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Vesicle and Vesicle-Free Extracellular Proteome of *Paracoccidioides brasiliensis*: Comparative Analysis with other Pathogenic Fungi

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Abstract

Microorganisms release effector molecules that modulate the host machinery enabling survival, replication, and dissemination of a pathogen. Here we characterized the extracellular proteome of *Paracoccidioides brasiliensis* at its pathogenic yeast phase. Cell-free culture supernatants from the Pb18 isolate, cultivated in defined medium, were separated into vesicle and vesicle-free fractions, digested with trypsin and analyzed by liquid chromatography-tandem mass spectrometry. In vesicle and vesicle-free preparations we identified, respectively, 205 and 260 proteins with two or more peptides, including 120 overlapping identifications. Almost 70% of the sequences were predicted as secretory, mostly using non-conventional secretory pathways, and many have previously been localized to fungal cell walls. A total of 72 proteins were considered as commonly transported by extracellular vesicles, considering that orthologues have been reported in at least two other fungal species. These sequences were mostly related to translation, carbohydrate and protein metabolism, oxidation/reduction, transport, response to stress, and signaling. This unique proteomic analysis of extracellular vesicles and vesicle-free released proteins in a pathogenic fungus provides full comparison with other fungal extracellular vesicle proteomes and broadens the current view on fungal secretomes.

Keywords

Extracellular vesicle proteome; *Paracoccidioides brasiliensis*; pathogenic fungus; proteomic analysis; vesicle-free released proteins

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Supporting Information:

Supplementary tables containing the complete set of results. This material is available free of charge via the Internet at <http://pubs.acs.org>. All LC–MS/MS raw datafiles were deposited on Tranche database (www.proteomecommons.org) under hash: 3CQXyXQ9XtCZNse0wa0i0CH4yHCHUizwN8VxRUynHYWCAqHUtFn9o9f3W0d/qAMmz w +INzkob2KonE8cRAL3E9Go08AAAAAAACDA==

INTRODUCTION

Paracoccidioidomycosis (PCM) is a systemic granulomatous mycosis that occurs as an active disease in up to 2% of infected individuals living in endemic areas of Latin America.¹ The disease is caused by isolates of the *Paracoccidioides brasiliensis* complex² and of the newly classified species *P. lutzii*.³ The PCM infection occurs upon inhalation of fungal conidia produced by environmental mycelia, after subsequent transformation to the yeast pathogenic phase in the pulmonary alveoli. Lungs are generally the main organs affected; however, dissemination to other sites is also common. In juvenile forms of PCM, progression is rapid throughout organs of the lymphatic system. Modulation of the immune system is a key element to determine the infection onset and cellular immunity is the main source of host protection.⁴ Fungal components that elicit immune response and/or promote other types of interaction with the host are also responsible for shaping the virulence of different isolates.

Bacteria, fungi, and parasites release effector molecules that modulate the immune system and the host-parasite relationship, enabling survival, replication, and dissemination of a pathogen within the host. Classically, secreted proteins in eukaryotic cells bear an *N*-terminal signal sequence that targets to the endoplasmic reticulum-Golgi pathway of secretion.⁵ Leaderless or non-classical released proteins use different routes of transportation outside the plasma membrane that have partially been unraveled.⁶ One alternative route is through exosomes, which derive from intracellular multivesicular bodies.⁷ We have recently characterized extracellular vesicles isolated from culture supernatants of *P. brasiliensis* at the pathogenic yeast phase cultivated in defined medium.⁸ We observed that 100,000g-vesicle pellets contain antigenic components revealed by immunoblots with sera from PCM patients. We demonstrated that these vesicles carry highly immunogenic α -galactopyranosyl (α -Gal) epitopes, which are localized both at the vesicle surface and inside the lumen. At least part of these α -Gal residues is contained in *O*-linked oligosaccharides that ornament vesicle protein(s). Anti- α -Gal antibodies from patients with Chagas disease can agglutinate and evoke lysis of its etiological agent, the protozoan parasite *Trypanosoma cruzi*.⁹ Similarly to *P. brasiliensis*, *T. cruzi* also transports components carrying α -Gal epitopes in extracellular vesicles.¹⁰

Fungal extracellular vesicles have recently been recognized as important structures for the *trans*-cell wall transport of virulence factors that modulate host's immune response.¹¹ Electron microscopy analysis identified heterogeneous populations of extracellular vesicles originally in *Cryptococcus neoformans*,¹² which is an opportunistic Basidiomycete yeast that causes meningitis. Covering the polysaccharide cell wall that surrounds the plasma membrane in fungi, *C. neoformans* bears a thick capsule mainly composed by large chains of glucuronoxylomannan (GXM), which is the fungal most important virulence factor and immunomodulator. Not coincidentally, GXM is a major constituent of cryptococcal-released vesicles both in culture media and during *in vitro* macrophage infection.^{12,13} Melanin, which is a common virulence factor among fungi,¹⁴ and monohexosylceramide (CMH), a membrane glycolipid found in *C. neoformans* cell wall¹⁵ that is important in fungal infection,¹⁶ have also been detected in *C. neoformans* vesicles.^{12,17}

Besides polysaccharides, pigment, and glycolipids, fungal extracellular vesicles carry a multitude of proteins. Enzymatic activities of virulence factors, such as laccase (that synthesizes melanin), urease, and phosphatase, have been described in extracellular vesicles in *C. neoformans*.¹⁸ Proteomic analysis of extracellular vesicles by liquid chromatography-tandem mass spectrometry (LC-MS/MS) led to the identification of 101 proteins in *C. neoformans*¹⁸ and 279 proteins in *Histoplasma capsulatum*.¹⁹ *H. capsulatum* and *P. brasiliensis* are phylogenetically related thermo-dimorphic Ascomycetes that evoke

granulomatous systemic mycoses with comparable traits. Using a similar strategy, a comparative extracellular vesicle proteomic analysis was carried out in the non-pathogenic Ascomycete *Saccharomyces cerevisiae*.²⁰ The study addressed the biogenesis of fungal extracellular vesicles by comparing a set of mutants and their respective wild-type cells and resulted in the identification of 400 proteins. In all studies mentioned above, the authors identified proteins of diverse nature, e.g., related to metabolism, stress, translation, signaling, and defense mechanisms. Part of these proteins has been associated previously with *C. neoformans* virulence, supporting an important role for extracellular vesicles as transport vehicles of microorganism modulators to distant sites inside the host.¹¹

Reports on extracellular proteomes comparing extracellular vesicles and vesicle-free proteins are rare,²¹ and so far have not been described in fungi. In the present work we characterized by LC-MS/MS the proteins found in vesicle and vesicle-free preparations of culture supernatant fluids from yeast-phase *P. brasiliensis* cultivated in defined medium. We then compared the identified proteins in *P. brasiliensis* with orthologues previously reported in other fungal extracellular vesicles.¹⁸⁻²⁰ Proteomic analysis was facilitated by the genomic data currently available for isolates Pb18, Pb3 (*P. brasiliensis*), and Pb01 (*P. lutzii*) at the Dimorphic Fungal Database from the Broad Institute site (http://www.broadinstitute.org/annotation/genome/dimorph_collab/MultiHome.html). Pb18 is highly virulent in the mouse model⁴ and was used in our work. By comparing the current *P. brasiliensis* data with orthologues detected in other yeasts we not only provide an overall perspective on fungal vesicle proteins so far reported, but also help broaden the current view on in vitro fungal secretomes.^{22,23}

2. MATERIALS AND METHODS

2.1. *P. brasiliensis* and Growth Conditions

P. brasiliensis, isolate Pb18, recently recovered from BALB/c lungs after experimental infection, was maintained in the yeast phase at 36 °C in slants of modified YPD medium (0.5% yeast extract, 0.5% casein peptone, 1.5% glucose, pH 6.5). For isolation of extracellular vesicles, yeast cells were transferred from 7-day-old slants into Erlenmeyer flasks containing 200 mL of defined Ham's F12 medium (Invitrogen) supplemented with 1.5% glucose (F12/Glc) and cultivated for 4 days at 36 °C with shaking (log-phase pre-inoculum). Cells from four pre-inoculums were sedimented by gravity and the supernatants were discarded. The remaining cells were then inoculated into a single Erlenmeyer flask containing 500 mL of fresh medium and cultivated at high density for another 48 h for vesicle purification. At this point the cells were actively growing (98-99% viability as determined by Trypan blue). It is of note that in our previous publication⁸ a control of extracellular vesicle production using heat-killed cells as pre-inoculum did not show any detectable sterol bands in 100,000 g-pellets by high performance thin-layer chromatography (HPTLC), suggesting lack of background membrane debris.

2.2. Isolation of Extracellular Vesicles

Extracellular vesicles were isolated from culture supernatants following the protocol previously described.¹² All the steps were carried out on ice or at 4 °C to avoid vesicle rupture. Cell-free supernatants were obtained by centrifugation at 4,000 g (15 min), followed by centrifugation at 15,000 g (30 min) to remove smaller debris. Pellets were discarded and the cell/debris-free supernatants were 20-fold concentrated using an Amicon ultrafiltration system (100-kDa cutoff, Millipore). Concentrated supernatants were centrifuged at 15,000 g (30 min) to remove aggregates and the resulting supernatant was then ultracentrifuged at 100,000g for 1 h to precipitate vesicles. Vesicle pellets were washed once in phosphate-

buffered saline solution (PBS), and the final pellets were suspended in PBS and lyophilized for proteomic analysis.

2.3. Protein Digestion

We processed for proteomic analysis a pool of five preparations of: a) extracellular vesicles; b) ultrafiltration flow-through fluids; and c) ultracentrifugation supernatants. All samples were dissolved in water and precipitated with 10% trichloroacetic acid for 20 min on ice to remove salts from previous buffers. Proteins were recovered by centrifuging for 20 min at 16,000 g at 4 °C, and the pellet was washed once with cold acetone. Protein digestion was carried out in organic/aqueous solution as described elsewhere.²⁴ The three samples were denatured at 95 °C for 5 min in buffered methanol/50 mM of NH_4HCO_3 (60:40, v/v); the disulfide bonds were reduced by adding a final concentration of 5 mM dithiothreitol and incubating for 30 min at 37 °C. Cysteine residues were alkylated by adding iodoacetamide to a final concentration of 10 mM and incubating for 90 min at room temperature in the dark. The digestion was performed with 1 µg of sequencing-grade trypsin (Promega), for 16 h at 37 °C. The reaction was terminated by addition of 0.05% trifluoroacetic acid, and the resulting peptides were desalted in a POROS R2 (Applied Biosystems) microcolumn, as described²⁵ and dried in a vacuum concentrator prior to LC-MS/MS analysis.

2.4. LC-MS/MS Analysis and Protein Identification

Tryptic peptides were dissolved in 20 µL 0.1% formic acid (FA), and 5 µL were loaded into a reversed-phase trap column (1 cm × 75 µm, Luna C18, 5 µm, Phenomenex). LC was performed in a capillary reversed-phase column (20 cm × 75 µm, Luna C18, 5 µm, Phenomenex) coupled to a nanoHPLC (1D Plus, Eksigent). Bound peptides were eluted in a linear gradient (5 to 40%) of solvent B (solvent A: 5% acetonitrile, ACN/0.1% FA; solvent B: 80% ACN/0.1% FA) for 200 min and directly analyzed in an electrospray ionization-linear ion trap-mass spectrometer (LTQ XL/ETD, Thermo Fisher) equipped at the front end with a Triversa NanoMate nanospray source (Advion). The nanospray was set at 1.45 kV and 0.25 psi N_2 pressure using a chip A (Advion). MS spectra were collected in centroid mode at the 400-1700 m/z range and the ten most intense ions were subjected twice to collision-induced dissociation (CID) with 35% normalized collision energy, before being dynamically excluded for 60 s.

All MS/MS spectra from peptides with 800 to 3,500 Da, more than 10 counts, and at least 15 fragments were converted into DTA files using Bioworks v.3.3.1 (Thermo Fisher). DTA files were submitted to database search using TurboSequest (Bioworks 3.3.1, Thermo Fisher Scientific). The database used in the analyses consisted of three *Paracoccidioides* genome sequences

(http://www.broadinstitute.org/annotation/genome/dimorph_collab.1/MultiHome.html, Pb01 v1.0; Pb18 v1.0; Pb03 v1.0), besides human (International Protein Index, IPI human v 3.63) and porcine trypsin sequences from the GenBank, forming a database with 219,740 sequences. All the sequences were used in the correct and reverse orientations to enable the estimation of false-discovery rates (FDR). The database search parameters included: i) trypsin cleavage in both peptide termini with one missed cleavage site allowed; ii) carbamidomethylation of cysteine residues as a fixed modification; iii) oxidation of methionine residues as a variable modification; and iv) 2.0 Da and 1.0 Da for peptide and fragment mass tolerance, respectively. TurboSequest outputs were filtered with $\text{DCn} \geq 0.05$, peptide probability ≤ 0.05 , and $\text{Xcorr} \geq 1.5$, 2.0, and 2.5 for singly-, doubly-, and triply-charged peptides, respectively. After filtering, the files were exported into XML formats and the peptide sequences were assembled into proteins using an in-house written script.²⁶ Redundant protein hits were assembled into protein groups. The protein hits were re-filtered with the sum of peptide $\text{Xcorr} \geq 3.0$. The FDR was estimated as described previously.^{18,19}

Five hundred twenty-four proteins were *P. brasiliensis* proteins identified with 0.5% FDR (Table S1, Supporting information). Proteins defined by the highest number of peptides and the spectra detected are shown in Table S2, Supporting Information. To avoid overestimation of enriched functions based on redundant sequences or isoforms, proteins with $\geq 90\%$ identity were removed from our dataset for further analyses. Proteins identified with more than one peptide in at least one fraction were used for further analysis and are shown in Table S3, Supporting Information.

2.5. Bioinformatic Analysis

Venn diagrams were prepared using either the Venn tool, available at <http://omics.pnl.gov/software/VennDiagramPlotter.php>, or the Power Point program for manual drawing. Protein sequences were submitted to glycosylphosphatidylinositol (GPI)-anchor prediction analysis using the FragAnchor algorithm²⁷ available at <http://navet.ics.hawaii.edu/~fraganchor/NNHMM/NNHMM.html>, as described.²⁸ Putative secretory proteins were predicted by the Fungal Secretome Database (FSD; <http://fsd.snu.ac.kr/>), which contains data of putative secreted proteins identified by a pipeline composed of nine prediction programs for secreted proteins in several fungal genomes²⁹. After removing potential false positives, the proteins were predicted as secretory of the following types: SP or SP3, bearing a signal peptide; SL, proteins with predicted subcellular localization; and NS, non-classical secretory proteins according to the SecretomeP 1.0f. This program³⁰ uses a combination of common features from experimentally proven secreted proteins that predict their probability of being extracellular, independent of the mechanism involved. The two best discriminatory features are the number of atoms and the number of positively charged residues, among others like the presence of predicted pro-peptides, regions of low complexity and presence of transmembrane helices.³⁰

All protein sequences were submitted to Gene Ontology (GO) annotation,³¹ using the Blast2GO algorithm <http://www.blast2go.de/>.³² Enrichment analyses of functional clusters of Pb18 proteins in comparison with the genome were carried out using the orthologues from *Aspergillus fumigatus* and matched against the DAVID database v6.7 (Database for Annotation, Visualization and Integrated Discovery)³³ available at <http://david.abcc.ncifcrf.gov/>. Identification of orthologous groups of vesicle proteins from *P. brasiliensis*, *C. neoformans*, *H. capsulatum*, and *S. cerevisiae* was performed using the OrthoMCL software v2.0.2,³⁴ <http://www.orthomcl.org/cgi-bin/OrthoMclWeb.cgi>, with percent match cutoff = 50 and E-value exponent cutoff = -5.

2.6. Enzymatic Assays

Different concentrations of vesicle preparations, expressed as sterol contents in their membranes (estimated with the Amplex Red Cholesterol Assay kit, Invitrogen), were incubated in the presence of 0.2% (10 mM) L-DOPA in PBS for laccase activity determination; or 5 mg/ml of *p*-nitrophenyl phosphate in acetate buffer (pH 5.0) for phosphatase activity determination. The medium for urease activity contained 4% urea, 0.02% yeast extract, 0.002% phenol red, 0.273% KH₂PO₄, and 0.285% Na₂HPO₄. All reaction mixtures were incubated overnight at room temperature in the dark and the results recorded in a spectrophotometer at 405 nm (phosphatase), 450 nm (laccase), or 540 nm (urease). All experiments were performed in triplicates and differences between samples were statistically evaluated by the one-tailed Student's *t*-test.

3. RESULTS

3.1. Overall Characteristics of *P. brasiliensis* Extracellular Proteins

In order to gain information about overall proteins exported to the extracellular milieu by *P. brasiliensis* we analyzed culture supernatants from the Pb18 isolate. Cell-free spent media from yeasts cultivated in defined medium were separated into vesicle (ves) and vesicle-free (ves-free) fractions, digested with trypsin and analyzed by LC-MS/MS. Vesicle proteome data correspond to the analysis of a pool containing five extracellular vesicle whole preparations (ves). Vesicle-free proteome is presented as combined data obtained from an aliquot of a pool of the corresponding a) ultrafiltration flow-through fluids and b) ultracentrifugation supernatants (details in Materials and Methods). Proteins identified with more than one peptide in either ves or ves-free fractions are denoted as “Yes” in Table S3, Supporting Information. We believe that by using a cutoff of one peptide for protein identification we are excluding eventual intracellular minor contaminants. Eighty-five proteins were identified exclusively in vesicle fractions, against 140 that were detected solely in ves-free fractions; we also found 120 sequences that have overlapped both fractions (Figure 1A and Table S3, Supporting Information).

We then searched the FSD²⁹ for predicted secretory proteins. Predicted secretion types are shown for each protein listed on Table S3, Supporting Information. Figure 1B shows that 69% of the ves-free proteins and 61% of the vesicle proteins were rated as secretory; however, only about 10% of the sequences in each fraction had a predicted signal peptide. These percentages take into consideration total proteins identified in each pool, i.e., 205 (ves) and 260 (ves-free). Most of the secretory proteins have been identified as using non-classical pathways of secretion. It is notable that a higher proportion of vesicle (39%) than ves-free (31%) proteins were predicted as non-secretory, suggesting that vesicular transport might be the sole route of extracellular delivery for part of these proteins. In Table S3, Supporting Information, we can observe that many proteins classified as non-secretory by FSD are prevalent in vesicle fractions, especially those related to protein and carbohydrate metabolism, response to stress, signaling process, cell division, transport, and other/unknown functions.

Glycosylphosphatidylinositols are glycolipid anchors that attach proteins to the cell surface. Potential GPI-anchored modified proteins contain a 15-30-mer peptide sequence of mainly hydrophobic amino acids at the C-terminus, preceded by three small amino acid residues that are recognized by the transamidase enzymatic complex, which transfers the glycolipid anchor to the mature polypeptide. In *S. cerevisiae*, GPI-anchored proteins were 4-fold enriched in vesicle preparations when compared to the genome background, suggesting that GPI-anchor modifications could target proteins to be transported within vesicles.²⁰ We presently performed a GPI-anchoring prediction analysis using the FragAnchor algorithm.²⁷ The results showed that 0.98% of *P. brasiliensis* released vesicle proteins (CRAL/TRIO domain-containing protein, PADG_07508, and zinc-regulated transporter 2, PADG_06417) had high probability of being GPI-anchored. This is almost 2-fold higher than the percentage predicted for Pb18 genome proteins. We performed a two-tailed Fisher test with GraphPad (<http://www.graphpad.com/quickcalcs/contingency1.cfm>) that showed that there was no significant enrichment ($p = 0.4011$).

Released proteins identified in *P. brasiliensis* were classified into functional categories using a Blast2GO tool.³¹ This analysis resulted in the annotation of 85.5% of the proteins into one or more GO (gene ontology) categories. Proteins listed in Table S3, Supporting Information, were grouped according to one of their GO categories. Following this classification, *P. brasiliensis* released proteins have diverse functions, such as protein, carbohydrate, lipid, and nucleotide metabolism, translation, response to stress, signaling, cell

division, oxidation/reduction, transport, besides nuclear and cytoskeleton proteins (Table S3, Supporting Information). Signaling, cell division, and transport proteins were concentrated in vesicle-exclusive sequences, probably because they are either transmembrane or tend to be membrane-associated, like signaling proteins.³⁵

3.2. Fungal extracellular vesicle orthologues

We next compared *P. brasiliensis* extracellular vesicle proteins with orthologues present in vesicles from *C. neoformans*,¹⁸ *H. capsulatum*,¹⁹ and *S. cerevisiae*.²⁰ The analysis was performed with the OrthoMCL software that uses the Markov Clustering (MCL) algorithm,³⁴ which was designed to group orthologous sequences in a genome-scale basis; it identifies both orthologues between species and functionally redundant, or “recent”, paralogs within species. The comparison has to be carefully considered due to variations in the LC-MS/MS conditions and amount of sample used in each study, thus although we only focused on the identified proteins, we cannot state that orthologues are not present in the other species. In addition, we compared *P. brasiliensis* sequences identified with two or more peptides with fungal proteins identified with one or more peptides.¹⁸⁻²⁰ We found that 63% of the *P. brasiliensis* vesicle-associated sequences had orthologues in other fungal extracellular vesicles (Figure 2; Table S3, Supporting Information). Among them, 72 were common to *P. brasiliensis* and at least two other species, while 26 were identified in all four species analyzed. Nearly half of the *P. brasiliensis* released vesicle proteins listed in Table S3, Supporting information has vesicle orthologues in *H. capsulatum*. Most of the common vesicle proteins were also detected in ves-free fractions of *P. brasiliensis* supernatants (overlapping group), while 61 ves-free exclusive sequences had orthologues identified in vesicle preparations from the other fungal species. It is also notable that vesicular fungal orthologues were rarely found among hypothetical/predicted proteins of the *P. brasiliensis* “other/unknown” group. A complete list of vesicle orthologue sequences from other fungi, excluding *P. brasiliensis*, can be visualized in Table S4, Supporting Information. Note that only two proteins were described in three other fungal species, but not in *P. brasiliensis* (malate synthase and a protein component of the large (60S) ribosomal subunit).

The 72 *P. brasiliensis* extracellular vesicle sequences mentioned previously that were commonly reported in at least two other fungal species are shown in Table 1. An enrichment analysis of functional clusters of these proteins in comparison with genome proteins using DAVID databases³³ (Figure 3A) showed that groups involved in carbohydrate catabolic process, cell redox homeostasis, translation, citrate cycle, nucleotide/GTP binding and transport were enriched in vesicles over 2-fold when compared to their proportion in the genome. In terms of number of sequences within GO categories, translation and carbohydrate metabolism proteins were the most represented, followed by oxidation/reduction, transport, protein metabolism, response to stress, and signaling processes (Table 1).

Some of these fungal proteins might have clear roles during infection. Proteins with antioxidant activities, such as superoxide dismutase (PABG_03387), mitochondrial peroxiredoxin (PADG_03095), and thioredoxin (PADG_05504), can defend microorganisms against reactive-oxygen species and are important for the survival of intracellular pathogens during infection.³⁶ An orthologue of mitochondrial peroxiredoxin PRX1 (PADG_03095), for e.g., was differentially localized to the cell surface in the pathogenic hyphal phase of *C. albicans*.³⁷ The protein was also localized to the cell wall of *P. brasiliensis* yeasts (Longo et al., unpublished).

It was interesting to observe that cell division proteins (septins) were so far described basically in *P. brasiliensis* alone (Table S3, Supporting Information). Two of them have recently been characterized in *P. brasiliensis*: cdc42³⁸ (cell division control protein,

PABG_04744) interferes in the yeast cell polymorphism and virulence; cdc12³⁹ (septin 4, PABG_05403) has been localized to the cell surface with a punctuated pattern suggestive of delivery in vesicles.

3.3. Detection of Enzymatic Activities in Extracellular Vesicles from *P. brasiliensis*

We tested whether *P. brasiliensis* releases vesicles containing active laccase, phosphatase, and urease. We found dose-dependent activities of phosphatase (Figure 4), whereas urease and laccase activities were not observed. Moreover, we could not find urease and laccase sequences in the extracellular vesicle proteome, but we identified a protein with predicted acid phosphatase activity (PADG_02403). In *P. brasiliensis*, a 75-kDa protein was localized at the cell surface bearing phosphatase activity;⁴⁰ however, since its identity is unknown we could not correlate it with our proteomic data. The presence of laccase activity would be expected, since dopamine-derived melanin has been described in *P. brasiliensis* yeasts.¹⁴ On the other hand, extracellular urease activity has been described by our group as contributing to increased culture supernatant pH during late-log phase of growth.⁴¹

4. DISCUSSION

This is an original description of a fungal extracellular proteome taking into consideration individual proteomic analyses of extracellular vesicle and vesicle-free preparations of culture supernatants. Our fungal model was *P. brasiliensis*, whose results were then compared with orthologous sequences described so far in extracellular vesicle proteomes from other fungi.¹⁸⁻²⁰ Among the *P. brasiliensis* culture supernatant proteins here predicted to be secretory by the FSD,²⁹ 50% would use non-classical means of transportation (leaderless proteins). Based on the current knowledge, Nickel (2005)⁴² proposed four mechanisms for leaderless protein secretion: (i) export through the release of exosomes, (ii) direct translocation from the cytoplasm mediated by plasma membrane transporters, (iii) lysosomal secretion, and (iv) export mediated by plasma membrane blebbing. Fungal extracellular vesicle preparations are composed of heterogeneous populations differing in size and probably also in content,^{11,20} which we now show to partially vary among species as well. In addition, recent results pointed to the existence of multiple pathways of vesicle-mediated unconventional secretion in fungi, specifically, classical Golgi-derived exocytosis, exosome-related secretion, autophagy- and vesicle-dependent unconventional secretion.^{20,43} In *S. cerevisiae*, about 25% of vesicle proteins were predicted to bear a signal peptide, which represented an enrichment of about 3-fold when compared to whole cells. That added evidence that the Golgi apparatus could be involved in released vesicle biogenesis.²⁰ In our overall analysis of fungal extracellular vesicle proteins (Table 1), 12.5% of the sequences had predicted signal peptide, including, for instance, an aminopeptidase (PAAG_09004), GTP-binding protein rho1 (PADG_08337), and superoxide dismutase (PABG_03387), thus corroborating this hypothesis.

Among the *P. brasiliensis* extracellular proteins here identified, 35% were detected in both vesicle and vesicle-free preparations. We believe that at least part of the overlapped sequences might be the result of vesicle-free proteins specifically bound, or possibly aggregated to, vesicles during the preparation process, especially during culture supernatant concentration. Very likely, though, is that proteins could have been released free from damaged vesicles during the purification process or even during growth. It is a fact that the yield of vesicle preparations is low and that the amount of protein in vesicle-free preparations is much higher. On the other hand, we now show that the extracellular vesicle content, which probably varies among different heterogeneous populations,^{11,20} partially varies among species as well. The existence of multiple pathways of vesicle-mediated unconventional secretion, as discussed above, would justify the vesicle protein heterogeneity observed intra- and inter-fungal species. That might also help to explain why we presently

verified a number of orthologues of *P. brasiliensis* vesicle-free exclusive sequences in vesicle preparations from other yeasts. It is also possible that not all vesicle populations are pelleted after 1 h of ultracentrifugation. This hypothesis has to be tested, since it could justify the finding of membrane proteins in vesicle-free fractions. An enrichment in extracellular vesicles of proteins that are predominantly cytoplasmic may also occur during the inward budding process of multivesicular body formation that randomly engulfs small portions of the cytosol,⁷ as well as through a vesicle budding process that happens at the plasma membrane level in case of microvesicles.⁴⁴ On the other hand, many of the extracellular proteins described here have mitochondrial function, and that could be the result of an autophagic delivery of cargoes.⁴⁵ Note that the above speculations are far from trying to define vesicle biogenesis, considering we are probably detecting vesicle proteins deriving from diverse pathways.

Our proteomic analysis of culture supernatants from the *P. brasiliensis* pathogenic phase revealed a numerous and complex composition of proteins with diverse biological functions such as protein and carbohydrate metabolism, translation, response to stress, signaling, cell division, oxidation/reduction, and transport, corroborating previous reports.¹⁸⁻²⁰ By comparing the current *P. brasiliensis* data with fungal orthologues detected in these reports we not only provided an overall perspective on fungal extracellular vesicle proteins so far reported, but also helped to broaden the current understanding of in vitro fungal secretomes from pathogenic fungi,^{22,23,46} considering that we analyzed vesicle and non-vesicle released proteins. Secretomes of whole cell-free supernatants have recently been reported in pathogenic *H. capsulatum*²² and *Candida albicans*.^{23,46} Holbrook et al. (2011)²² used very stringent criteria to define 33 proteins as extracellular in the *H. capsulatum* yeast phase with high confidence degree. Among them, 23 are orthologous to sequences here detected in both vesicle and vesicle-free preparations. Sorgo et al. (2010)²³ compared *C. albicans* proteins from various growth conditions, differing in pH, temperature and presence of the hyphal inducer *N*-acetylglucosamine. The analysis revealed that growth conditions strongly affected the composition of the secretome, suggesting that released proteins could be involved in the adaptation and survival in different environments. Out of 51 extracellular proteins reported in *C. albicans*, only 13 are orthologous to those described here for *P. brasiliensis*, including glycosidases, proteinases, and superoxide dismutase.

We propose that the orthologues similarly detected in at least three distinct yeast extracellular vesicle proteomes (Table 1) commonly use this means of transportation towards the cell surface in different fungal species. It is worth mentioning that so far we have been unable to detect vesicles in the supernatants of the mycelium phase of *P. brasiliensis* (Silva and Puccia, unpublished results) and we have not yet characterized the mycelium-released proteins either. Included in these yeast vesicle proteins are proteins that could neutralize host defense mechanisms, as mentioned earlier for oxidation/reduction components, and others that could potentially help fungal dissemination, like proteases and lipases. A subtilase-type proteinase psp3 (PADG_07422) was common to three fungal released vesicle proteomes. We have previously characterized an exocellular serine-thiol (subtilase type) proteinase activity (PbST) in the yeast phase of *P. brasiliensis* that can selectively cleave components of the basal membrane of the extracellular matrix,⁴¹ therefore showing potential role in fungal dissemination. The identity of PbST could be proteinase psp3, since it shows a free cysteine residue in its sequence; however, this possibility still needs further experimental evidence. We can also cite four proteins within the carbohydrate metabolism GO group that have been microscopically localized to the *P. brasiliensis* cell wall and are implicated in adhesion to matrix-associated components, specifically, glyceraldehyde-3-phosphate dehydrogenase (PADG_02411),⁴⁷ enolase (PADG_04059),⁴⁸ gp43 (PADG_07615),^{49,50} and triosephosphate isomerase (PADG_06906).⁵¹

Gp43 is the best studied molecule in *P. brasiliensis* for its antigenic, immunomodulatory and protective properties. Although the molecule is in the group of beta-1,3-exoglucanases, it is not enzymatically active, and it has peculiar characteristics seen only in isolates of the *P. brasiliensis* complex.⁵² Gp43 has a signal peptide and can be abundantly found in the culture supernatants of some isolates, as well as partly on the cell wall.⁵³ Transmission electron microscopy images show clusters of immunogold labeling with monoclonal antibodies outward from the cell wall, suggestive of being inside vesicles. A similar profile is depicted for histone 2B near the *H. capsulatum* cell wall.⁵⁴ Orthologues of both molecules were identified in fungal supernatant vesicles (Table 1).

Extracellular vesicles seem to also export enzymes implicated in the carbohydrate architecture of the cell wall. We identified a cell wall alpha-1,3-glucan synthase MOK1-like (PADG_03169) in *P. brasiliensis* vesicles that has not been described in other fungal vesicle proteomes, in spite of the fact that both *H. capsulatum* and *C. neoformans* have high proportions of alpha-1,3-glucans in their cell wall. In addition, signaling GTP-binding protein Rho1 (PADG_08337) was found in all yeast vesicle proteomes; it is involved in cell wall integrity pathways as regulator of beta-1,3-glucan synthases in *C. neoformans*,⁵⁵ *S. cerevisiae*,⁵⁶ and *P. brasiliensis*.⁵⁷

In this study we described an original proteomic analysis of extracellular vesicles and vesicle-free released proteins in a pathogenic fungus, which also provided full comparison with other fungal vesicle proteomes. We therefore believe that our work broadens the current view on fungal secretomes and helps understand the finding of some proteins with intracellular functions on the cell wall.^{58, 59, 60}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A, Venn diagrams showing the number of proteins detected in vesicle and vesicle-free (ves-free) fractions of the Pb18 extracellular proteome. **B**, Proportion of proteins in vesicle (ves) and ves-free preparations classified as secretory or not (**no**) when using the FSD: **SP** (containing signal peptide identified by SignalP 3.0), **SP3** (bearing signal peptide predicted by SigPred, SigCleave or RPSP), **SL** (sub-cellular localization predicted by PSort II and/or Target 1.1b), and **NS** (non-classical, predicted by SecretomeP 1.0f).

Figure 2. Analysis of orthologous proteins found in fungal extracellular vesicles. The Venn diagram shows the number of proteins detected in extracellular vesicle preparations from *P. brasiliensis* (this work) and orthologues described in vesicles from *H. capsulatum*¹⁹, *C. neoformans*¹⁸ and *S. cerevisiae*.²⁰ Overlapping groups are shown in gray scale.

Figure 3.

Functional categorization of fungal extracellular vesicle proteins commonly found in *P. brasiliensis* and at least two other species (total of 72, Table 1); * $p \leq 0.05$. The figure shows enriched functional clusters in comparison with the genome, represented by bars of fold-increase.

Figure 4. Phosphatase activity (A_{405}) at different Pb18 extracellular vesicles concentrations expressed as equivalents of sterol contents. Background values were subtracted. * $p \leq 0.05$.

Table 1

List of *P. brasiliensis* extracellular vesicle (ves) non-redundant sequences identified with two or more peptides in Pb18 culture supernatants, and commonly found in at least two other fungal species. The groups were organized according to GO functions. Accession number of *P. brasiliensis* sequences and their orthologues in reported vesicle proteins from *H. capsulatum*¹⁹, *C. neoformans*¹⁸, and *S. cerevisiae*²⁰ are indicated. The presence (Yes) or absence (NF) in vesicle-free fractions of *P. brasiliensis* culture supernatants is indicated. ND*, identified with one peptide. The FSD classification (see Figure 1 for details) is included. The gray scale indicates proteins exclusively detected in *P. brasiliensis* vesicles (dark gray), or in both fractions (medium gray). (accession number*) indicates that there are other orthologues in the group.

FSD class	<i>P. brasiliensis</i>	Protein identification	ves	ves-free	<i>H. capsulatum</i>	<i>C. neoformans</i>	<i>S. cerevisiae</i>
protein metabolism							
1	-	PABG_04656	Yes	NF*	HCAG_07418	CNAG_04601	YLR058C
2	SP3	PAAAG_09004	Yes	NF*	HCAG_06935		YHR047C*
3	NS	PABG_07587	Yes	NF*	HCAG_05565	CNAG_01890	YER091C
4	SP	PADG_07422	Yes	Yes	HCAG_00635		YEL060C
5	NS	PADG_06488	Yes	Yes	HCAG_04485*		YDR155C*
6	NS	PADG_01605	Yes	Yes	HCAG_06019	CNAG_00370	
7	NS	PADG_01404	Yes	Yes	HCAG_08678		YLR027C
carbohydrate metabolism							
8	NS	PADG_04604	Yes	NF*	HCAG_05000	CNAG_00965	YBR117C*
9	NS	PADG_00668	Yes	NF*	HCAG_00010		YKL060C
10	-	PADG_06906	Yes	Yes	HCAG_02511		YDR050C
11	NS	PADG_02411	Yes	Yes	HCAG_04910	CNAG_06699	YGR192C*
12	-	PADG_04059	Yes	Yes		CNAG_03072	YGR254W*
13	SP	PADG_07615	Yes	Yes		CNAG_00799	YLR300W
14	NS	PADG_07950	Yes	Yes	HCAG_03191		YCL040W
15	-	PADG_01896	Yes	Yes	HCAG_03385	CNAG_03358	YCR012W
16	-	PADG_07420	Yes	Yes	HCAG_00638		YLR354C
17	NS	PADG_04898	Yes	Yes	HCAG_05266		YLR304C
18	-	PADG_08387	Yes	Yes	HCAG_06981	CNAG_00061	YNR001C
19	NS	PADG_00451	Yes	Yes	HCAG_08202		YBR196C
20	-	PADG_04148	Yes	Yes	HCAG_04277		YGL156W

FSD class	<i>P. brasiliensis</i>	Protein identification	ves	ves-free	<i>H. capsulatum</i>	<i>C. neoformans</i>	<i>S. cerevisiae</i>
lipid metabolism							
21	NS	PADG_01363 conserved hypothetical protein (107 aa)	Yes	Yes	HCAG_08732	CNAG_06140	
translation							
22	NS	PABG_03350 60S ribosomal protein L35 (127 aa)	Yes	NF*	HCAG_03415	CNAG_00771	
23	NS	PADG_02329 60S ribosomal protein L9-A (193 aa)	Yes	NF*		CNAG_00034	YGL147C
24	NS	PADG_04731 ribosomal protein L14 (148 aa)	Yes	NF*		CNAG_04799	YKL006W
25	NS	PADG_07685 40S ribosomal protein S13-1 (152 aa)	Yes	NF*		CNAG_01153	YDR064W
26	NS	PADG_07803 60S ribosomal protein L12 (166 aa)	Yes	NF*		CNAG_01480	YDR418W
27	NS	PADG_00942 40S ribosomal protein S7 (202 aa)	Yes	NF*	HCAG_06613		YOR096W
28	-	PADG_01220 conserved hypothetical protein (218 aa)	Yes	Yes	HCAG_07708	CNAG_06095	
29	NS	PADG_01427 40S ribosomal protein S12 (152 aa)	Yes	Yes	HCAG_06308		YOR369C
30	NS	PADG_02828 60S ribosomal protein L10a (218 aa)	Yes	Yes	HCAG_01850	CNAG_02144	
31	NS	PADG_06265 elongation factor 1-gamma 1 (408 aa)	Yes	Yes		CNAG_00417	YKL081W
32	NS	PADG_02445 40S ribosomal protein S15 (154 aa)	Yes	Yes		CNAG_06633	YOL040C
33	NS	PADG_05721 60S ribosomal protein L4-A (373 aa)	Yes	Yes	HCAG_00468	CNAG_04762	YBR031W
34	-	PAAAG_00594 elongation factor 2 (832 aa)	Yes	Yes	HCAG_05988		YOR133W
35	-	PADG_00692 elongation factor 1-alpha (461 aa)	Yes	Yes	HCAG_08798	CNAG_06125	YPR080W
36	NS	PADG_02142 ribosomal protein L5 (300 aa)	Yes	Yes	HCAG_08444		YPL131W
37	SP	PADG_02446 hypothetical protein (114 aa)	Yes	Yes		CNAG_05762	YDR382W
38	-	PADG_01407 40S ribosomal protein S3 (271 aa)	Yes	Yes	HCAG_00214		YNL178W
response to stress							
39	-	PADG_07715 heat shock protein (672 aa)	Yes	Yes	HCAG_04686	CNAG_06150	YMR186W*
40	-	PADG_08369 heat shock protein (596 aa)	Yes	Yes	HCAG_06961	CNAG_03891	YLR259C
41	-	PADG_08118 hsp70-like protein (655 aa)	Yes	Yes	HCAG_05805	CNAG_00334*	YAL005C*
42	SP	PADG_03562 hsp70-like protein (678 aa)	Yes	Yes	HCAG_05805	CNAG_00334*	YAL005C*
43	NS	PADG_00430 heat shock protein SSC1 (681 aa)	Yes	Yes	HCAG_08176		YJR045C
44	-	PADG_02785 heat shock protein Hsp88 (728 aa)	Yes	Yes	HCAG_00783		YBR169C*
signaling process							

FSD class	<i>P. brasiliensis</i>	Protein identification	ves	ves-free	<i>H. capsulatum</i>	<i>C. neoformans</i>	<i>S. cerevisiae</i>
45	SP3	PADG_01243 rab GDP-dissociation inhibitor (469 aa)	Yes	NF*	HCAG_07830		YER136W
46	NS	PADG_02833 ADP-ribosylation factor (184 aa)	Yes	NF*	HCAG_04840		YDL192W
47	SP3	PADG_08337 GTP-binding protein rho1 (192 aa)	Yes	NF*	HCAG_05560	CNAG_03315	YGR152C
48	NS	PADG_07524 nucleoside diphosphate kinase (153 aa)	Yes	Yes	HCAG_00544	CNAG_04577	YKL067W
49	-	PABG_01564 inorganic pyrophosphatase (465 aa)	Yes	Yes	HCAG_04307		YBR011C
50	-	PADG_04440 14-3-3-like protein 2 (245 aa)	Yes	Yes	HCAG_04527	CNAG_05235	YDR095W*
oxidation reduction							
51	SP	PABG_03387 superoxide dismutase (214 aa)	Yes	NF*	HCAG_01543*		YHR008C
52	NS	PADG_03651 6-phosphogluconate dehydrogenase (492 aa)	Yes	NF*	HCAG_05884	CNAG_02620	YGR256W*
53	NS	PADG_03095 mitochondrial peroxiredoxin PRX1 (223 aa)	Yes	Yes	HCAG_06210	CNAG_03482*	YML028W
54	SP	PAAAG_00986 disulfide isomerase Pdi1 (539 aa)	Yes	Yes	HCAG_03630		YCL043C
55	NS	PADG_07210 malate dehydrogenase (341 aa)	Yes	Yes	HCAG_03969	CNAG_03225	YKL085W
56	-	PABG_04154 NADP-specific glutamate dehydrogenase (458 aa)	Yes	Yes	HCAG_05651	CNAG_01577	YAL062W*
57	NS	PADG_05504 thioredoxin (118 aa)	Yes	Yes		CNAG_02801	YGR209C
58	-	PADG_06671 3-isopropylmalate dehydrogenase A (365 aa)	Yes	Yes	HCAG_02260	CNAG_00450	YCL018W
transport							
59	NS	PADG_08342 GTP-binding protein YPTM2 (162 aa)	Yes	NF*	HCAG_06999		YFL038C
60	-	PADG_08391 plasma membrane ATPase (930 aa)	Yes	NF*	HCAG_06977	CNAG_06400	YGL008C
61	NS	PABG_07782 conserved hypothetical protein (311 aa)	Yes	NF*	HCAG_07936		YMR183C
62	-	PAAAG_08620 ADP-ATP carrier protein (310 aa)	Yes	Yes	HCAG_06283	CNAG_06101	YBL030C
63	NS	PAAAG_06268 cytochrome c (112 aa)	Yes	Yes	HCAG_05938		YJR048W
64	-	PADG_07042 ATP synthase subunit 5 (231 aa)	Yes	Yes	HCAG_03815	CNAG_01204	
65	-	PADG_02561 ATPase alpha subunit (557 aa)	Yes	Yes	HCAG_02813	CNAG_05750	YBL099W
66	NS	PADG_08349 ATP synthase subunit beta (514 aa)	Yes	Yes	HCAG_06944	CNAG_05918	YJR121W
cytoskeleton proteins							
67	NS	PAAAG_03031 tubulin beta chain (448 aa)	Yes	Yes		CNAG_01840	YFL037W
68	NS	PADG_06295 actin (347 aa)	Yes	Yes	HCAG_08210	CNAG_00483	YFL039C
nuclear proteins							

FSD class	<i>P. brasiliensis</i>	Protein identification	ves	ves-free	<i>H. capsulatum</i>	<i>C. neoformans</i>	<i>S. cerevisiae</i>
69	-	PADG_07134 histone H4.2 (104 aa)	Yes	Yes	HCAG_03885	CNAG_01648	YBR009C
70	NS	PADG_05907 histone H2B type 1-A (142 aa)	Yes	Yes	HCAG_03525		YBL002W
other/ unknown function							
71	-	PADG_01509 DUF907 domain-containing protein (746 aa)	Yes	NF*	HCAG_03638		YAL053W*
72	NS	PADG_04288 L-PSP endoribonuclease family protein (Hmf1) (168 aa)	Yes	Yes	HCAG_04397		YER057C*