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Original article

Comparative proteomics in the three major human pathogenic species of the genus *Sporothrix*

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ABSTRACT

Sporotrichosis is a subcutaneous mycosis of humans and other mammals, caused by dimorphic species of the genus *Sporothrix*. In Brazil, human disease is broadly linked to transmission by infected cats and is mainly caused by *Sporothrix brasiliensis*, *Sporothrix schenckii* and *Sporothrix globosa*. In this study, we used a nanoscale liquid chromatography coupled with tandem mass spectrometry approach to provide the yeast proteomic profiles of *S. brasiliensis*, *S. schenckii* and *S. globosa*. From a total of 247 identified proteins, 137 were found as differentially expressed. Functional classification revealed that most are related to carbohydrate and amino acid metabolism as well as stress response. Our data indicate that *S. brasiliensis* metabolism is distinct of that of *S. schenckii* and *S. globosa,* mainly regarding amino acid metabolism and cell wall remodeling, which are induced in the former. Enzymes belonging to glycolytic pathway are, on the other hand, up-regulated in *S. schenckii* and *S. globosa*. These findings may explain the previously described more virulent character of *S. brasiliensis*. Besides complementing genomic comparisons already published, this first comparative proteomic study provided information that indicates new aspects of *Sporothrix* species metabolism as well as offers information that may be useful in the development of prospective functional studies.

Several *Sporothrix* pathogenic species cause sporotrichosis, a subcutaneous cosmopolitan mycosis, with high incidence in Latin America and East Asia. These are thermo-dimorphic fungi that grow as a mycelial form in environmental conditions and as a yeast-like form during parasitism or when cultured in enriched media at 37 °C. In Brazil, the cases of sporotrichosis have significantly increased in the last two decades, especially in the metropolitan area of Rio de Janeiro, Brazil, and they are specially caused by the species *Sporothrix brasiliensis*, *Sporothrix schenckii* sensu stricto and *Sporothrix globosa* [1]. More recently, sporotrichosis has spread to several other areas of the country, including states from Northeast and Mid-Western Brazil [2].

Classically, cutaneous sporotrichosis develops following traumatic inoculation of the skin with soil, plant material, or organic material harboring *Sporothrix* spp. [3]. Humans can also acquire sporotrichosis from infected animals. In Brazil, a large number of cases comes up due to fungal transmission from naturally infected cats to humans [4]. Sporotrichosis has a wide spectrum of clinical manifestations, including the most frequently observed lymphocutaneous and fixed cutaneous forms [5] as well as unusual clinical forms, such as disseminated cutaneous form without an underlying immunosuppressive condition [6], pulmonary infection [7], hypersensitivity manifestations [5], and involvement of the central nervous system [8]. In immunocompromised patients, sporotrichosis can cause severe infection resulting in high rates of hospitalization and death [9].

The broad range of clinical manifestations of sporotrichosis is postulated to be associated with host- and fungal-related factors.

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Among the fungal-related factors, the inoculum size and fungal virulence are the most noteworthy aspects affecting the outcome of sporotrichosis [10]. Some studies have demonstrated differences in virulence potential among the major human pathogenic species of the genus *Sporothrix*: *S. brasiliensis* is considered the most virulent species, while *S. globosa* is the less virulent one. *S. schenckii,* on the other hand, has an intermediate virulence phenotype [11]. Moreover, some differences in clinical manifestations and therapeutic response were observed between sporotrichosis cases caused by *S. brasiliensis* and *S. schenckii* [12]. The genome information of these three major sporotrichosis agents became available in the past years, although only annotated for *S. brasiliensis* and *S. schenckii* [13]. Nevertheless, studies regarding a comparative analysis of gene expression or metabolism of these three sporotrichosis agents, which would explain the differences in virulence and clinical manifestations observed among them, are still scarce.

Some proteomic studies on *Sporothrix* species have been performed, especially with *S. brasiliensis* and *S. schenckii*. Even before the complete *S. schenckii* genome had been published, a proteomic methodology employing two-dimensional (2D) gel electrophoresis was used to study dimorphic transition in this fungus [14]. Moreover, several immunogenic molecules were found in *Sporothrix* yeast cells using 2D gel electrophoresis and pooled sera from individuals with sporotrichosis. A total of eight specific proteins were identified among pathogenic and non-pathogenic isolates such as *S. brasiliensis*, *S. schenckii*, *S. globosa*, and *Sporothrix mexicana*, the ancestral environmental fungus. In the same study, 2D-DIGE characterization suggested that post-translational modifications occur in the major antigen of sporotrichosis, the Gp70 protein, only in the pathogenic species [15]. In the same sense, using proteomics and sera from infected cats, the cross-reactivity among *S. brasiliensis* and *S. schenckii* antigens were detected showing that epitopes may be conserved among closely related species [16]. Additionally, in another immunoproteomic approach, the efficiency of the peptides derived from *S. brasiliensis* immunogenic proteins was tested as vaccines [17]. Proteomic and genomic approaches were also used to compare *Sporothrix* species. The proteome analysis focused on proteins exclusively expressed in *S. brasiliensis* and *S. schenckii*, totalizing 60 and 87 unique proteins in each of these species, respectively. Genome analysis revealed more than 6000 clusters of orthologs conserved in this five *Sporothrix* species: *S. brasiliensis*, *S. schenckii*, *S. globosa*, *Sporothrix pallida,* and *Sporothrix insectorum* [18]. Also, a proteomic descriptive study was performed on extracellular vesicles produced by *S. brasiliensis* and *S. schenckii* yeast cells, which revealed 63 and 40 proteins in vesicles produced by these species, respectively, being four of them common to both fungi [19].

The aim of this study was to provide the large yeast proteomic profiles of the three major pathogenic *Sporothrix* species, using nanoscale liquid chromatography coupled with tandem mass spectrometry approach (NanoUPLC-MS^E). The results depicted an overview of biochemical processes in pathogenic *Sporothrix* fungi, not yet published. This strategy highlighted the potential biological differences among the main agents of sporotrichosis.

1. Material and methods

1.1. Strains and culture conditions

S. brasiliensis (IPEC16490/CBS120339), *S. schenckii* (IPEC27722) and *S. globosa* (IPEC27135), previously characterized at species level by molecular methods [20–22], were used throughout this study. The yeast phase of each strain was maintained in Brain Heart Infusion broth (Difco, Detroit, USA) at 35 °C for 7 days. For protein extraction, 10^6 yeasts/ml of each strain were used to inoculate 250 ml Sabouraud dextrose broth (Difco, Detroit, USA). Cultures were incubated at 35.5 °C in a rotary shaker (BioLab, mod. SL222, Brazil) with constant orbital agitation (150 rpm) for 72 h. Cells were collected by centrifugation (10,000 *g* for 15 min at 4 °C), checked under a brightfield light microscope (Zeiss, mod. Axiolab, Germany) for the presence of at least 95% of yeast cells in the samples, and stored at −80 °C until protein extractions. For growth curves, the three species grown in BHI were transferred to Sabouraud dextrose broth, as described above. Each species was cultured in quadruplicate and cultures were monitored for 144 h, in 12 h intervals, by measuring the optical density at 530 nm in a SpectraMax Plus 384 absorbance microplate reader (Molecular Devices, San Jose, CA, USA) at 35.5 °C.

1.2. Sample preparation and NanoUPLC-MS^E data acquisition

Biological triplicates of *S. brasiliensis*, *S. schenckii* and *S. globosa* were used for protein extraction and proteomic analysis. Cells collected by centrifugation were harvested, resuspended in a solution containing 20 mM Tris−HCl, pH 8.8, 2 mM $CaCl_2$, and disrupted using glass beads and bead beater apparatus (BioSpec, Oklahoma, OK, USA) in five cycles of 30 s, while on ice. Subsequently, the samples were washed with 50 mM ammonium bicarbonate using a 3-kDa molecular weight cut off in ultracel-regenerated membrane (Amicon Ultra centrifugal filter, Millipore, Bedford, MA, USA), in order to change the buffer. The protein concentrations were determined via Bradford assay. Equimolar amounts of proteins from each sample (100 μg) were digested and prepared for nanoscale liquid chromatography coupled with tandem mass spectrometry (NanoUPLC-MS^E) as previously described, in three experimental replicates [23]. Briefly, 50 mM ammonium bicarbonate was added to the samples, followed by addition of 50 μl of RapiGEST™ (0.2% v/v) (Waters Corporation, Milford, MA, USA). The mixture was vortexed and incubated at 80 °C for 15 min. Then, the disulfide bonds were reduced by adding a total of 2.5 μl of 100 mM DTT, followed by incubation at 60 °C for 30 min. In order to alkylate the cysteine residues, the sample was then cooled at room temperature and a total of 2.5 μl of 300 mM iodoacetamide was added, followed by new incubation in dark room for 30 min. A 20 μl aliquot of a 50 ng μl^{-1} solution of trypsin (Promega, Madison, WI, USA), previously prepared using 50 mM ammonium bicarbonate was added to the samples, slightly vortexed, and proteins were allowed to digest at 37 °C, overnight. Following the digestion, a total of 20 μl of 5% (v/v) trifluoroacetic acid was added, at 37 °C for 90 min, in order to hydrolyze the remaining RapiGEST™. The samples were centrifuged at 18,000 *g* at 6 °C for 30 min, and the supernatant was dried in speed vacuum. After that, the peptide mixture was resuspended in 20 mM ammonium formate buffer, and transferred to a Waters Total Recovery vial (Waters Corp). A solution of 1 pmol μl^{-1} MassPREP Digestion Standard [rabbit phosphorylase B (PHB)] (Waters Corp) was used to prepare the final concentration of 400 fmol μl^{-1} of the PHB. The digested peptides were then analyzed via NanoUPLC-MS^E system (Waters Corporation, Manchester, UK). To ensure adequate coverage of the proteome from each sample, the same amount of peptides (in nanograms) of each sample was injected to each mass spectrometric run, in three technical replicates for each sample. Digested peptide mixtures were loaded onto a nanoAcquity UPLC system coupled to the mass spectrometer operated in the MS^E data-independent acquisition mode, as previously described [24]. The first dimension chromatography included a 5 μm NanoEase™ BEH130 C18, 300 μm × 50 mm column (Waters Milford MA, USA). The bound peptides were separated into five fractions eluting at 10, 14, 16, 25 and 65% (v/v) acetonitrile/0.1% (v/v) formic acid gradient. In-line, eluted fractions were trapped in 5 μm Symmetry® C18,

180 μm × 20 mm column (Waters Milford MA, USA). Second dimension chromatography was carried out with a 1.7 μm NanoEase™ BEH130 C18, 100 μm × 100 mm analytical column (Waters Milford MA, USA). All analyses were performed with nanoelectrospray ionization in the positive ion mode nanoESI(+) with a NanoLockSpray source. The double charged ($[M + 2H]^{2+} = 785.8426$) precursor ion $[Glu]^1$-Fibrinopeptide B (GluFib) (Sigma, St. Louis, USA) at 200 fmol.ul^{-1} solution was delivered through the reference sprayer of the NanoLockSpray source and the MS/MS fragment ions of GluFib were used to obtain the final instrument calibration. Data-independent scanning (MS^E) experiments were performed with a Synapt™ G1 HDMS™ System mass spectrometer (Waters, Manchester, UK) with a hybrid quadrupole/time-of-flight (TOF). The radiofrequency applied to the quadrupole mass analyzer was adjusted, such that the ions from *m/z* 50 to 2000 were efficiently transmitted. Moreover, the spectrometer was automatically programmed to switch between low collision energy MS (3 eV) and elevated collision energies MS^E (12−40 eV). The transfer collision cell was adjusted to 1 eV with a scan time of 1.0 s, both in low energy and in high energy, to give a minimum of 10 points in low and high energy above 10% of peak capacity. The TOF analyzer was operated in mode "V" reflection [24,25].

1.3. Proteome data processing

The MS/MS spectra obtained from NanoUPLC-MS^E analysis were processed and searched using the ProteinLynx Global Server version 3.0 (PLGS) (Waters, Manchester, UK). Protein identifications were carried out using algorithms previously described [26,27] and a search against the *S. brasiliensis* genome database [28] available in the NCBI database (https://www.ncbi.nlm.nih.gov/genome/). MS spectra were collected in centroid, de-isotoped, and charge-state-reduced mode to obtain associated product ions and a monoisotopic mass for all peptides. Protein identification criteria included: (i) minimum number fragments ion matches per peptide (2), (ii) minimum number fragments ion matches per protein (5), (iii) minimum number peptide matches per protein (1), (iv) maximum protein mass (600 kDa), (v) trypsin was chosen as the primary digest reagent, (vi) carbamidomethylation of cysteine residues as a fixed modification, (vii) methionine oxidation and phosphoryl STY as a variable modification, (viii) and a maximum 4% false-positive discovery rate, in at least two out of three technical replicate injections. Correct and reversed sequences databases were used to estimate false-positive rates (FPR). Using protein identification replication as a filter, the false positive rate was minimized because false positive protein identifications, i.e., chemical noise, have a random nature and do not tend to replicate across injections. For the analysis of the protein identification and quantification level, the observed intensity measurements were normalized to the intensity measurement of the identified peptides of the digested internal standard $[Glu]^1$-Fibrinopeptide B (GluFib) (Sigma, St. Louis, USA). Peptides and protein tables were generated by ProteinLynx Global Server (PLGS), as previously described [25]. The resulting peptide and proteins were analyzed related to the dynamic range of the experiment, the peptide parts per million error (ppm) and peptide detection type using the softwares MassPivot v1.0.1. and Spotfire v8.0. Microsoft Excel (Microsoft, USA) was also used for table manipulations [25].

1.4. Differentially expressed proteins

In order to determine the differentially expressed proteins, for each species, the protein quantification data were analyzed using the updated version of the software R [29]. For each protein, the obtained quantification (in fentomols) was converted to log2(y+1) scale. Then, the ANOVA and subsequently the Tukey tests were used to compare the protein expression levels among the species, always accepting 0.05 as a significant threshold. The results were demonstrated in tables and heat maps, which were generated by the MultiExperiment Viewer software V.4.8 (www.tm4.org/mev/). All scripts can be obtained on request. Proteins were functionally classified using Pedant on MIPS (http://pedant.gsf.de/) and Uniprot databases (http://www.uniprot.org/), as previously described [23]. To non-characterized proteins, the annotation was performed by homology from the NCBI database (http://www.ncbi.nlm.nih.gov/).

2. Results

2.1. Proteomic analysis

The protein extracts from *S. brasiliensis*, *S. schenckii* and *S. globosa* grown in Sabouraud dextrose broth for 72 h (Fig. S1) were analyzed using nanoscale liquid chromatography coupled with tandem mass spectrometry, NanoUPLC-MS^E. The resulting peptide and protein data generated by the PLGS process are shown in Fig. S2, Fig. S3 and Fig. S4. These experiments resulted in 2512; 1731 and 1332 identified peptides in *S. brasiliensis*, *S. schenckii* and *S. globosa*, respectively. From these, a total of 64.6%, 50.4% and 47.9% of peptides were obtained from peptide match type data (PepFrag1) to *S. brasiliensis*, *S. schenckii* and *S. globosa*, respectively (Fig. S2). A total of 11.7%, 11.4% and 10.7% of total peptides were identified by a missed trypsin cleavage, whereas an in-source fragmentation rate was of 15.0%, 26.1% and 26.8% for *S. brasiliensis*, *S. schenckii* and *S. globosa*, respectively (Fig. S2). Fig. S3 shows the peptide parts per million error (ppm) indicating that the majority, 94.5%, 83.5% and 93.2%, of identified peptides were detected with an error of less than 10 ppm to *S. brasiliensis*, *S. schenckii* and *S. globosa* samples, respectively. The dynamic range detection for all samples was also obtained. The results showed that a 3-log range concentration was considered as a good detection distribution of high and low molecular weights for all samples and fractions, as shown by the graph profile (Fig. S4).

A total of 247 proteins were identified by search against the *S. brasiliensis* genome database. The quantification (*fmol*) obtained for each protein was then converted to the log2(y+1) scale. In order to compare the protein expression levels among the three *Sporothrix* species, ANOVA was employed, followed by Tukey's multiple comparison test. Considering 0.05 as a critical level of significance, 137 proteins were found as differentially expressed (Table S1). Table S2 depicts the expression values as a result of the subtraction between each average of Table S1 and the average of expression of the protein in all the species. The expression values were then used for the elaboration of the heat map shown in Fig. S5.

2.2. Proteomic profile of *Sporothrix* species

Eight of the ten enzymes involved in the glycolytic pathway, except for phosphofructokinase-1 and phosphoglycerate mutase, were identified in the three species. Seven of them, including pyruvate kinase (KIH90234.1), fructose-bisphosphate aldolase (KIH88089.1), enolase (KIH90372.1), triosephosphate isomerase (KIH88287.1), glucose-6-phosphate isomerase (KIH86517.1), phosphoglycerate kinase (KIH87097.1) and glyceraldehyde 3 phosphate dehydrogenase (KIH93501.1), were more abundant in *S. schenckii* and *S. globosa*, when compared to *S. brasiliensis*. Most of these differences were more than 2-fold higher (Fig. 1). Three alcohol dehydrogenases were identified in the *S. brasiliensis* proteome, while two of them were expressed by *S. schenckii* and *S. globosa*. One of the enzymes belonging to the pyruvate dehydrogenase

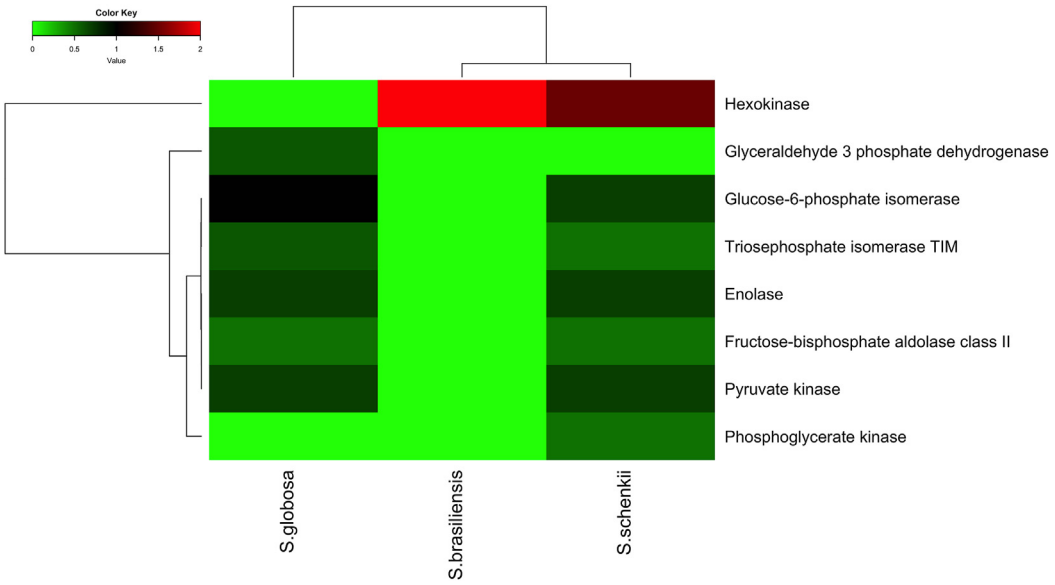

Fig. 1. Expression profile of glycolysis proteins in *S. brasiliensis, S. schenckii* and *S. globosa*. Seven enzymes of glycolytic pathway were induced in *S. schenckii* and *S. globosa*, when compared to *S. brasiliensis*. Green represents down-regulation ratios, while red represents up-regulation.

complex (PDH), the pyruvate dehydrogenase E2 component dihydrolipoamide acetyltransferase (KIH92633.1), as well as four enzymes of the tricarboxylic acid (TCA) cycle (citrate synthase KIH91553.1, aconitate hydratase KIH88979.1, malate dehydrogenase KIH92656.1 and fumarate hydratase KIH91518.1) were expressed by all three *Sporothrix* species, although preferentially in *S. schenckii* and *S. globosa*. The E1 (KIH95018.1) and E2 (KIH89101.1) components of the 2-oxoglutarate dehydrogenase complex were also identified; the first only in *S. brasiliensis* and the second in both *S. brasiliensis* and *S. schenckii* (Fig. 2). Additionally, a thiamine biosynthetic enzyme was preferentially expressed in both *S. schenckii* and *S. globosa*. As thiamine is the precursor of thiamine pyrophosphate, the coenzyme necessary for function of PDH, these findings are in agreement. Enzymes of both oxidative and non-oxidative reactions of the pentose-phosphate pathway, 6-phosphogluconate dehydrogenase (KIH95114.1), transaldolase (KIH91383.1) and transketolase (KIH88049.1), were also more abundant in *S. schenckii* and *S. globosa.* Moreover, as all three *Sporothrix* species induce at least one component of respiratory chain/ATP synthesis, we can assume that they have an aerobic metabolism.

The enzyme UDP-N-acetylglucosamine pyrophosphorylase (KIH89477.1), whose activity generates the precursor for chitin synthesis, was identified in all the proteomes analyzed, although being more abundant in *S. schenckii* and *S. globosa*. Three enzymes involved in the hydrolysis of glucosidic bonds (beta-glucosidase KIH89315.1, endo-1,3(4)-glucanase KIH94423.1 and 1,3-beta-glucosidase KIH89494.1) were found exclusively or with higher

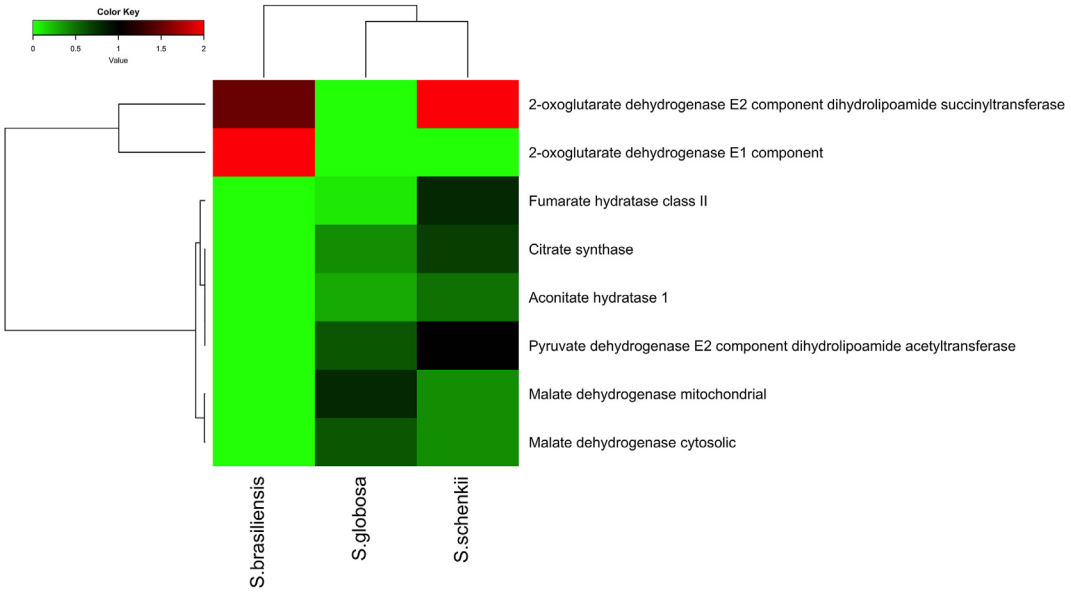

Fig. 2. Differential expression of TCA cycle proteins in *Sporothrix* species. One enzyme of PDH complex as well as some belonging to TCA cycle were up-regulated in *S. globosa* and *S. schenckii*. Green: down-regulation ratios; red: up-regulation.

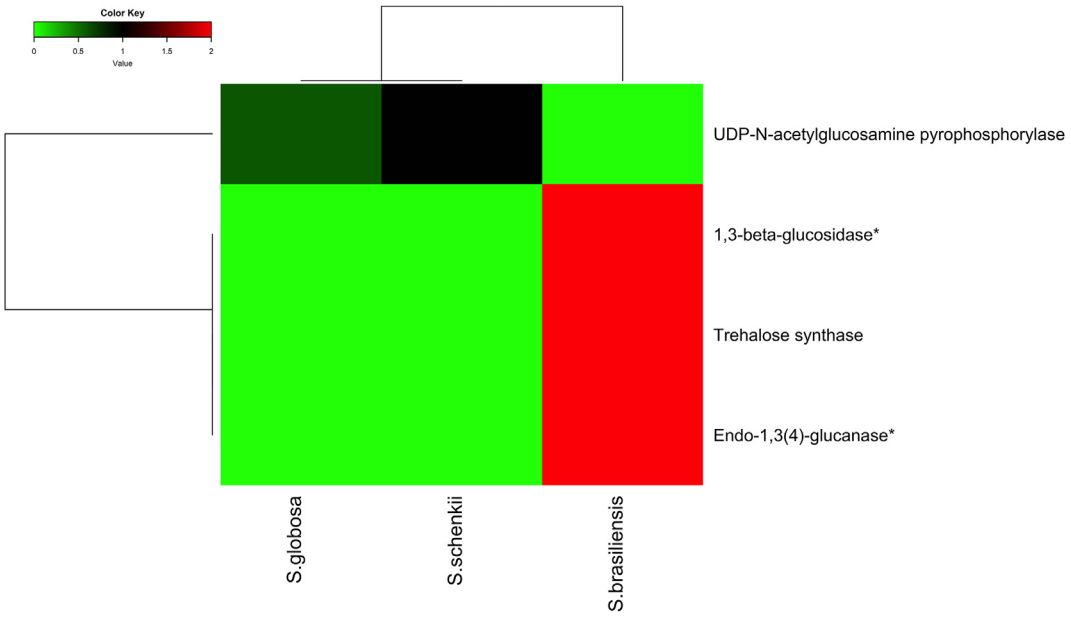

Fig. 3. Expression profile of cell wall related proteins in *Sporothrix* species. Hierarchical clustering showing protein expression levels of enzymes related to the cell wall remodeling. Green represents down-regulation ratios, while red represents protein induction. *: Protein annotation by homology from NCBI database (http://www.ncbi.nlm.nih.gov/).

expression in *S. brasiliensis* (Fig. 3), the only species which expressed trehalose synthase (KIH86407.1).

Nineteen differentially expressed proteins belonged to the amino acid metabolism category. From those, six were exclusively identified in the *S. brasiliensis* proteome and are mainly related to the metabolism of branched chain amino acids (Fig. 4). In general, most of the other proteins are enzymes related to cysteine and methionine metabolism. Cystathionine beta synthase (KIH87885.1) and cystathionine gamma lyase (KIH89602.1), both responsible for the two steps pathway that synthesizes cysteine from homocysteine and serine, were detected in *S. brasiliensis* and *S. schenckii* proteomes, being more abundant in the latter. Of special note was the identification of 4-hydroxyphenylpyruvate dioxygenase (KIH95230.1) in the *S. brasiliensis* proteome. This enzyme is involved in the synthesis of pyomelanin, a soluble melanin that results from tyrosine catabolism [30]. Concerning protein modification, the peptidases Xaa-Pro dipeptidase (KIH90522.1), metallopeptidase MepB (KIH86351.1), peptide-methionine S S oxide reductase (KIH93232.1), Xaa-Pro aminopeptidase (KIH92945.1) and dipeptidyl-peptidase III (KIH86924.1) were overexpressed in *S. brasiliensis*, which also exclusively expressed urease (KIH89566.1).

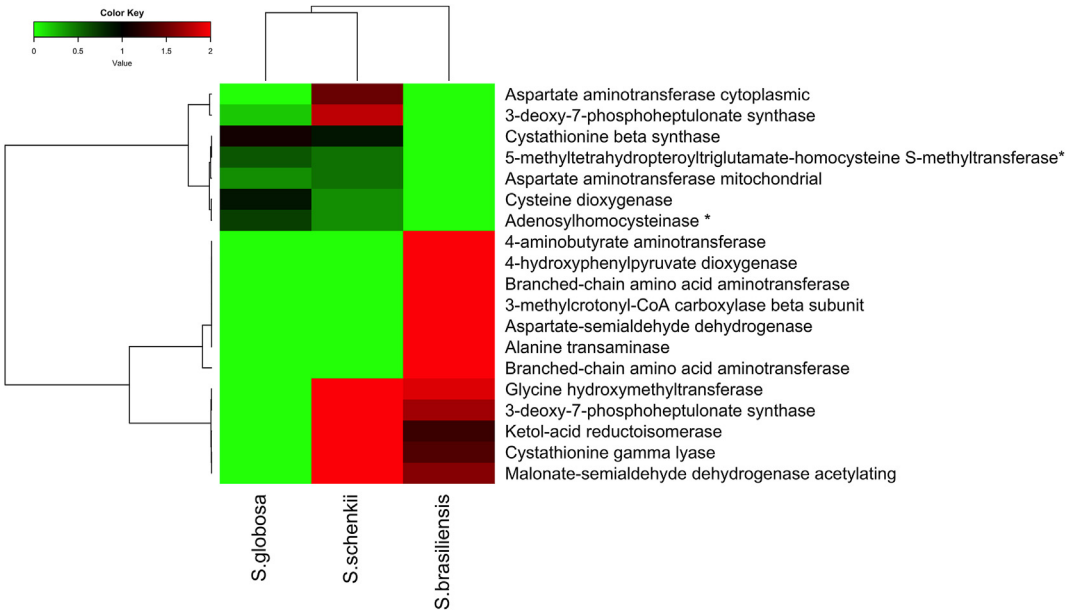

Fig. 4. Amino acid metabolism is induced in *S. brasiliensis*. Most of enzymes involved in the metabolism of amino acids are more abundant in *S. brasiliensis*. Green represents down-regulation ratios, while red represents up-regulation. *: Protein annotation by homology from NCBI database (http://www.ncbi.nlm.nih.gov/).

The three enzymes related to the metabolism of lipids with differential expression among the analyzed species were down-regulated in *S. globosa*. Diphosphomevalonate decarboxylase (KIH90026.1) was more abundant in *S. brasiliensis* and *S. schenckii*, while leukotriene-A4 hydrolase (KIH87873.1) and acetyl-CoA acyltransferase (KIH94193.1) were exclusive of *S. brasiliensis*.

The *Sporothrix* species analyzed here express enzymes involved in the response to oxidative stress, such as catalase (KIH91664.1), superoxide dismutase (KIH91379.1) and peroxidase (KIH91934.1), which were more abundant in *S. globosa*. However, expression of glutathione reductase (KIH90720.1) and glutathione S transferase (KIH91032.1) was identified only in the *S. brasiliensis* proteome. Six heat shock proteins also presented differential expression between the *Sporothrix* species. In general, all of them were down-regulated in *S. brasiliensis*, with a higher expression in *S. schenckii* and *S. globosa*. Levels of chaperonin GroEL (KIH94969.1) were slightly higher in *S. schenckii* than in *S. globosa*, chaperonin GroES (KIH93032.1) presented similar expression in these two species, and heat shock 70 kDa protein 4 (KIH88952.1), molecular chaperone HtpG (KIH87017.1), heat shock 70 kDa protein 5 (KIH94177.1) and heat shock 70 kDa protein 18 (KIH91446.1) were slightly up-regulated in *S. globosa*, in comparison with *S. schenckii*. These proteins are chaperones typically associated to stress conditions, having their expression increased during exposure to heat, cold, ultraviolet light and compounds with antifungal properties [31].

3. Discussion

The genetic and phenotypic differences already reported in the *Sporothrix* genus begun to be better understood after the release of the complete genome sequences of some species. The protein expression pattern is, however, the bottleneck for a most accurate interpretation of such differences. This is the first report of a comparative analysis at the protein level among the three main causative agents of sporotrichosis, since their genomic data became available. Most of the differentially expressed proteins are related to carbohydrate and amino acid metabolism as well as stress response.

A study comparing the cell wall composition of *S. brasiliensis* and *S. schenckii* demonstrated qualitative similarities among polysaccharide and amino acid contents, with a few quantitative differences, depending on the time of culture incubation [32]. The composition changes on dependence of cell morphology, being the percentage of N-acetylglucosamine, the basic unit of chitin, slightly higher in *S. schenckii* yeast-like cells, when compared to the same morphotype of *S. brasiliensis* [33]. This finding may explain the higher abundance of UDP-N-acetylglucosamine pyrophosphorylase in *Sporothrix schenkii* proteome, when compared to *S. brasiliensis*. Enzymes involved in the hydrolysis of glucosidic bonds were overexpressed by *S. brasiliensis*, which is the only species that expresses trehalose synthase whose activity produces trehalose, a disaccharide involved in the resistance to osmotic stress [34]. These findings suggest that cell wall remodeling may be more pronounced in *S. brasiliensis*. Given that *S. brasiliensis* was reported as the most pathogenic species followed by *S. schenckii* and *S. globosa* [11], the cell wall remodeling could be a relevant aspect faced by *Sporothrix* in the environmental adaptation, reflecting in its virulence.

Taking into account that the identified proteins related to amino acid metabolism were all found in *S. brasiliensis* proteome, some in an exclusive way, it seems that this fungus utilizes amino acids as carbon sources more pronouncedly when compared to the other two species. Evidences for this hypothesis include: down regulation of glycolytic enzymes, overexpression of several peptidases as well as the exclusive expression of urease. An *in vitro* proteolytic activity was observed in some strains of *S. brasiliensis*, which was more pronounced in yeast-like cells in comparison with the mycelial form of the fungus. This *in vitro* activity was not detected in the *S. schenckii* or *S. globosa* strains tested [35]. Using a different approach, activities of serine- and cysteine proteases were also observed for a *S. schenckii* strain [36], which would act on the cleavage of peptides, yielding an amino acid supply for this species, which is able to produce some enzymes related to amino acid metabolism. Some peptidases were also detected as immunogenic proteins in *Sporothrix* species [15], highlighting the relevance of this class of proteins to the *Sporothrix* spp. metabolic responses.

S. brasiliensis strains produce higher levels of urease when compared to *S. schenckii* [35]. This molecule has an impact on virulence in other pathogenic fungi. Urease activity confers *Coccidioides posadasii* the ability to exacerbate damage to host lung tissue. Additionally, mutant strain for *URE* gene is easily cleared by mice infected intranasally when compared to the wild type strain [37]. In the non-dimorphic fungus *Cryptococcus neoformans* urease is important for central nervous system invasion since it facilitates the crossing of blood–brain barrier [38]. Moreover, survival of mice infected with the knockout strain was lower than those infected with the wild type strain [39].

We found components of respiratory chain/ATP synthesis in the three *Sporothrix* species under study. Some of these proteins were induced in *S. brasiliensis,* when compared to *S. schenckii* and *S. globosa*. Interestingly, one of them, the F-type H + -transporting ATPase subunit beta, was detected as one of the immunogenic proteins in *Sporothrix* species in a previous study that involved proteomic and human sporotrichosis pooled sera to investigate relevant antigens of sporotrichosis [15]. Taking into account that most amino acids are not a fermentable carbon source, these findings reinforce the suggestion that amino acids metabolism is more pronounced in *S. brasiliensis*.

Another interesting aspect of amino acids metabolism is related to tyrosine. Genes required for tyrosine catabolism are induced under infection conditions in the dimorphic fungus *Talaromyces marneffei* (formerly *Penicillium marneffei*), suggesting that tyrosine may provide an important source of carbon and/or nitrogen during infectious growth [40]. A previous study demonstrated that the tyrosine catabolic cluster gene *hpdA*, which encodes the enzyme 4-hydroxyphenylpyruvate dioxygenase, is induced in *T. marneffei* yeasts, the pathogenic cell type [41]. 4-hydroxyphenylpyruvate dioxygenase, also known as 4-HPPD, catalyzes the conversion of 4-hydroxyphenylpyruvate, the first product of tyrosine catabolism, to 2,5-dihydroxyphenylacetate (homogentisate) [42], and it was detected only in the *S. brasiliensis* proteome in this study (Table S1). Deletion of *hpdA* gene restricts germination of *P. marneffei* conidia inside macrophages [40]. Additionally, the use of a specific inhibitor of 4-HPPD activity blocked the transition of mycelium to yeast in the dimorphic fungus *Paracoccidioides brasiliensis*, suggesting that 4-HPPD activity is required for this conversion [43].

The homogentisate produced as a result of tyrosine catabolism is a substrate in the synthesis of the soluble compound pyomelanin, a type of melanin belonging to a large group of pigment macromolecules that protect cells from environmental stress. Pyomelanin is involved in the oxidative stress response in *Aspergillus fumigatus* [44] and *T. marneffei* [40]. Additionally, it is produced by yeasts of *Histoplasma capsulatum*, suggesting a possible involvement in fungal virulence [45]. The identification of 4-HPPD in *S. brasiliensis* proteome agrees with studies conducted by our group. We have shown that the growth of a *S. brasiliensis* strain in presence of tyrosine promoted a protective effect against UV exposure, nitrogen- and oxygen-derived oxidants as well as resistance to amphotericin B [46] and terbinafine [47]. These findings suggested

that pyomelanin might play a role in virulence of *Sporothrix* spp. In this sense, the detection of 4-HPPD in *S. brasiliensis* proteome points to the involvement of this molecule in fungus success during cell transition processes and survival in the host comparing to both *S. schenckii* and *S. globosa*.

Together with the preferential amino acid metabolism in *S. brasiliensis*, the overexpression of enzymes involved in lipid metabolism shows that the yeast-like form of this species is highly adapted for the utilization of nutrients available during parasitism. This fact would explain the efficiency of *S. brasiliensis* to infect a broad-range of mammalian species in comparison with *S. globosa* [4].

Despite the lack of an expression pattern among the three *Sporothrix* species, it is clear that all of them are able to deal with reactive oxygen species (ROS). The first line of defense against reactive oxygen species is the superoxide dismutase/catalase system, followed by the glutathione and thioredoxin systems. The proteomic data reveal that *S. brasiliensis* and *S. globosa* face these free radicals in different ways. It is speculated that the expression of catalase is considered a virulence factor during infection and functions as a protector against environmental ROS [48]. In fact, the catalase/peroxidase protein was reported as immunogenic in fungi for the first time in *T. marneffei* showing be crucial to this fungus during the oxidative stress response [49]. Facing free radicals may grant a pathogenic potential to *S. globosa*, despite its low thermotolerance. *S. brasiliensis* is highly adapted to high temperatures, with the ability to efficiently infect cats (*Felis catus*) that present normal body temperature around 38–39 °C, whereas *S. globosa* growth is severely impaired in temperatures higher than 35 °C [20], which may explain the differences in heat shock protein expression between them. Among the *Sporothrix* heat shock proteins, it has been described that the chaperone HtpG, also known as HSP90, is essential for the maintenance of the yeast form of the fungus [50]. This particular chaperone is more abundant in *S. globosa*, followed by *S. schenckii*, indicating that *S. brasiliensis* probably has other mechanisms involved in its dimorphism.

The proteomic approach provided insights to believe that the parasitic yeast-form of *S. brasiliensis* has its metabolism differentiated from *S. schenckii* and *S. globosa* (Fig. 5). These differences may explain its higher virulence, reported in previous works, and capacity to infect a broad-range of mammalian species. The proteomic results complement the genomic data and, additionally,

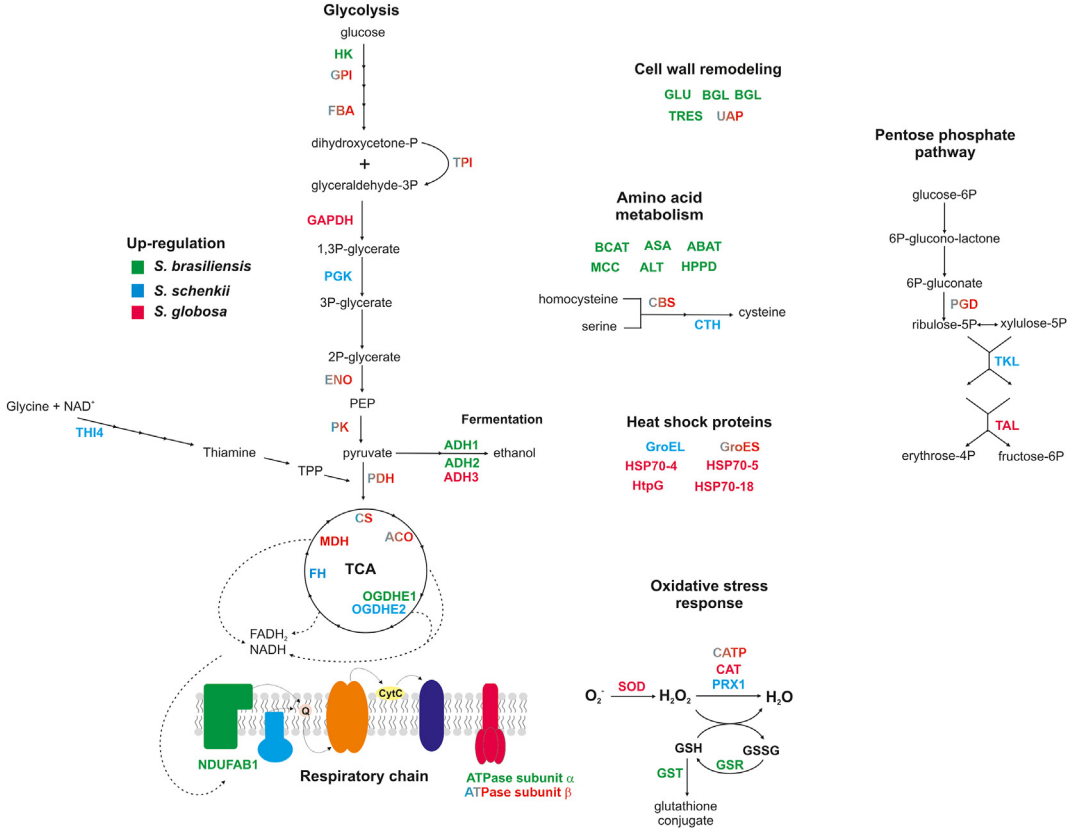

Fig. 5. Differentially expressed proteins in *Sporothrix* species. Induced proteins in each specie belong to the following categories: glycolytic pathway HK (hexokinase), GPI (glucose-6-phosphate isomerase), FBA (fructose biphosphate aldolase), TPI (triosephosphate isomerase) GAPDH (glyceraldehyde 3 phosphate dehydrogenase), PGK (phosphoglycerate kinase), ENO (enolase), PK (pyruvate kinase); TCA cycle PDH (pyruvate dehydrogenase), CS (citrate synthase), ACO (aconitase), OGDHE1 (2-oxoglutarate dehydrogenase E1 component), OGDHE2 (2-oxoglutarate dehydrogenase E2 component), FH (fumarate hydratase), MDH (malate dehydrogenase); vitamin biosynthesis THI4 (thiamine biosynthetic enzyme); fermentation ADH (alcohol dehydrogenase); respiratory chain NDUFAB1 (NADH dehydrogenase ubiquinone 1 alpha beta subcomplex 1); pentose phosphate pathway PGD (6-phosphogluconate dehydrogenase), TKL (transketolase), TAL (transaldolase); amino acid metabolism BCAT (branched-chain amino acid aminotransferase), ASA (aspartate-semialdehyde dehydrogenase), ABAT (4-aminobutyrate aminotransferase), MCC (3-methylcrotonyl-CoA carboxylase beta subunit), ALT (alanine transaminase), HPPD (4-hydroxyphenylpyruvate dioxygenase), CBS (cystathionine beta synthase), CTH (cystathionine gamma lyase); cell wall remodeling GLU (Endo-1,3(4)-glucanase), BGL (beta-glucosidase), TRES (trehalose synthase), UAP (UDP-N-acetylglucosamine pyrophosphorylase); oxidative stress response SOD (superoxide dismutase), CATP (catalase/peroxidase), CAT (catalase), PRX1 (peroxiredoxin), GSR (glutathione reductase), GST (glutathione S transferase); heat shock response GroEL (chaperonin GroEL), GroES (chaperonin GroES), HSP70-4 (heat shock 70 kDa protein 4), HSP70-5 (heat shock 70 kDa protein 5), HtpG (molecular chaperone HtpG), HSP70-18 (heat shock 70 kDa protein 18). Acronyms with two colors represent induction of the protein in two species.

contribute to the understanding of already existent and further investigations regarding adaptation of *Sporothrix* species to the host.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micinf.2020.09.008.

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