Biochemical characterization of potential virulence markers in the human fungal pathogen *Pseudallescheria boydii*

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The ubiquitous *Pseudallescheria boydii* (anamorph *Scedosporium apiospermum*) is a saprophytic filamentous fungus recognized as a potent etiologic agent of a wide variety of infections in immunocompromised as well as in immunocompetent patients. Very little is known about the virulence factors expressed by this fungal pathogen. The present review provides an overview of recent discoveries related to the identification and biochemical characterization of potential virulence attributes produced by *P. boydii*, with special emphasis on surface and released molecules. These structures include polysaccharides (glucans), glycopeptides (peptidorhamnomannans), glycolipids (glucosylceramides) and hydrolytic enzymes (proteases, phosphatases and superoxide dismutase), which have been implicated in some fundamental cellular processes in *P. boydii* including growth, differentiation and interaction with host molecules. Elucidation of the structure of cell surface components as well as the secreted molecules, especially those that function as virulence determinants, is of great relevance to understand the pathogenic mechanisms of *P. boydii*.

**Keywords** *Pseudallescheria boydii*, peptidorhamnomannans, glucans, glucosylceramides, hydrolytic enzymes, virulence

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**Introduction**

*Pseudallescheria boydii* (anamorph *Scedosporium apiospermum*) is a ubiquitous ascomycetous fungus found worldwide in soil, manure, decomposing vegetable materials and multiple water sources including creeks contaminated with sewage and standing water [1]. *P. boydii* causes human infections by inhalation or after traumatic subcutaneous implantation, allowing hyphal fragments and conidial forms to penetrate the skin. The clinical manifestations of *P. boydii* infections, collectively termed pseudallescheriasis, are quite varied since these infections can affect practically all the organs of the body. The most common manifestation is mycetoma, a chronic granulomatous infection of the skin and subcutaneous tissue [2,3]. Following this subcutaneous infection, the lungs are the most common site of *P. boydii* infection. Pulmonary infection ranges from colonization of bronchiectatic and tuberculous cavities to invasive necrotizing pneumonia [3]. For instance, *P. boydii* is among the most common filamentous fungi...
colonizing the lungs of patients with cystic fibrosis with a frequency ranging from 0.7–9% [4]. The lungs also appear to be the site of entry for most cases of disseminated pseudallescheriasis. Nonmycetoma \textit{P. boydii} infections occur almost exclusively in compromised hosts. These infections have been known for a long time, but in recent years, a marked increase in severe invasive infections has been noticed, mainly in immunocompromised hosts [5]. The optimal treatment for these infections is unknown, and the mortality rate is very high despite aggressive antifungal treatment [3].

The role of \textit{P. boydii} in fatal infections may be underestimated due to the present lack of detailed diagnostics. Diagnosis of a \textit{Pseudallescheria/Scedosporium} infection is difficult, because clinical features and histopathology are similar to those of aspergillosis, fusariosis, and other relatively common hyalohyphomycosis [6,7]. On the basis of nuclear DNA-DNA reassociation, some studies have proved that important genetic variation exists in \textit{P. boydii} [4,8,9]. Other authors have reported considerable differences with respect to growth and sporulation [10–13]. In addition, a high variability in antifungal susceptibility of the different isolates and in their clinical response has been observed [14,15]. This could be explained by the fact that \textit{P. boydii} does not represent a single species but instead is a complex comprising at least six known species (\textit{P. boydii}, \textit{Pseudallescheria angusta}, \textit{Pseudallescheria ellipsoidea}, \textit{Pseudallescheria fusoidea} and \textit{Scedosporium aurantiacum}) and two cryptic species represented by clades 3 and 4 as described by Gilgado et al. [16]. The discovery of new antifungal agents thus remains an important challenge for the scientific community, which is further complicated by the similarity between fungal and mammalian cells. Like mammalian cells, fungi are eukaryotic, so they share many structures and metabolic pathways with mammalian cells, making it more difficult to identify specific targets for the development of new antifungals.

Despite the growing importance of \textit{Pseudallescheria Scedosporium} infections, very little is known about the physiology and biochemistry of this intriguing human pathogenic fungus. A brief glimpse at the PubMed database (www.pubmed.com) corroborates this statement in that it reveals that around 70% of the works are concerned with clinical case reports and/or epidemiology studies, while papers on biochemistry and/or physiology correspond to less than 4%. In addition, few studies on innate and adaptive immune response against the \textit{P. boydii} complex have recently been published [17–19]. Collectively, these data reinforce the relevance of biochemical studies in \textit{Pseudallescheria Scedosporium}. In this context, the characterization of cell wall and other surface components, as well as secreted molecules are relevant to the development of new antifungal drugs and for an understanding of the fungus’ pathogenic mechanisms. In the present review, we summarize the current knowledge on the biochemical markers expressed by \textit{P. boydii}, including molecules which have been implicated in some fundamental cellular processes including growth, differentiation and interaction with host molecules.

**Peptidorhamnomannan: a potential antigen involved in adhesion**

Polysaccharides and peptidopolysaccharides are especially relevant for the architecture of the fungal cell wall, but several of them are immunologically active compounds with great potential as regulators of pathogenesis and the immune response of the host. In addition, some of these molecules can be specifically recognized by antibodies in patients’ sera, suggesting that they can be also useful in the diagnosis of fungal infections [20].

In the search for structures that could be helpful in the diagnosis of pseudallescheriasis, much attention has been paid to the study of \textit{P. boydii} cell wall antigens. Peptidopolysaccharides have been isolated from its mycelial form and characterized using chemical and immunological methods. Hot aqueous extraction, followed by treatment with Cetavlon in the presence of sodium borate, provided a precipitate of peptidorhamnomannan (PRM) [21]. Sugar component analysis of the PRM molecule showed the presence of rhamnose (Rha), mannose (Man), galactose (Gal) and glucose (Glc) in a ratio of 29:5:60:5:5:4:5, respectively. Methylation analysis and \textsuperscript{1}H- and \textsuperscript{13}C-nuclear magnetic resonance (NMR) spectra indicated the presence of a rhamnomannan with a structure distinct from that of similar components isolated from \textit{Sporothrix schenckii} [22]. Specifically the former consists in \(\alpha\)-rhamnopoyranosyl-(1 \textendash 3)\(\alpha\)-rhamnopoyranose side-chain epitopes linked (1 \textendash 3) to a (1 \textendash 6) linked \(\alpha\)-mannopyranosyl core [21]. This PRM reacted poorly with an antiserum raised against whole cells of \textit{S. schenckii} and strongly with one against \textit{P. boydii} hyphae. These characteristics and immunological differences suggest that this major rhamnose-containing antigen of \textit{P. boydii} may be useful for the specific diagnosis of infections attributable to this fungus [21].

PRM from \textit{P. boydii} mycelia is a complex glycoconjugate consisting of a peptide chain substituted with both \textit{O}- and \textit{N}-linked glycans. \textit{O}-linked oligosaccharides were released by \(\beta\)-elimination under mild alkaline reducing conditions [23]. Three oligosaccharide
fractions were obtained and the major oligosaccharide, a hexasaccharide, was characterized by methylation analysis, $^1$H- and $^{13}$C-NMR spectroscopy, matrix-assisted laser desorption ionization mass spectrometry (MALDI MS) and electrospray ionization quadrupole time-of-flight tandem mass spectrometry (ESI MS/MS). It was a branched structure, with a main chain of $\alpha$-rhamnopyranosyl-(1→3)$\alpha$-rhamnopyranosyl-(1→3)$\alpha$-mannopyranosyl-(1→2)-mannitol substituted at O-6 of mannitol with a $\alpha$-glucopyranosyl-(1→4)-b-galactopyranosyl group [23]. Immunofluorescence analysis using a polyclonal anti-PRM antibody demonstrated that the PRM molecule is expressed in both conidia and mycelia of P. boydii (Fig. 1A).

The O-linked oligosaccharides (Fig. 1B) may account for a significant part of the antigenicity of PRM, because de-O-glycosylation decreased by around 80% its activity. The immunodominance of the O-linked oligosaccharide chains was evaluated by testing their ability to inhibit the reactivity between PRM and anti-P. boydii rabbit antiserum in an enzyme-linked immunosorbent assay (ELISA) hapten system. Up to 75% inhibition was obtained with the hexasaccharide fraction [23]. Similar results were obtained with the peptidogalactomannan from Aspergillus fumigatus [24] and PRM from S. schenckii [25]. Besides the contribution of O-linked oligosaccharides to the antigenicity of PRM, O-glycosylation is critical for fungal adhesion to host cells, because de-O-glycosylation of PRM efficiently inhibits the adhesion of P. boydii conidia to epithelial cells, as well as preventing their endocytosis [26]. Corroborating this result, the treatment of conidia with a polyclonal anti-PRM antibody reduced both adhesion and endocytosis by epithelial cells [26] (Fig. 1C). In a similar way, the pre-incubation of epithelial cells with soluble PRM drastically diminished the interaction process [26] (Fig. 1C). Interestingly, P. boydii PRM binds to a polypeptide of 25 kDa on...
the HEp2 cell surface, suggesting its participation as an adhesin molecule [26].

Studies on the interaction between \textit{P. boydii} and HEp2 cells demonstrated that conidia attached to, and were ingested by, epithelial cells in a time-dependent process [26]. Similarly, the same interaction pattern was observed during the interaction with A549 cells, an epithelial lung lineage (Fig. 1D). In \textit{P. boydii}, conidia differentiation into mycelia is a crucial event during its life cycle. In this context, after 2–4 h of interaction, the conidia produced a germ-tube projection, which was able to penetrate the epithelial cell membrane, leading to the cell’s death [26] (Fig. 1D). Interestingly, when conidia were incubated alone in culture medium (e.g., DMEM or Sabouraud), the germ-tube formation was observed only after 6 h of incubation [26]. The differentiation of conidia into mycelia is an important step observed in other opportunistic fungi during the interaction, invasion and dissemination processes [27].

Glucosylceramides: an antigenic molecule linked to fungal differentiation

Glycosphingolipids are amphipathic molecules consisting of a ceramide lipid moiety linked to a glycan chain of variable length and structure. The ceramide monohexosides (CMHs) glucos- and galactosylceramides are the main neutral glycosphingolipids expressed in almost all fungal species [28]. A few exceptions include \textit{Torulaspora delbrueckii}, \textit{Candida glabrata}, \textit{Saccharomyces cerevisiae}, \textit{Kluveromyces polysporus} and \textit{Kluveromyces yarrowii} [29]. Fungal cerebrosides have conserved structures in which modifications include different sites of unsaturation, as well as the varying length of fatty acid residues in the ceramide moiety. These molecules have been related with sorting of molecules to cell surface, cell differentiation, growth and pathogenicity [30–37].

CMHs were purified from lipidic extracts of \textit{P. boydii} and analyzed by high-performance thin-layer chromatography (HPTLC), gas chromatography coupled to mass spectrometry (GC-MS), fast atom bombardment–mass spectrometry (FAB-MS), and NMR. This combination of techniques allowed the identification of CMHs from \textit{P. boydii} as molecules containing a glucose residue attached to 9-methyl-4,8-sphingadienine in amide linkage to 2-hydroxyoctadecanoic or 2-hydroxyhexadecanoic acids [34] (Fig. 2A).

\textit{P. boydii} CMHs are antigens recognized by antibodies from a rabbit infected with this fungus. These antibodies were purified and used in indirect immunofluorescence, which revealed that CMHs are detectable on the surface of mycelia and pseudohyphae but not conidial forms of \textit{P. boydii}, suggesting a differential expression of glucosylceramides according to the morphological phase of the fungus [34]. Biosynthesis, expression or chemical structures of CMHs seem to be modified during the conidium to mycelium transition, which suggests a role for CMHs in fungal differentiation. In agreement with this hypothesis is the observation that antibodies against CMH were able to inhibit the differentiation of conidia into mycelia in \textit{P. boydii} (Fig. 2B), but did not influence mycelial growth. Similarly, cellular differentiation in \textit{C. albicans} was affected by antibodies against GlcCer [34]. The mechanisms by which anti-CMH antibodies inhibit fungal growth and/or differentiation remain to be established, but there is a possibility that CMHs are associated with enzymes involved in the hydrolysis and synthesis of the cell wall and/or with glycosylphosphatidylinositol-anchor precursors during cell differentiation and division. In this context, binding of antibodies to CMHs could impair the action of CMH-associated functional proteins, and inhibit cell wall synthesis.

Anti-GlcCer antibodies are not exclusive molecules that can bind to GlcCer and impair the fungal growth. Thevissen et al. [38] showed that the antifungal peptide RsAFP2, a defensin purified from radish seeds, presented a potent fungicidal action. A series of experiments revealed that the cell target of the RsAFP2 defensin was fungal GlcCer. Interestingly, mammalian GlcCer was not recognized by this defensin. In addition, the GlcCer-lacking yeast, \textit{S. cerevisiae}, was resistant to the antifungal effects of the RsAFP2 defensin. These results suggest that, most probably, association of RsAFP2 with fungal GlcCer activates signaling pathways leading to cell death rather than causing direct membrane permeabilization. Further experiments in fact revealed that the RsAFP2-GlcCer association activates the production of endogenous reactive oxygen species (ROS) by \textit{C. albicans} [39].

Glycosphingolipid synthesis has also been proposed as an attractive target for development of new antifungal drugs [40]. The administration of a fungal glucosylceramide synthase (GCS) inhibitor could be effective in prophylaxis and/or therapy. However, compounds that inhibit the mammalian GCS enzyme [41] do not inhibit the fungal enzyme perhaps because of a different substrate specificity between the fungal enzyme and the mammalian one [42].

Glucans: structural molecules that bind to Toll-like receptor

In a recent study, Bittencourt et al. [19] determined the chemical structure of an \( \alpha \)-glucan extracted from the
*P. boydii* cell wall employing a combination of techniques including gas chromatography, $^1$H-TOCSY, $^1$H- and $^{13}$C-NMR spectroscopy and methylation analysis. This polysaccharide consists of a glycogen-like structure with linear 4-linked $\alpha$-D-glucopyranosyl residues substituted at O-6 with $\alpha$-D-glucopyranosyl units. The $^1$H-NMR spectrum of the purified $\alpha$-glucan also confirmed the similarity of the glucan of *P. boydii* with glycogen from other species including *A. fumigatus* [43], *Mycobacterium bovis* [44] and rabbit liver [45].

The host immune response to fungi is in part dependent on activation of evolutionarily conserved receptors able to recognize pathogen-associated molecular patterns (PAMPs), among them, the phagocytic receptors and the toll-like receptors (TLR). TLRs are present in a variety of human cells, including the gastrointestinal tract and cell lineages involved in the immune response [reviewed in 46]. In fungi, the (1→3)-$\beta$-glucans have a well-characterized role as a ligand for these receptors [47,48] and as an activator of immune response [49,50]. Although cell wall $\alpha$-glucans are also of interest because of their role in the virulence of several important fungal pathogens [51–53], the significance of these polysaccharides in the processes of interaction with host immunological cells is poorly known. In order to investigate whether the $\alpha$-glucan of *P. boydii* could be involved in the phagocytic process, macrophages were incubated with conidia in the

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Fig. 2 (A) Chemical structure of the ceramide monohexosides (CMHs) extracted from *Pseudallescheria boydii* mycelia. (B) The incubation of conidial cells for 48 h in the presence of anti-CMH antibodies (+ anti-CMH) blocked the conidia to hyphal transition in Sabouraud medium (− anti-CMH).
absence or in the presence of increasing concentrations of the polysaccharide [19]. Phagocytosis of conidia was inhibited in a dose-dependent way, where an α-glucan concentration of 100 μg/ml caused approximately 50% inhibition of phagocytosis [19] (Fig. 3A). The role of α-glucan in the internalization of conidia was further characterized comparing the phagocytic index of conidia submitted to treatment with α-amylglucosidase with that from untreated conidia. Removing of α-glucan from conidial surface by enzymatic treatment caused a significant decrease in the phagocytic index, suggesting that α-glucan present in the P. boydii surface plays an essential role in the internalization of conidia by macrophages [19].

Absence of intracellular signaling upon TLR engagement by PAMPs, in a MyD88-dependent pathway, results in increased susceptibility to a wide variety of microorganisms including fungal pathogens, such as C. albicans [54]. Despite the progress in understanding the interaction of some fungal PAMPs with TLR receptors, the molecular nature of these fungal ligands responsible for cell activation is still in its beginning.

Distinct from the glycogen and others α-glucans from lichens and oysters, α-glucan of P. boydii induced TNF-α secretion by mice peritoneal macrophages in vitro. The secretion of inflammatory cytokines, like TNF-α and IL-12, by mice macrophages and dendritic cells stimulated by α-glucan of P. boydii, is a mechanism that involves TLR2, CD14 and MyD88 adapters [19] (Fig. 3B). Recognition of α-glucan might have relevance in the immunomodulation during fungal infection favoring the host resistance through IL-12 secretion and consequent induction of a Th1 phenotype, or, alternatively, contributing to the pathology through TNF-α release provoking tissue injury.

The presence of (1→3)-β-D-glucans in P. boydii cell wall have yet to be described. These polysaccharides are components of the cell wall of a wide variety of fungi [55] and the enzyme β(1→3)-glucan synthase, a well-conserved component of fungal morphogenetic machinery, is a target for human antifungal therapy. Measurement of β-glucan has emerged as an adjunct diagnostic strategy for invasive fungal infections [47,56]. Recent data indicate that β-glucans are released from fungal cell walls into the systemic circulation of patients with invasive fungal infections who were or where not receiving antifungal medication. Its presence in blood or other body fluids could be a serological marker of fungal sepsis in patients with fungaemia, aspergillosis, candidiasis, fusariosis, trichosporonosis or infections caused by Pneumocystis jiroveci (carinii), Acremonium spp. or Saccharomyces spp. [reviewed in 48]. Interestingly, Scedosporium spp. and P. boydii, produced and extracellularly released low β-glucan levels [57]. In addition, Kahn et al. [58] demonstrated that caspofungin inhibits β-glucan synthesis and reduces in vitro growth of clinical members of the genera Alternaria, Curvularia, Acremonium, Bipolaris, Trichoderma and Scedosporium. Among the new antifungal drugs, inhibitors of β-glucan synthesis, as well as

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Fig. 3 (A) Ingestion of Pseudallescheria boydii conidia by mouse peritoneal macrophages in the absence (upper panel) or in the presence (lower panel) of α-glucan. Note that macrophages treated with the polysaccharide presented a reduction in the number of intracellular conidia in comparison with the non-treated ones. The arrows show the conidia ingested by macrophage cells. (B) Schematic representation of cytokine production by cells of the innate immune system by the α-glucan of P. boydii, in a mechanism involving TLR2, CD14 and MyD88.

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second-generation azole and triazole derivatives, have characteristics that render them potentially suitable for use against some resistant fungi [59]. Fungal β-glucans exist both in soluble and in particulate forms and are believed to have several functions. Cytoplasmic and exocellular β-glucans probably act as carbon storage materials, which may be re-utilized by the fungus under conditions of carbon limitation, suggesting an important survival function [60]. Soluble forms of β-glucans may inhibit phagocytosis by monocytes, by blocking the receptors for β-glucans present on phagocytes; according to Miyazaki et al. [47], this could explain the development of a fungal infection.

Proteolytic enzymes: cleavage of key host components

The pathogenic filamentous fungi use extracellular enzymes to degrade the structural barriers of the host. In animal tissues, these barriers are mostly composed of proteins, therefore the fungus requires proteolytic enzymes to invade them. For this reason, it is logical to assume that this class of hydrolytic enzymes could act by making this tissue invasion easier, but they could also participate in the infection by eliminating some mechanisms of the immune defense and/or helping in the obtaining of nutrients [61–63]. Despite the importance of proteases in fungus-host interaction, little is known about these molecules in P. boydii.

Evidence about the expression of protease in this fungus was first reported by Larcher et al. [64], who had purified and characterized an extracellular protease produced by a clinical isolate of P. boydii. The highest yield of enzyme production was obtained when the fungus was cultivated in modified Czapek-Dox liquid medium supplemented with 0.1% bacteriological peptone and 1% glucose as nitrogen and carbon sources respectively. Analysis of the purified enzyme by SDS-PAGE revealed a single polypeptide chain with an apparent molecular mass of 33 kDa. The inhibition profile and N-terminal amino acid sequencing confirmed that this enzyme belongs to the subtilisin family of serine proteases that has numerous biochemical and physical similarities with those of the previously described serine protease of A. fumigatus [65]. Interestingly, the 33-kDa serine protease is able to degrade human fibrinogen, suggesting an action as a mediator of the severe chronic bronchopulmonary inflammation from which cystic fibrosis patients suffer [64].

Recently, our group described the secretion of new proteolytic enzymes related to the metalloprotease class. By means of SDS-PAGE containing bovine serum albumin (BSA) as co-polymerized substrate, Silva et al. [66] identified a 28-kDa proteolytic enzyme released to the extracellular environment by mycelia of P. boydii. This protease was detected during the growth of this fungus in Sabouraud dextrose medium for 13 days and reached its maximal production on day 7. The 28 kDa protease was active in acidic pH and had its activity completely blocked by 1,10-phenanthroline, a potent zinc-metalloprotease inhibitor [66]. In addition, in an effort to know more about some biochemical properties of this enzyme and possible other proteolytic enzymes induced after growth on Sabouraud medium for 7 days, mycelia of P. boydii were incubated for additional 20 h in PBS-glucose and then analyzed for proteolytic activity. The cell-free PBS-glucose supernatant was submitted to SDS-PAGE and 12 secreted polypeptides were observed. Two of them of 28 and 35 kDa presented proteolytic activity when BSA was used as a copolymerized substrate. These extracellular proteases were also most active in acidic pH (5.5) and fully inhibited by 1,10-phenanthroline. Other metallo, cysteine, serine and aspartic proteolytic inhibitors did not significantly alter the proteolytic activities. To confirm that these enzymes belong to the metallo-type proteases, the apoenzymes were obtained by dialysis against chelating agents, and supplementation with different cations, especially Cu²⁺ and Zn²⁺, restored their activities. Except for gelatin, both metalloproteases hydrolyzed various copolymerized substrates, including human serum albumin, casein, hemoglobin and immunoglobulin G. Additionally, the metalloproteases were able to cleave different soluble proteinaceous substrates such as extracellular matrix components (laminin and fibronectin) and sialylated proteins (mucin and fetuin). Collectively, these properties could help the fungus to escape from natural human barriers and defenses [67]. Interestingly, the major 28 kDa secreted metalloprotease was also detected in cellular extracts from mycelia and conidia [68]. However, quantitative protease assay, using soluble albumin, showed a higher metalloprotease production in mycelial cells in comparison with conidia. In this sense, conidia synthesized a single protease of 28 kDa, while mycelia yielded 6 distinct metalloproteases ranging from 28 to 90 kDa [68]. The regulated expression of proteases in the different morphological stages of P. boydii represents potential target for isolation of stage-specific proteolytic enzymes and their biochemical and immunological analysis.

Ecto-phosphatases

Fungal pathogens have developed a diversity of strategies to interact with host cells, manipulate their
behaviors, and thus survive and propagate. During the process of pathogenesis, phosphorylation of proteins on hydroxyl amino acids (serine, threonine and tyrosine) occurs at different stages, including cell-cell interaction, as well as adherence and changes in host cellular structure and function induced by infection. The phosphorylation reactions are catalyzed in a reversible fashion by specific protein kinases and phosphatases that belong to either the invading fungal cells or the infected host cells [69,70].

Ecto-phosphatase activities were characterized in intact mycelial forms of P. boydii, which are able to hydrolyze, with different specificities the artificial substrates p-nitrophenylphosphate (p-NPP), β-glycerophosphate and phosphoaminoacids such as phosphoserine, phosphotyrosine and phosphothreonine [71]. MgCl₂, MnCl₂ and ZnCl₂ were able to increase the p-NPP hydrolysis while CdCl₂ and CuCl₂ inhibited it. High sensitivity to specific inhibitors of alkaline and acid phosphatases suggests the presence of both acid and alkaline phosphatase activities at the surface of P. boydii mycelia. Cytochemical localization of the acid and alkaline phosphatase showed electron-dense cerium phosphate deposits on the whole cell wall, as visualized by transmission electron microscopy. However, the alkaline conditions favored the detection, suggesting the predominance of phosphatases with alkaline characteristics on the cell surface of this fungus. The enhancement of p-NPP hydrolysis by increasing pH values (2.5–8.5) over an approximately 5-fold range corroborate this finding. For some cells, the electron-dense precipitate indicative of the alkaline phosphatase activity was also found apart from the cells; however, under the experimental conditions employed in that work, no enzymatic activity was biochemically detected in culture supernatant [71].

Several biological roles for extracytoplasmic phosphatases have been proposed. In Candida parapsilosis, a surface phosphatase activity was described to be involved with fungal adhesion to host cells [72] and in C. albicans, the endocytosis by vascular endothelial cells is associated with tyrosine phosphorylation of specific host cell proteins [73]. These ecto-enzymes have also been associated with cell differentiation [74,75] and may also have a role as ‘safeguard’ enzymes to protect the cells from acidic conditions by buffering the periplasmic space with phosphate released from polyphosphatases [76]. From a general standpoint, the demonstration of a direct relationship between protein phosphorylation on serine/threonine/tyrosine and fungal virulence represents a novel concept of great importance in deciphering the molecular and cellular mechanisms that underlie pathogenesis. However, knowledge of the relevance of these enzymes in P. boydii biology or pathogenesis is still very limited.

**Superoxide dismutase**

In spite of their diversity, all the primary or opportunistic pathogenic fungi have to face the first line of host defense against fungal infection: oxidative response of phagocytes. Evolution of antioxidant systems could therefore be a key process determining fungal adaptation and resistance in the host environment, contributing to the emergence of pathogenicity among fungi. Many enzymatic, e.g., catalase, glutathione peroxidase and superoxide dismutase (SOD), and non-enzymatic systems, e.g., melanin and mannitol, participate in reactive oxygen species elimination [77]. SODs are ubiquitous metalloenzymes, catalyzing the dismutation of toxic superoxide anions into hydrogen peroxide. Three main isoforms are described, depending on their metal cofactor: manganese- (MnSOD), iron- (FeSOD), or copper/zinc- (Cu/ZnSOD) SODs. Mn- and Fe-dependent SODs are found in bacteria, whereas eukaryotic cells usually present both Mn-SODs in mitochondria and Cu,Zn-SODs located in the cytoplasm [78]. SODs have already been characterized from various organisms, including pathogenic fungi such as C. albicans, C. neoformans, Trichophyton mentagrophytes var. interdigitale, A. fumigatus, Aspergillus flavus, Aspergillus nidulans and Aspergillus terreus [79–83]. Recently, a Cu,Zn-SOD was characterized from P. boydii mycelial form [84]. The purified enzyme presented a relative molecular mass of 16.4 kDa under reducing conditions and was inhibited by potassium cyanide and diethyldithiocarbamate, which are two well-known inhibitors of Cu,Zn-SODs. The encoding gene was sequenced and a database search for sequence homology revealed for the deduced amino acid sequence 72 and 83% identity rate with Cu,Zn-SODs from A. fumigatus and Neurospora crassa, respectively [84]. 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 [85]. Similarly, iron starvation leads to an increased production of the SOD enzyme in P. boydii [84]. Interestingly, under conditions of low iron availability, most fungi excrete siderophores in order to mobilize extracellular iron and stimulate SOD levels to a protective antioxidant role [86].

**Conclusion**

Pseudallescheria/Scedosporium species are a well-recognized cause of serious fungal infections that are
difficult to treat. Infections due to these opportunistic fungi are usually marked by a poor response to antifungal therapy, in agreement with the in vitro resistance of the fungus to most available antifungal agents. In addition, several key steps of fungal pathogenesis are still unknown, which motivates new studies on the characterization of fungal molecules with key roles in cell growth, differentiation and in the interaction with the host.

In this context, several studies have focused on understanding how the fungal cell wall is assembled. The cell wall is an excellent target for the action of antifungal agents, since most of its components are absent in mammalian cells. Over recent years, polysaccharides, peptidopolysaccharides, O-linked oligosaccharides, glycosphingolipids and several hydrolytic enzymes (e.g., proteases and phosphatases) have been identified in *Pseudallescheria*/*Scedosporium*. The role of each molecule on fungal physiology and pathogenesis is only beginning to be elucidated. In this context, *P. boydii* PRM molecules have been shown to be useful for diagnostic purposes and also to influence the interaction of this fungus with the host cells. An α-glucan, resembling a glycogen-like polysaccharide, is accessible at the surface of conidia and mediates their interaction with receptors of cells of the host innate immune system. However, the partial effect of α-glucan on inhibition of phagocytosis indicates that other putative phagocytic ligands may be present on *P. boydii* conidia. Besides its involvement in phagocytosis of conidia, α-glucan also stimulates the secretion of inflammatory cytokines by macrophages and dendritic cells by a mechanism involving TLR-2, CD14 and MyD88. So, it is possible that this polysaccharide is a TLR2-activating molecule representing typical PAMP in *P. boydii*. Glucosyleceramides having highly conserved structures are involved in fungal development. In this context, specific antibodies against *P. boydii* CMHs were able to arrest the fungal growth and differentiation. Assuming the concept that sphingolipid ordered membrane microdomains contribute to the differential trafficking of cell surface components [87], it is reasonable that antibodies to CMHs could impair the transport to the cell wall of molecules involved in conidia to hyphae transition. Extracellular proteases described in *P. boydii* were able to cleave human serum proteins, extracellular matrix components as well as sialylated proteins. These hydrolytic properties could help the fungus to obtain amino acids for its nutrition, to escape from human defenses, and to disseminate through host barriers. Ecto-phosphatase activities were also characterized in intact mycelial forms of *P. boydii*, but the relevance of these enzymes in biology or pathogenesis of this fungus is still unclear. In addition, the Cu, Zn-SOD enzyme can help the fungus during the oxidative response generated by the host phagocytic cells, minimizing the toxic effect of superoxide anions.

Determination of structural and functional aspects of the abovementioned molecules could contribute to the design of new agents capable of inhibiting fungal growth and/or differentiation and will provide critical information in understanding the nature of host-fungus interactions.

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