43/5e-13
29	XP_002542702.1	hypothetical protein UREG_02218 (contains a mannitol dehydrogenase domain)	<i>Uncinocarpus reesii</i> 1704	FYEWVVDK (1084.58 Da) <sup>a</sup>	8.21	110580.00	n.d./3e-4
29	EGO59636.1	mannitol-1-phosphate 5-dehydrogenase	<i>Neurospora tetrasperma</i> FGSC 2508 (and several others)	AVHFGAGNIGR (1097.63 Da) <sup>b</sup>	5.33	44490.00	104/2e-5
29	EGO59636.1	mannitol-1-phosphate 5-dehydrogenase	<i>Neurospora tetrasperma</i> FGSC 2508 (and several others)	(314.20 Da)PHLADAVER (1320.75 Da) <sup>a</sup>	5.33	44490.00	n.d./3e-3
29	XP_003009490.1	mannitol-1-phosphate 5-dehydrogenase	<i>Verticillium albo-atrum</i> VaMs.102	IVPAQDPNSGLDVK (1451.81) <sup>b</sup>	5.29	43574.18	104/9e-8
29	XP_003009490.1	mannitol-1-phosphate 5-dehydrogenase	<i>Verticillium albo-atrum</i> VaMs.102	(211.98 Da)EVGTEGTEITISGYR (1952.05 Da) <sup>a</sup>	5.29	43574.18	n.d./8e-6
29	EFX05323.1	mannitol-1-phosphate dehydrogenase	<i>Grosmannia clavigera</i> kw1407	(377.12 Da)VNTGHATAAYHGYAMK (2050.09 Da) <sup>a</sup>	5.36	44054.45	n.d./9e-9
29	XP_003238212.1	mannitol-1-phosphate dehydrogenase	<i>Trichophyton rubrum</i> CBS 118892 (and others)	(693.34 Da)AVIACENAIGATDSAIVA(308.25 Da) (2727.50 Da) <sup>a</sup>	5.33	44334.10	n.d./2e-8
30	EGS22126.1	phosphoglycerate mutase-like protein	<i>Chaetomium thermophilum</i> var. <i>thermophilum</i> DSM 1495 (and others)	QTHLYALLR (1113.69 Da) <sup>b</sup>	5.28	56796.00	84/1e-4
30	EGR49197.1	phosphoglycerate mutase	<i>Trichoderma reesei</i> QM6a (and several others)	YAHVTFFNGGVEK (1615.83 Da) <sup>b</sup>	5.06	58467.08	84/5e-10
32	XP_003191411.1 / XP_566812.1	sterol metabolism-related protein	<i>Cryptococcus gattii</i> WM276 / <i>Cryptococcus neoformans</i> var. <i>neoformans</i> JEC21	GGSYNMMAGR DASR (1486.70 Da) <sup>b</sup>	9.18 / 8.49	25640.20 / 23536.93	35/2e-10
34	XP_002381142.1	Ran-specific GTPase-activating protein 1	<i>Aspergillus flavus</i> NRRL3357 (and several others)	FANSENANLFK (1253.63 Da) <sup>b</sup>	5.13	53168.16	132/2e-6
34	EFQ25293.1	RanBP1 domain-containing protein	<i>Glomerella graminicola</i> M1.001	SWVWNAAADVSEGEAEAVTLAIR (2444.28 Da) <sup>b</sup>	5.02	25996.91	132/2e-19
42	AAP32823.1	aspartyl proteinase	<i>Paracoccidioides brasiliensis</i> (and several others)	EPGLAFAFGR (1063.59 Da) <sup>b</sup>	5.28	43577.03	78/5e-5

Table 1. Continued

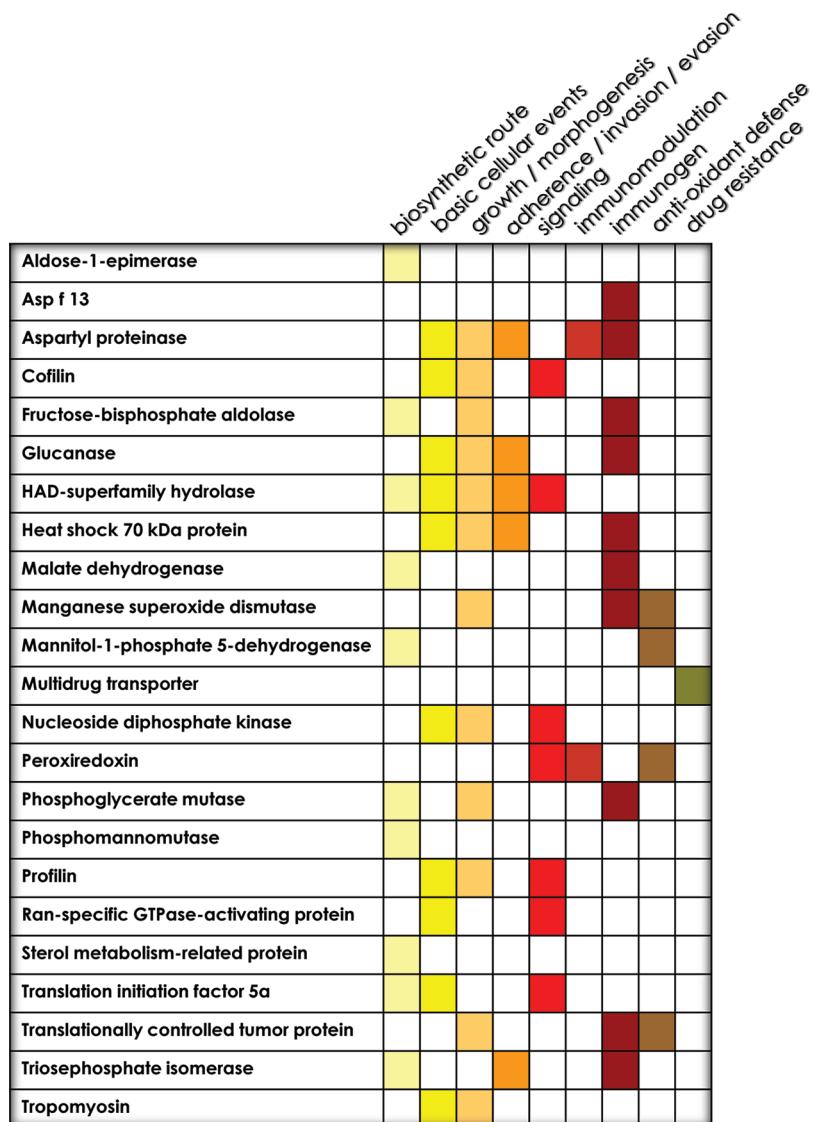
Spot num	Access number	Protein name	Orthologous to	Peptide sequence (Mr)	Theoretical pI	Mr (Da)	Score <sup>d</sup> / E value <sup>e</sup>
42	AAP32823.1 / EGS22121.1	aspartyl proteinase	<i>Paracoccidioides brasiliensis</i> / <i>Chaetomium thermophilum</i> var. <i>thermophilum</i> DSM 1495	FDGILGLGYDTISVNR (1738.94 Da) <sup>b</sup>	5.28 / 5.06	43577.03 / 42911.82	78/2e-11
42	XP_002479865.1 / XP_002143541.1	aspartyl proteinase Pep2	<i>Talaromyces stipitatus</i> ATCC 10500 / <i>Penicillium marneffei</i> ATCC 18224	DQDFAEATKEPGLAFAFGR (2068.05 Da) <sup>b</sup>	4.80 / 4.85	42657.00 / 42830.18	109/4e-13
44	XP_747995.1	nucleoside diphosphate kinase	<i>Aspergillus fumigatus</i> Af293 (and others)	GLIGPIISR (924.63 Da) <sup>b</sup>	7.77	16922.00	184/1e-3
44	XP_747995.1	nucleoside diphosphate kinase	<i>Aspergillus fumigatus</i> Af293 (and others)	GDFADIVGR (948.52 Da) <sup>b</sup>	7.77	16922.00	184/8e-4
44	XP_747995.1	nucleoside diphosphate kinase	<i>Aspergillus fumigatus</i> Af293 (and several others)	TILGATNPLASAPGTIR (1652.01 Da) <sup>b</sup>	7.77	16922.00	184/4e-11
44	XP_001543498.1	nucleoside diphosphate kinase	<i>Ajellomyces capsulatus</i> NAm1 (and others)	acetylSASEQTFTIAKPDGVQR (1888.05 Da) <sup>b</sup>	7.77	16748.00	133/8e-12
45	XP_962235.1	Profilin	<i>Neurospora crassa</i> OR74A (and others)	(413.17 Da)GAYVDSSLIGTG(512.32 Da) (2046.08 Da) <sup>a</sup>	6.03	13963.00	n.d./1e-4
46	ADF43946.1 / ADF43928.1	manganese superoxide dismutase	<i>Thielavia microspore</i> / <i>Corynascus sepedonium</i>	QMNTALAGIQGSGWAWLVK (2013.15 Da) <sup>b</sup>	6.03 / 6.03	14714.00 / 14723.50	35/1e-15
46	AAU04427.1	manganese superoxide dismutase	<i>Scedosporium prolificans</i>	QMNTALAGIQGSGWAWLVK (2013.15 Da) <sup>b</sup>	5.89	14017.54	n.d./9e-13
47	XP_961911.2	eukaryotic translation initiation factor 5A	<i>Neurospora crassa</i> OR74A (and several others)	VHLVAIDIFTGK (1311.84 Da) <sup>b</sup>	5.48	18137.00	n.d./2e-7
50	XP_002546993.1	heat shock 70 kDa protein C precursor	<i>Candida tropicalis</i> MYA-3404 (and several others)	FELTGIPPAPR (1196.74 Da) <sup>b</sup>	4.82	74635.97	31/2e-6
50	XP_381014.1	HS70 NEUCR heat shock 70 kDa protein (HSP70)	<i>Gibberella zeae</i> PH-1	(697.37 Da)DNQQQATR (1528.85 Da) <sup>a</sup>	5.02	71301.00	n.d./2e-2

<sup>a</sup> de novo sequencing. <sup>b</sup> Mascot analysis. <sup>c</sup> Peptide sequence after SPITC derivatization ( $M_r + 214.97$  Da). <sup>d</sup> Mascot protein score. <sup>e</sup> Blast analysis. <sup>f</sup> n.d., not determined by the Mascot software. Spots 15, 17, 16, 19, 20, 21, 22, 23, 31, 33, 35, 36, 37, 38, 39, 40, 41, 43, 48, and 49 generated MS with low quality and/or nonidentified peptide sequence, precluding their identifications.

this mycosis (Figure 1D). Five major reactive bands were observed with relative molecular masses corresponding to 120, 90, 50, 35, and 27 kDa (Figure 1D). On the other hand, no reactive polypeptides were observed using a pool of sera from normal individuals (data not shown). In parallel, the addition of *P. boydii* secretion on the confluent monolayer of pulmonary epithelial cells (A549 lineage) caused a significant loss of viability ( $CC_{50} = 0.24 \mu\text{g}/\mu\text{L}$ ), resulting in cell destruction that begins with the rounding of the epithelial cells, followed by their detachment from the plastic substrate (Figure 1E).

Figure 2A shows the bidimensional electrophoresis pattern of the proteins secreted by mycelia of *P. boydii*. All the spots selected are located in the 4–7 pI range, scattered all over the molecular mass interval, and present variable staining intensities. We detected around 100 spots, from which we have randomly selected 50 spots to be used in the present work (Figure 2B). All of the proteins that were subsequently identified are indicated by arrows, and the associated numbers correspond to those found in Table 1. In order to identify these selected proteins,

spots were picked from 2-DE gels and subjected to MALDI-ToF/ToF analysis. Of out 50 spots selected, 30 (60%) spots were consistently identified, while 20 (40%) spots generated peptides that showed no resemblance to any known protein from other fungi and/or MS with low quality (Figure 2B). The proteins identified in the culture filtrate of *P. boydii* could be classified into several major functional categories, and a potential function could be assigned due to the presence of conserved domains detected by the NCBI CDD algorithm: energy and/or carbohydrate metabolism (phosphomannomutase, triosephosphate isomerase, malate dehydrogenase, fructose-1,6-bisphosphate aldolase, phosphoglycerate mutase, mannitol-1-phosphate 5-dehydrogenase, and aldose-1-epimerase), fatty acid metabolism (sterol metabolism-related protein), cell wall remodeling (glucanase), RNA processing (Ran-specific GTPase-activating protein 1), nucleotide metabolism (nucleoside diphosphate kinase), protein degradation/nutrition (aspartyl proteinase), translation machinery (initiation factor 5A), drug elimination/detoxification (major facilitator superfamily multidrug

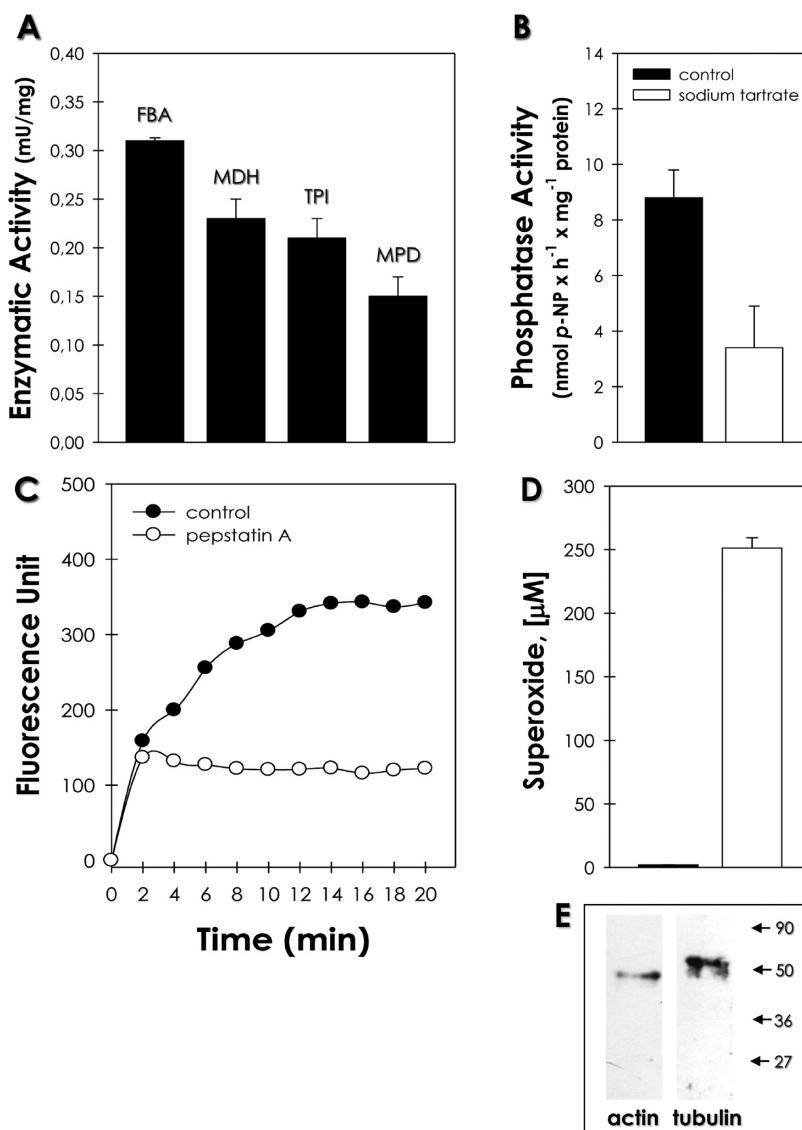


**Figure 3.** Distribution of the identified proteins in the secretome of *Pseudallescheria boydii* in different functional categories. The different colored squares indicate the possible distinct roles of each identified protein in the *P. boydii* secretions. The present analysis was based on the well-known roles of similar/homologous proteins published for other several pathogenic fungi. Note that almost all proteins presented more than one recognized biological function, corroborating the biochemical flexibility of fungal molecules.

transporter, peroxiredoxin, manganese superoxide dismutase, and translationaly controlled tumor protein), environmental stress (heat shock protein), cytoskeleton/movement proteins (cofilin, profilin, and tropomyosin), allergen (Asp f13-like protein), miscellaneous reactions (haloacid dehalogenase-superfamily hydrolase), and hypothetical proteins without known function but with a characterized domain/region (Forkhead associated domain involved in signaling events and ABC-type transport system region). It is obvious that some of these proteins can be used by fungal cells in different social contexts, and for this reason, different roles can be assigned to each protein, as summarized in Figure 3.

As already mentioned, the genome of *P. boydii* is completely unknown. For these reasons, we performed some protein/activity identification in order to test the proteomic findings. In this context, we measured some enzymatic activities in the secretions of *P. boydii*, including the following: mannitol-1-phosphate 5-dehydrogenase, triosephosphate isomerase, malate

dehydrogenase, fructose-1,6-bisphosphate aldolase (Figure 4A), sodium tartrate-sensitive phosphatase activity (Figure 4B), pepstatin A-sensitive aspartyl proteinase (Figure 4C), and superoxide dismutase activity (Figure 4D). Subsequently, we searched in the PubMed database for deposited genes belonging to the *Pseudallescheria/Scedosporium* complex, and only a few genes were until now sequenced, including the following: 18S rRNA,  $\beta$ -tubulin, manganese superoxide dismutase (Mn-SOD), elongation factor 1 $\alpha$ , and calmodulin genes. The unique similar protein detected in the secretome analysis was the Mn-SOD, which presented high identity with the Mn-SOD of *Scedosporium prolificans* (*E*-value threshold of  $9 \times 10^{-13}$ ). As described earlier, the secretion in fungal cells requires vesicular transport and, consequently, the active participation of cytoskeleton and/or movement proteins. Taking this fact in mind, we searched for the presence of tubulin and actin in *P. boydii* secretions. By means of Western blotting assay, the anti-actin antibody reacted with a 45 kDa protein and a protein of 55 kDa was revealed by the anti-tubulin antibody (Figure 4E).



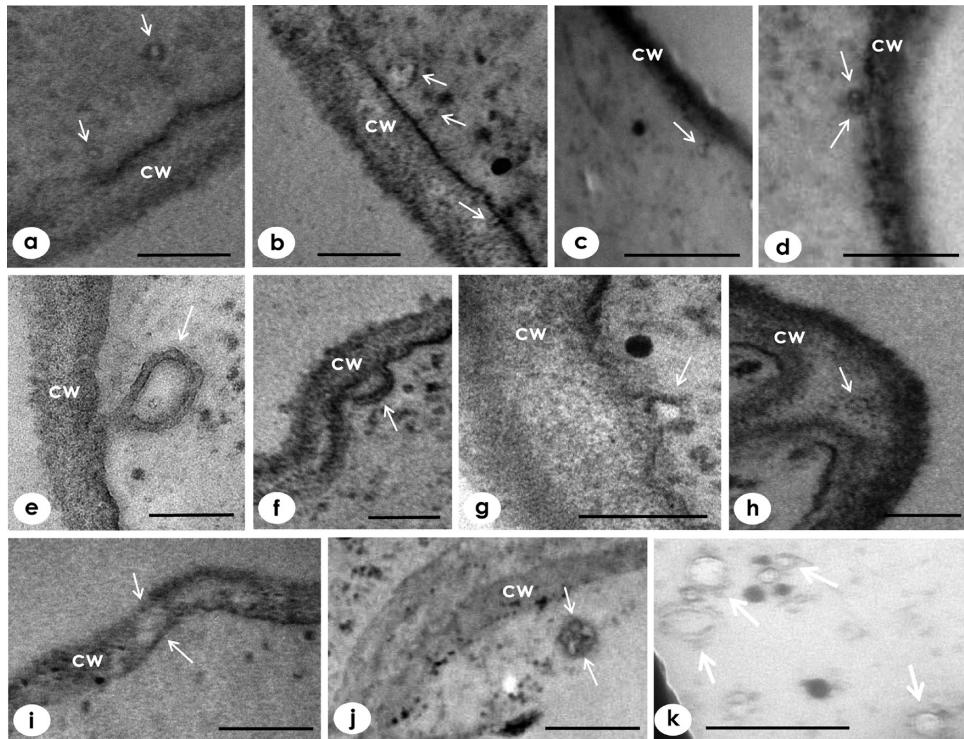
**Figure 4.** Measurement of enzymatic activity and immunodetection of some proteins identified in the *Pseudallescheria boydii* secretions. (A) Detection of fructose-1,6-bisphosphate aldolase (FBA), malate dehydrogenase (MDH), triosephosphate isomerase (TPI), and mannitol-1-phosphate 5-dehydrogenase (MPD). (B) Phosphatase activity was measured in the absence (control) or in the presence of 15 mM sodium tartrate (which inhibits the enzymatic activity around 60%). (C) Aspartyl proteinase activity was detected by the cleavage of cathepsin D fluorogenic substrate. As expected, pepstatin A at 1 μM inhibited (~65%) the hydrolytic activity. (D) Superoxide dismutase activity was determined using the method that involves generation of superoxide and reduction of the tetrazolium dye MTT to formazan in PBS (control, black bar) and PBS-conditioned medium (white bar). (E) Immunoblotting showing the recognition of both actin and tubulin molecules after incubation with anti-actin and anti-tubulin antibodies, respectively. The numbers on the right indicate the relative molecular mass markers expressed in kilodaltons.

The mechanisms by which macromolecules such as proteins reach the extracellular environment and how they are transported through the cell wall, however, have not been rigorously explored in fungi. Recently, some researchers described that vesicular secretion is a key and common mechanism of extracellular delivery in several fungi.<sup>26</sup> Since the profile of protein detection in the *P. boydii* secretions resembled those found in mammalian exosomes and the content of extracellular vesicles purified from different pathogenic fungi,<sup>26</sup> ultrathin sections of *P. boydii* mycelial cells were searched for the presence of vacuole-like structures. TEM images revealed the presence of vesicles close to the cell surface (Figure 5a–d), which were sometimes found in fusion with the plasma membrane (Figure 5e–g), inside the cell wall (Figure 5h and i), or free in the extracellular environment (Figure 5j and k).

These results add *P. boydii* to the list of human pathogenic fungi that are able to extracellularly release vesicle-like structures.

#### 4. DISCUSSION

Protein secretion is a universal process of fundamental importance for various aspects of cell physiology. However, the protein secretion pathways used by fungal cells are particularly complex and still not well understood, especially in filamentous fungi. Typical studies have focused on the identification, purification, and characterization of single secreted proteins, but studies on the global analysis of filamentous fungal extracellular proteomes are just beginning to take place. This is quite surprising when considering the clear importance of fungi to mankind.



**Figure 5.** Transmission electron micrographs showing the vesicle-like structures in *Pseudallescheria boydii*. The images revealed the presence of vesicles close to the plasma membrane (a–d), which were sometimes found in fusion with this structure (d–g), inside the cell wall (h, i), or free in the extracellular medium (j, k). White arrows evidence the vesicular bodies. cw, cell wall. Bars: 0.1  $\mu$ m.

Fungal secreted proteins perform several important functions, such as provision of nutrients, cell-to-cell communication, substrate colonization, detoxification of the environment, and killing of potential competitors (ecological interactions). Moreover, secreted proteins of pathogenic fungi seem to play critical roles in virulence. In this sense, fungal secreted proteins can be directly and/or indirectly involved in the molecular dialogue with the host cells, enabling their survival, multiplication, and dissemination. Consequently, the secretome can be considered as a real biological compartment, playing a key role in the fungal infection strategy. Herein, we performed a first comprehensive proteome-level study to identify secreted proteins by mycelial cells of *P. boydii*, in order to gain a more thorough understanding of this human fungal opportunistic pathogen with unknown genome. Having a comprehensive list of *P. boydii* mycelia secreted proteins will facilitate our understanding about the complex relationships between this intriguing fungus and the host. Corroborating the relevance of this study, some of the secreted proteins were recognized by antibodies presented in a serum from a patient with pseudallescheriosis, which supports our hypothesis that these proteins are produced during human infection. Also, these secreted molecules induced irreversible damage in pulmonary epithelial cells, which are target cells during the infection by *P. boydii*, culminating in cell death.

A rigorous experimental method to produce a secretome-containing medium, which reflected the *P. boydii* active export molecules, was developed. Nevertheless, some proteins were identified with known intracellular locations and functions, but it was not surprising, since the presence of these proteins is a common hallmark of published secretome maps of different fungi. In this sense, enzymes belonging to the energy metabolism

represented the most abundant category in the secretome of *P. boydii*, which accounts for 27% of the total proteins identified, including (i) glycolytic enzymes, such as triosephosphate isomerase and phosphoglycerate mutase, (ii) gluconeogenesis enzymes, such as fructose-1,6-bisphosphate aldolase, (iii) general carbohydrate metabolism enzymes, such as aldose-1-epimerase (which catalyzes the interconversion of  $\alpha/\beta$ -anomers of aldoses, such as glucose and galactose) and phosphomannomutase (which catalyzes the conversion of mannose-6-phosphate to mannose-1-phosphate, which is further converted into guanosine diphosphate (GDP)-mannose, which is required as a mannose source for the synthesis of oligosaccharide chains in the glycosylation pathway),<sup>27</sup> (iv) tricarboxylic acid cycle enzymes, such as malate dehydrogenase (which catalyzes the interconversion of oxaloacetate and malate linked to the oxidation/reduction of dinucleotide coenzymes),<sup>28</sup> and (v) mannitol metabolism enzymes, such as mannitol-1-phosphate 5-dehydrogenase.<sup>29</sup> Interestingly, mannitol is an acyclic hexitol that is found in most fungi and is usually the most abundant of all the soluble carbohydrates within the mycelium. Consequently, the physiological role of mannitol has been widely studied over many years. These roles include carbohydrate storage; a reservoir of reducing power, high temperature, and oxidative stress tolerance; and spore dislodgement/dispersal.<sup>30</sup>

The earlier cited and several other energy/carbohydrate metabolism enzymes have been detected in secretome maps of human pathogenic fungi, including *Candida albicans*,<sup>31</sup> *Cryptococcus neoformans*,<sup>32</sup> *Aspergillus fumigatus*,<sup>33</sup> *Histoplasma capsulatum*,<sup>34</sup> and *Paracoccidioides brasiliensis*.<sup>35</sup> In fungi, proteins frequently have more than a single function and are found in different cellular compartments. For instance, in addition to a

cytoplasmic location, triosephosphate isomerase of *P. brasiliensis* is present at the fungal cell wall and is an adhesin putatively involved in binding to both epithelial cells and extracellular matrix components (e.g., laminin and fibronectin).<sup>35</sup> Corroborating these unpredicted roles, some enzymes required for energy metabolism in fungi are highly immunogenic and/or allergenic (Figure 3).<sup>31,33,35</sup>

During tissue invasion, the fungal cells are exposed to oxidative stress and need to deal with highly toxic reactive oxygen species produced by immune cells. Toxic molecules such as superoxide ( $O_2^{\bullet-}$ ) and hydrogen peroxide ( $H_2O_2$ ) can alter membrane properties and disrupt membrane-bound proteins and biological macromolecules, leading to cell death.<sup>36</sup> *Pseudallescheria/Scedosporium* species respond to antifungal phagocytic function to various degrees.<sup>37</sup> For instance, serum opsonization of hyphae of *S. apiospermum* resulted in a higher level of superoxide anion released by human polymorphonuclear leukocytes (PMNs) in response to SA54A (an amphotericin B resistant strain) than that seen in response to SA1216 (amphotericin B susceptible strain); however, PMNs and mononuclear leukocytes induced less hyphal damage to SA54A than to SA1216.<sup>37</sup> Therefore, *Pseudallescheria/Scedosporium* has to maintain its redox homeostasis for survival. Scavenger proteins that play important roles in cell survival during oxidative stress, such as Mn-SOD, peroxiredoxin, and translationally controlled tumor protein (TCTP) [this last protein can act as an antioxidant in a nonenzymatic manner], were identified in *P. boydii* secretions. Lima and co-workers purified from mycelial cells of *S. apiospermum* a Cu,Zn-superoxide that is stimulated by iron starvation.<sup>38</sup> Iron availability has been suggested as a significant controlling factor on expression levels of antioxidant enzymes in various microorganisms such as *A. fumigatus* and *A. nidulans*.<sup>39</sup> TCTP is involved in important cellular processes, such as cell growth and cell cycle progression, and it can protect cells from various stress conditions and apoptosis due to its biological properties. Interestingly, TCTP was identified as a glucose-regulated protein,<sup>40</sup> and this was the condition in which *P. boydii* cells were submitted to obtain its secretion content. TCTP can complex with microtubules and actin microfilaments regulating the cell shape in cells. TCTP is also believed to be involved in a variety of inflammatory processes: secretion of histamine and cytokines such as IL-4, IL-8, and IL-13 thereby potentially regulates allergic phenomena.<sup>41</sup> For instance, *Cladosporium herbarum* TCTP is an IgE-binding antigen and is associated with disease severity.<sup>42</sup> TCTP produced by the fungus *Madurella mycetomatis*, which causes a disease characterized by tumorous swellings in eumycetoma patients, is a predominant immunogenic antigen that is secreted *in vitro* into the culture medium. *In vivo*, TCTP was found to be expressed on hyphae present in developing stages of the eumycetoma characteristic black grain. Significant IgG and IgM immune responses, against the TCTP of *M. mycetomatis*, were determined, and the antibody levels correlated with lesion size and disease duration.<sup>43</sup>

Another interesting and unexpected aspect of the *P. boydii* secretome was the presence of the putative eukaryotic translation initiation factor 5A (eIF5A), which is a highly conserved and essential protein present in all organisms from archaea to mammals, but not in eubacteria.<sup>44</sup> Despite being highly conserved and essential, the critical cellular role of eIF5A remains unclear. Frigeri and co-workers<sup>45</sup> revealed that eIF5A was localized in both soluble and membrane fractions of *S. cerevisiae*, and its membrane association was ribosome-dependent. Those

authors also proposed a link between translation and vesicular trafficking by using yeast cells mutated in several secretion pathways.

The secretion in fungal cells has been proposed as a result of vesicular transport. Rodrigues and co-workers<sup>32</sup> showed similarities in the protein content of extracellular vesicles released by living *C. neoformans* cells and mammalian exosome-like structures, which usually contain cytoplasmic proteins such as elongation factors, tubulin, actin, actin-binding proteins, annexins, Rab protein, molecules responsible for signal transduction, and heat shock proteins such as Hsp70 and Hsp90.<sup>46</sup> Sorting of cytosolic proteins into exosomes is normally explained by a random engulfment of small portions of cytosol during the inward budding process of multivesicular bodies.<sup>46</sup> Supporting this statement, transmission electron microscopic images evidenced the exosome-like structures in cryptococcal cells, suggesting that the extracellular vesicles originate from fungal exosomes.<sup>32</sup> A similar vesicular transport mechanism was subsequently reported in other yeasts and filamentous fungi, suggesting that this exosome-like structure is a common and well-preserved way to extracellularly release molecules by fungal cells.<sup>26</sup> Similarly, vesicles corresponding to endosomes and/or exosomes are frequently seen in the cytoplasm in close proximity to the cell surface of *P. boydii* or free in the extracellular medium.

As it is well-known, the fungal cell wall is a barrier to movement events. However, the cell wall is a dynamic structure. Wall expansion during growth requires continuous remodeling of the cell wall polysaccharide network.<sup>47</sup> Synthesis and remodeling of the cell wall polysaccharide skeleton require not only synthases but also enzymes (hydrolases and transglycosidases) outside the plasma membrane that interconnect and remodel individual cell wall polymers to create a strong but flexible network of macromolecules,<sup>47</sup> which permits the passage of vesicles through the cell wall polymers. At least a glucanase was identified in the secretions of *P. boydii*.

As expected, cytoskeleton and movement proteins must participate of the complex vesicular transport in fungal cells. Herein, we detected some of these proteins: cofilin, profilin, tubulin, and actin. The organization of the actin cytoskeleton is important for various cellular events, including morphogenesis, migration, and cytokinesis. Eukaryotic cells express various kinds of proteins that control the architecture and behavior of the actin cytoskeleton.<sup>48</sup> Yeast cells contain two prominent actin structures, cables and patches, both of which are rapidly assembled and disassembled by operation of other movement proteins, such as cofilin and profilin, which bind to actin monomers (G-actin) and filaments (F-actin) and stimulate depolymerization and fragmentation of F-actin, and tropomyosin, which inhibits the dissociation of actin subunits from the pointed end of an F-actin.<sup>48</sup> In another way, cofilin phosphorylation/dephosphorylation at Ser3 acts as a simple switch for actin assembly and disassembly/severing.<sup>49</sup>

Signaling events are essential mechanisms to adaptation of cells to several conditions and stimuli. Fungal cells respond to extracellular stimuli by signaling pathways that coordinate processes involved in the activation and/or synthesis of protein kinases, protein phosphatases, second messengers, and transcription factors. Membrane-bound protein phosphatases were characterized in *P. boydii* mycelial cells.<sup>50</sup> Proteins potentially involved in signaling cascades were also detected herein in the extracellular content of *P. boydii*, including a phosphatase inhibited by sodium tartrate. However, the physiological roles of these membrane and secreted phosphatases have not been well established yet. In fungi such as *C. neoformans*, *C. parapsilosis*, and

*Fonsecea pedrosoi*, phosphatase was implicated in the adhesive process to epithelial cells.<sup>51,52,24</sup> Moreover, the protein sequence analysis revealed a putative adhesion member of the haloacid dehalogenase (HAD) superfamily of hydrolases, which includes dehalogenases, phosphonatases, phosphomutases, phosphatases, and ATPases,<sup>53</sup> in the *P. boydii* secretome. An interesting work evaluated the relevance of a 32 kDa protein (PbHad32p), a putative protein belonging to the HAD superfamily of hydrolases, in the virulence of *P. brasiliensis*. Knockdown of PbHad32 did not alter cell vitality or viability but induced morphological alterations in yeasts as well as significantly affected their capacity to adhere to human epithelial cells and presented decreased virulence in a mouse model of infection.<sup>54</sup> The role of nucleoside diphosphate (NDP) kinase in signal transduction systems is well recognized.<sup>55</sup> NDP kinases catalyze the transfer of the  $\gamma$ -phosphate from a nucleoside triphosphate to a nucleoside diphosphate and are important in nucleotide metabolism, providing NTPs as a housekeeping enzyme to sustain growth and differentiation; they were also identified in *P. boydii*. The GTPase Ran that belongs to the superfamily of small Ras-like GTP-binding proteins is one of the key player molecules regulating the processes inside the eukaryotic nucleus. So far it has been shown to participate in nuclear transport, nuclear pore complex assembly, mitotic spindle assembly, and DNA replication.<sup>56</sup> Here, we detected a Ran-binding protein 1(RanBP1) that in *S. cerevisiae* has a major role in the cytoplasm, both in recycling of transport receptors and in release of export cargo.<sup>57</sup> Consistent with this view, *S. cerevisiae* YRB1, encoding yeast RanBP1, is essential for cell viability and is required for both nuclear protein import and poly(A)<sup>+</sup> RNA export.<sup>58</sup>

The heat shock proteins (Hsp) are not restricted to heat shock protection; they also play a role in protein folding, translocation of proteins across membranes, and gene regulation. Interestingly, microbial Hsps are major targets of host immune responses. In particular, members of the 70 kDa Hsp (Hsp70) family are among the most immunogenic proteins of human pathogenic microorganisms, including fungi.<sup>59</sup> *P. boydii* mycelial cells were able to secrete Hsp70 as well as other immunogenic molecules: the allergen Asp 13 and aspartyl proteinase. Especially, aspartyl-type proteinases are multifaceted molecules carrying out "housekeeping" tasks common to many eukaryotes as well as functions highly specific to the fungal life cycles, including nutrition, proliferation, differentiation, adhesion to either abiotic or biotic substrates, and escape of host immune responses.<sup>60</sup> Several pathogenic fungi secrete aspartyl proteinases such as *Aspergillus* spp. (named aspergillopepsin), *Penicillium* spp. (penicillopepsin), *Rhizopus* spp. (rhizopuspepsin), and *Candida* spp. (Saps).<sup>60</sup>

In yeasts, including the pathogenic *Candida*, an up-regulation of multidrug transporter genes belonging to either the ATP Binding Cassette (ABC) or Major Facilitator Superfamily (MFS) is frequently observed in the cells exposed to the drugs leading to the phenomenon of multidrug resistance (MDR).<sup>61</sup> MDR is a ubiquitous biological phenomenon causing serious problems in the treatment of human cancers and infections of bacterial and fungal origin. The detection of multidrug transporters can be correlated with the intrinsic resistance of *P. boydii* to several antifungal drugs, including amphotericin B and more-recently introduced antifungal compounds such as voriconazole and posaconazole.<sup>62</sup>

A picture of the proteins secreted by *P. boydii* in cell-free culture provides a basis for investigation of effector proteins that may be active in host cells. Targeting of virulence factors into host surroundings has been shown to be an effective strategy

used by fungal pathogens in order to remodel the environment and to influence host cell function. Appreciation of the multi-functional nature of the *P. boydii* secreted proteins can lead us to speculate that these molecules may play ancillary roles in pathogenesis or pathogen survival. Furthermore, this first global survey on secreted proteins of *P. boydii* appeared before the genome of *Pseudallescheria/Scedosporium*, making protein identification remarkably challenging. However, the authors really hope that this initial analysis can stimulate the researchers around the world to work together in order to obtain the complete genome sequence of *P. boydii*. Undoubtedly, the knowledge that will come from sequencing will lead to a better understanding of pathogenic mechanisms expressed by this fungus as well as possible candidates in an antidisease strategy that include either diagnostic or drug targets and potential vaccine candidates.

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