Transcriptional and Proteomic Responses to Carbon Starvation in *Paracoccidioides*

Patrícia de Sousa Lima¹,², Luciana Casaletti¹, Alexandre Melo Bailão¹, Ana Tereza Ribeiro de Vasconcelos³, Gabriel da Rocha Fernandes⁴, Célia Maria de Almeida Soares¹*  

¹Laboratório de Biologia Molecular, Instituto de Ciências Biológicas, Universidade Federal de Goiás, Goiânia, Goiás, Brazil, ²Programa de Pós Graduação em Patologia Molecular, Faculdade de Medicina, Universidade de Brasília, Brasília, Distrito Federal, Brazil, ³Laboratório Nacional de Computação Científica (LNCC), Petrópolis, Rio de Janeiro, Brazil, ⁴Laboratório de Bioinformática, Universidade Católica de Brasília, Brasília, Distrito Federal, Brazil

Abstract

**Background:** The genus *Paracoccidioides* comprises human thermal dimorphic fungi, which cause paracoccidioidomycosis (PCM), an important mycosis in Latin America. Adaptation to environmental conditions is key to fungal survival during human host infection. The adaptability of carbon metabolism is a vital fitness attribute during pathogenesis.

**Methodology/Principal Findings:** The fungal pathogen *Paracoccidioides* spp. is exposed to numerous adverse conditions, such as nutrient deprivation, in the human host. In this study, a comprehensive response of *Paracoccidioides*, Pb01, under carbon starvation was investigated using high-resolution transcriptomic (RNAseq) and proteomic (NanoUPLC-MS²) approaches. A total of 1,063 transcripts and 421 proteins were differentially regulated, providing a global view of metabolic reprogramming during carbon starvation. The main changes were those related to cells shifting to gluconeogenesis and ethanol production, supported by the degradation of amino acids and fatty acids and by the modulation of the glyoxylate and tricarboxylic cycles. This proposed carbon flow hypothesis was supported by gene and protein expression profiles assessed using qRT-PCR and western blot analysis, respectively, as well as using enzymatic, cell dry weight and fungus-macrophage interaction assays. The carbon source provides a survival advantage to *Paracoccidioides* inside macrophages.

**Conclusions/Significance:** For a complete understanding of the physiological processes in an organism, the integration of approaches addressing different levels of regulation is important. To the best of our knowledge, this report presents the first description of the responses of *Paracoccidioides* spp. to host-like conditions using large-scale expression approaches. The alternative metabolic pathways that could be adopted by the organism during carbon starvation can be important for a better understanding of the fungal adaptation to the host, because systems for detecting and responding to carbon sources play a major role in adaptation and persistence in the host niche.

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* E-mail: cmasoares@gmail.com

Introduction

Metabolic adaptability and flexibility are important attributes for pathogens to successfully colonize, infect, and cause disease in a wide range of hosts. Therefore, they must be able to assimilate various carbon sources. Carbohydrates are the primary and preferred source of metabolic carbon for most organisms and are used for generating energy and producing biomolecules [1].

Studies have highlighted the importance of carbon metabolism in fungi [2,3]. Pathogens such as *Candida albicans* display sufficient metabolic flexibility to assimilate the available nutrients in diverse niches such as the skin, mucous membranes, blood, and biofilms [4,5]. The mucosal surface of the lung may provide a more nutrient-limited condition because it is not in direct contact with nutrients from food intake [6]. Additionally, in the lungs, macrophages rapidly phagocytize inhaled microorganisms supported by neutrophils and dendritic cells [7]. Macrophages are considered a glucose- and amino acid-poor environment [8,9] and may form extremely nutrient-limited conditions causing severe starvation [10]. In *C. albicans* and *Cryptococcus neoformans*, alternative carbon metabolism was detected after internalization by macrophages playing a role in fungal survival in these host cells [9,11,12]. In contrast, in the case of systemic infections, pathogens can reach different internal organs such as the liver, which is the main storage compartment of glucose in the form of glycogen. The bloodstream, for example, is the major carrier of nutrients, glucose, proteins, amino acids, and vitamins in larger quantities [10]. In this way, metabolic and stress adaptation represent vital fitness attributes that have evolved alongside virulence attributes in fungi [8–10,13].

Alternative carbon metabolism was also described to be important to protozoa and bacteria [14,15]. *Entamoeba histolytica*...
Author Summary

The species of the Paracoccidioides genus, a neglected human pathogen, represent the causative agents of paracoccidioidomycosis (PCM), one of the most frequent systemic mycoses in Latin America. Despite being phagocytosed, the fungus conidia differentiate into the parasitic yeast form that subverts the normally harsh intraphagosomal environment and survives and replicates into murine and human macrophages. It has been suggested that alternative carbon metabolism plays a role in the survival and virulence of Paracoccidioides spp. within host cells. We used large-scale transcriptome and proteome approaches to better characterize the responses of Paracoccidioides, Pb01, yeast parasitic cells, to carbon starvation. We aimed to identify important molecules used by the fungus to adapt to these hostile conditions. The shift to a starvation mode, including gluconeogenesis and ethanol increases, activation of fatty acids, and amino acid degradation are the strategies used by the pathogen to persist under this stress. Our study provides a detailed map of Paracoccidioides spp. responses under carbon starvation conditions and contributes to further investigations of the importance of alternative carbon adaptation during fungus pathogenesis.

uses an alternative source of energy when the microorganism is exposed to glucose starvation. In the specific pyruvate-to-ethanol pathway in E. histolytica, acetyl-CoA is converted to acetaldehyde, which is then reduced to ethanol [16]. Reduced levels of the long-chain fatty-acid-CoA ligase protein during glucose starvation conditions in E. histolytica may explain a mechanism by which acetyl-CoA is shuttled from the fatty acid metabolism into this pyruvate-to-ethanol pathway. In addition, the glucose starvation modulates the protozoa virulence, based on proteome analysis [15]. The transcriptome and large-scale proteome dynamics were also analyzed in Bacillus subtilis from glucose-starved cells. A direct consequence of glucose depletion on proteins was the switch from glycolytic to gluconeogenic metabolism and elevated abundance of proteins of the tricarboxylic cycle used for energy generation. Genes that are involved in exponential growth, amino-acid biosynthesis, purine/pyrimidine synthesis and the translational machinery were down-regulated in the bacteria cells under glucose starvation [14].

The species of the Paracoccidioides genus represent the causative agents of paracoccidioidomycosis (PCM), one of the most frequent systemic mycoses in Latin America [17]. The genus comprises four phylogenetic lineages (S1, PS2, PS3, and Pb01-like). The phylogenetic analysis of many Paracoccidioides isolates has resulted in the differentiation of the genus into two species: P. brasiliensis, which represents a complex of three phylogenetic groups, and P. lutzii, which represents the Pb01-like isolates [18–21]. Paracoccidioides spp. grows as a yeast form in the host tissue and in vitro at 36°C, while it grows as mycelium under saprobic condition and in culture at room temperature (18–23°C). As the dimorphism is dependent on temperature, when the mycelia/conidia are inhaled into the host lungs, the transition of the mycelia to the pathogenic yeast phase occurs [22].

One of the first lines of defense faced by Paracoccidioides spp. during host invasion is the lung resident macrophages. Despite being phagocytosed, the fungus conidia differentiate into the parasitic yeast form that subverts the normally harsh intraphagosomal environment and survives and replicates into murine and human macrophages [23]. It has been proposed for PCM and other systemic mycoses that the fungal intracellular parasitism is a major event for disease establishment and progression in susceptible hosts. The survival inside the macrophage may allow fungal latency and/or dissemination from the lungs to several organs such as observed in C. neoformans [24,25]. In this sense, Paracoccidioides spp. has evolved defense mechanisms to survive under nutritionally poor environments. It has been suggested that alternative carbon metabolism plays a role in the survival and virulence of Paracoccidioides spp. within the host [26,27], as occurs in C. albicans and C. neoformans [9,12]. The transcriptional analysis of Paracoccidioides spp. upon internalization by macrophages, as determined by a DNA microarray, consisting of 1,152 cDNA clones, showed that the fungus responds to the glucose-depleted environment found in the macrophage phagosome, by the expression of 119 classified genes, differentially transcribed. Genes involved in methionine biosynthesis (eustathione β-lactase), oxidative stress response (superoxide dismutase and heat shock protein 60), and cytochrome electron transport system (cytochrome oxidase c) were induced by the fungus. Moreover, Paracoccidioides spp. reduced the expression of genes that are involved in the glycolysis pathway such as the key regulatory phosphofructokinase (gfkA) and genes related to cell wall polysaccharides such as β-glucan synthase (βs) [27]. In addition, studies of the transcriptome profiling from yeast cells of Paracoccidioides spp. derived from mouse liver revealed that the fungus most likely uses multiple carbon sources during liver infection. Genes encoding enzymes, regulators, and transporters in carbohydrate and lipid metabolism were significantly overexpressed. Ethanol production was also detected, indicating that it may be particularly important during infection [28].

Here, we described the response of Paracoccidioides facing carbon starvation using a high-throughput RNA Illumina sequencing (RNAseq) and quantitative proteome NanoUPLC-MSE, a two-dimensional liquid chromatography-tandem mass spectrometry approach. RNAseq is a developed approach to transcriptome profiling that uses deep-sequencing technologies and has already been applied to organisms such as Saccharomyces cerevisiae, Arabidopsis thaliana, mouse, and human cells [29–33]. With regard to proteomic analysis, our group has developed detailed proteome maps of the process of the fungus dimorphism, the response to iron and zinc deprivation, the fungus exoproteome, and the response to oxidative stress as well as comparative proteome maps of members of Paracoccidioides phylogenetic species [34–39]. In this study, a comprehensive response of Paracoccidioides, isolate Pb01, under carbon starvation was performed by transcriptional and proteomic approaches. To the best of our knowledge, this is the first description of high-resolution transcriptomics and proteomics applied to study the response of Paracoccidioides spp. to carbon starvation. We believe that the obtained data can be relevant in the understanding of the fungal establishment in the host.

Materials and Methods

Paracoccidioides maintenance and carbon starvation

Paracoccidioides, Pb01 (ATCC MYA-826), was used in the experiments. The yeast phase was cultivated for 7 days, at 36°C in BHI semisolid medium added to 4% (w/v) glucose. When required, the cells were grown for 72 h at 36°C in liquid BHI, washed with PBS 1x, and incubated at 36°C in a McVeigh/Morton (MMcM) medium with the following composition per 100 mL: KH₂PO₄ 0.13 g; MgSO₄·7H₂O 0.05 g; CaCl₂·2H₂O 0.015 g; (NH₄)₂SO₄ 0.2 g; vitamin 1 mL and trace element supplements 0.1 mL. The stock vitamin solution contained, also per 100 mL: thiamine hydrochloride, 6.0 mg; niacin, 6.0 mg;
carbon source) or 0% of glucose (carbon starvation). The cells were inoculated in modified MMcM medium [40] with 4% (glucose, (NH4)6Mo7O24.4H2O, 3.6 mg; ZnSO4.7H2O, 79.2 mg, as described previously [40], except for removal of the amino acids. All components except the vitamin supplement were mixed, and the pH was adjusted to 7.0 with IN NaOH. The vitamin solution was filter sterilized and added after the remainder of the medium had been autoclaved at 121°C for 15 min and cooled.

Paracoccidioides yeast cells were subjected to carbon starvation as following. The P. brasiliensis yeast cells were grown for 72 h at 36°C in liquid BHI added to 4% (w/v) glucose. The cells were harvested and washed three times with PBS 1×. A total of 10^6 cells/mL were inoculated in modified MMcM medium [40] with 4% (glucose, carbon source) or 0% of glucose (carbon starvation). The cells were incubated at 36°C.

Quantitative real time PCR (qRT-PCR) analysis
Following Paracoccidioides incubation in carbon starving condition, cells were centrifuged at 1,500 × g, frozen in liquid nitrogen, and disrupted by maceration as described in [38]. Briefly, cells were treated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The manufacturer's protocol was followed to extract total RNA. The RNA was reversibly transcribed using the high capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA). We confirmed the specificity of each primer pairs for the target cDNA by the visualization of a single PCR product following agarose gel electrophoresis and melting curve analysis. The cDNA was quantified by qRT-PCR using a SYBR green PCR master mix (Applied Biosystems Step One Plus PCR System). qRT-PCR analysis was performed in biological triplicate for each cDNA sample as previously described [38]. The data were normalized using the constitutive gene encoding the 60S ribosomal L34 as the endogenous control. In order to analyze the reliability of the normalizer used in our qRT-PCRs we used data obtained from three different housekeeping genes and the software NormFinder (Aarhus University, Aarhus, Denmark). The software identify the most suitable reference genes as previously described in [41]. We used the actin (PAAG_00564), tubulin alpha-1 chain (PAAG_01647) and 60S ribosomal protein L34 (PAAG_00746) genes and the results show that the L34 is the best gene to be used as normalizer in our qRT-PCRs. It was demonstrated by the lower stability value by comparing with actin and tubulin genes after two different runs (Table S1). The 60S ribosomal L34 gene was amplified in each set of qRT-PCR experiments and was presented as relative expression in comparison to the experimental control cells value set at 1. Data were expressed as the mean ± standard deviation of the biological triplicates of independent experiments. Standard curves were generated by diluting the cDNA solution 1:5. Relative expression levels of genes of interest were calculated using the standard curve method for relative quantification [42]. Statistical comparisons were performed using the student’s t test and p-values≤0.05 were considered statistically significant. The specific primers, both sense and antisense, are described in Table S2.

Western blot analysis
Proteins were fractionated by 12% SDS-polyacrylamide gel electrophoresis, and stained with Coomassie Blue R or transferred to Hybond ECL membrane (GE Healthcare). Membranes were blocked for 1 h at room temperature in a solution containing 10% (w/v) skim milk powder and 0.1% Tween 20 in Tris-buffered saline (TBS-T). The primary polyclonal antibody anti-isocitrate lyase [43] was diluted in blocking solution and incubated with the membrane for 1 h at room temperature. Membranes were washed in Tris-buffered saline and then incubated with alkaline phosphatase conjugated secondary antibodies for another hour at room temperature. Labeled bands were revealed with 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium and negative controls were obtained with rabbit preimmune. Images from western blots were acquired with ImageMaster 2D Platinum 6.0 (Geneva Bioinformatics, GeneBio). Raw Tiff images were analyzed by densitometric analysis of immunoblotting bands using the software AphaEaseFC (Genetic technologies Inc.). Pixel intensity for the analyzed bands was generated and expressed as Integrated Density Values (IDV).

High-throughput mRNA sequencing (RNA-seq)
Following Paracoccidioides growth in the presence or not of carbon for 6 h, cells were treated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) to obtain RNA molecules, from biological replicates. The cDNAs libraries were prepared from poly(A)-fragment selected mRNA and processed on the Illumina HiSeq2000 Sequencing System (http://www.illumina.com/). As a result, approximately 40 million of reads of 100 bp paired-end sequencing were obtained for each sample. The sequencing reads were mapped to reference the Paracoccidioides genome (Pb01), (http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html), using the Bowtie 2 tool [44]. Mapped reads data was analyzed by the DEGseq package [45]. Briefly, each read was allowed to align in just one site of the genome and the reads were counted. The default parameters were used to perform the alignment. The number of mismatches allowed in seed alignment (−N) is 0, and the length of each seed (−L) is 20. The fold change selection method was used for differentially expressed genes selection using a Fisher exact test, and a p-value of 0.001 was considered to select the genes. From the selected genes, the 2-fold change cut-off was considered. Genes with log2 (fold change) higher than 1 or less than −1 were selected and classified as up- and down-regulated genes, respectively. Gene’s identifications and annotations were determined from the Paracoccidioides genome database (http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html). The biological processes were obtained using the Pedant on MIPS (http://pedant.helmholtz-muenchen.de/pedant3htmlview/pedant3view?Method = analysis&Db = p3_r4832 5_Par_brasiliensis_Pb01) which provides a tool to browse and search the Functional Categories (FunCat) of proteins. All scripts can be obtained on request.

Sample preparation and NanoUPLC-MS² data acquisition
Following Paracoccidioides cell incubation in carbon-starved media for up to 48 h, the cells were centrifuged at 1,500 × g, resuspended in a 50 mM ammonium bicarbonate pH 8.5 solution and disrupted using glass beads and bead beater apparatus (BioSpec, Oklahoma, USA) in 5 cycles of 30 sec, while on ice. The cell lysate was centrifuged at 10,000 × g for 15 min at 4°C and the supernatant was quantified using the Bradford reagent (Sigma-Aldrich) [46]. The samples were analyzed using nanoscale liquid chromatography coupled with tandem mass spectrometry. Sample aliquots (50 μg) were prepared for NanoUPLC-MS² as previously described [47,48]. Briefly, 50 mM ammonium bicarbonate was added and was followed by addition of 25 μL of RapiGEST (0.2% v/v) (Waters Corp, Milford, MA). The solution was vortexed and then incubated at 80°C for 15 min; 2.5 μL of a 100 mM DTT solution was then added and incubated for 30 min at 60°C. The sample was cooled at room temperature and 2.5 μL of 300 mM iodoacetamide was added following by sample incubation in a dark
room for 30 min. A 10 μL aliquot of trypsin (Promega, Madison, WI, USA) prepared with 50 mM ammonium bicarbonate to 50 ng/μL, was added. The sample was vortexed slightly and digested at 37°C overnight. Following the digestion, 10 μL of 5% (v/v) trichloroacetic acid was added to hydrolyze the RapIDEST, followed by incubation at 37°C for 90 min. The sample was centrifuged at 18,000× g at 6°C for 30 min, and the supernatant was transferred to a Waters Total Recovery Vial (Waters Corp). A solution of one pmol μL−1 MassPREP Digestion Standard [rabbit phosphorilase B (PHB)] (Waters Corp) was used to prepare the final concentration of 150 fmol μL−1 of the PHB. The buffer solution of 20 mM ammonium formate (AF) was used to increase ion detection, clustering, and log-scale parametric normalizations database (http://www.broadinstitute.org/annotation/ genome/paracoccidioides_brasilensis/MultiHome.html). The ion detection, clustering, and log-scale parametric normalizations were performed in PLGS with an ExpressionE license installed (Waters, Manchester, UK). The intensity measurements were typically adjusted for these components, i.e., the desoated and charge state reduced EMRTs that were replicated throughout the entire experiment for the analysis at the EMRT cluster level. STY phosphorylations were set as variable modification. Components were typically clustered with a 10 ppm mass precision and a 0.25 min time tolerance against the database-generated theoretical peptide ion masses with a minimum of one matched peptide. The alignment of elevated-energy ions with low-energy precursor peptide ions was performed with an approximate precision of 0.05 min. One missed cleavage site was allowed. The precursor and fragment ion tolerances were determined automatically.

The protein identification criteria also included the detection of at least three fragment ions per peptide, 7 fragments per protein and the determination of at least one peptide per protein. The maximum protein mass was set to 600 kDa and trypsin was chosen as the primary digest reagent. The identification of the protein was allowed with a maximum 4% false positive discovery rate in at least two out of three technical replicate injections. 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. Protein and peptides tables generated by PLGS were merged and the dynamic range of the experiments, peptides detection type, and mass accuracy were determined for each condition as described in [47] by setting the minimum repeat rate for each protein in all replicates to 2. Normalization was performed with a protein that showed no significant difference in abundance in all injections [52] to accurately compare the expression protein level to carbon and carbon-starved samples.

**Paracoccidioides cell dry weight assay**

Paracoccidioides yeast cells were grown for 72 h at 36°C in liquid BHI, washed with PBS 1×, and filtered using a nylon mesh filter to yield small and non-aggregated cells. A total of 5×10⁷ cells/50 mL were inoculated in modified MMcM medium [40] with carbon source (4% glucose) or under carbon starvation (absence of glucose) and were incubated at 36°C. In each time-point, 10 mL of culture were centrifuged at 1,500× g and the supernatants were carefully removed. The cells were resuspended in PBS 1× up to 500 μL and subjected to 95°C heating for 1 h. The cells were centrifuged, frozen in liquid nitrogen and bophilized for 24 h. Dry weight was determined. Data are expressed as the mean ± standard deviation of the triplicates of independent experiments. Statistical comparisons were performed using the Student’s t test and p-values≤0.05 were considered statistically significant.

**Paracoccidioides cell viability analysis**

The viability was determined by membrane integrity analysis using propidium iodide as dead cells marker as previously described [34,35]. Briefly, cell suspension (10⁹ yeast cells/mL) were centrifuged and the supernatant was discarded. The cells were stained by addition of the propidium iodide solution (1 μg/mL) for 20 min in the dark at room temperature. After dye incubation, stained cell suspension was immediately analyzed in a C6 Accuri flow cytometer (Accuri Cytometers, Ann Arbor, MI, USA). A minimal of 10,000 events per sample was acquired with the FL3-H channel. Data was collected and analyzed using FCS Express 4 Plus Research Edition software (Denovo Software, Los Angeles, CA, USA).

**Ethanol measurement assay**

The concentration of ethanol was quantified by enzymatic detection kit according to the manufacturer’s instruction (UV-test for ethanol, RBiopharm, Darmstadt, Germany). Ethanol is oxidized to acetaldehyde by the enzyme alcohol dehydrogenase, in the presence of nicotinamide-adenine dinucleotide (NAD). Acetaldehyde is quantitatively oxidized to acetic acid in the presence of aldehyde dehydrogenase, releasing NADH, which is determined by means of its absorbance at 340 nm. *Paracoccidioides P ú01 yeast cells were subjected or not to carbon starvation, and 10⁶ cells were used to assay. Briefly, cells were counted, centrifuged, and lysed using glass beads and bead beater apparatus (BioSpec, Oklahoma, USA) in 5 cycles of 30 sec, keeping the samples on ice. The cell lysate was centrifuged at 10,000× g for 15 min at 4°C and the supernatant was used for enzymatic assay according to the manufacturer’s instructions. The concentrations of ethanol were obtained in triplicate.

**Enzyme activity assays**

Following *Paracoccidioides* growth under carbon source (4% glucose) or carbon starvation (absence of glucose) the cells were centrifuged at 1,500× g, resuspended in a solution containing 20 mM Tris-HCl, pH 8.8, 2 mM CaCl₂ [53] and disrupted using glass beads and bead beater apparatus (BioSpec, Oklahoma, USA) in 5 cycles of 30 sec, while on ice. The cell lysate was centrifuged at 10,000× g for 15 min at 4°C and the supernatant was quantified using the Bradford reagent (Sigma-Aldrich) [46]. Formamidase activity was determined by measuring the amount of ammonia formation as previously described [38,54]. One μg of *Paracoccidioides* total protein extract prepared as described above was added to 200 μL of a 100 mM formamide substrate solution in 100 mM phosphate buffer containing 10 mM of EDTA, pH 7.4. Samples were incubated at 37°C for 30 min. A total of 400 μL of phenol-nitroprusside and the same volume of alkaline hypochlorite (Sigma Aldrich, Co.) were added on the tube. The samples were then incubated for 6 min at 50°C and the absorbance was read at 625 nm. The amount of ammonia
One unit (U) of formamidase specific activity was defined as the amount of enzyme required to hydrolyze 1 mmol of formamide (corresponding to the formation of 1 mmol of ammonia) per min per mg of total protein.

Glyoxylate-phenylhydrazone formation was determined by measuring the absorbance at 324 nm, using an extinction coefficient of 16.8 mM$^{-1}$ cm$^{-1}$, in a reaction mixture containing 2 mM threo-

Isocitrate lyase activity was determined by measuring the formation of glyoxylate as its phenylhydrazone derivative [55]. Glyoxylate-phenylhydrazone formation was determined by measuring the absorbance at 324 nm, using an extinction coefficient of 16.8 mM$^{-1}$ cm$^{-1}$, in a reaction mixture containing 2 mM threo-

Figure 1. Effect of carbon starvation in transcripts and protein expression in *Paracoccidioides* yeast cells. (A) The expression of fructose-1,6-biphosphatase, isocitrate lyase and 3-ketoacyl-CoA thiolase genes in Pb01 yeast cells grown in MMcM medium with 4% (carbon source) or absence of glucose (carbon starvation) were analyzed. The cells were incubated at 36°C for several time intervals. The data were normalized using the constitutive gene encoding the 60S ribosomal L34 gene as the endogenous control and are presented as relative expression in comparison to the experimental control cells value set at 1. Data are expressed as the mean ± standard deviation of the triplicates of independent experiments. * significantly different from the control, at a p value of ≤0.05. (B) Proteins (50 µg) of Pb01 yeast cells were incubated at 36°C in MMcM medium with 4% or 0% of glucose and the abundance of PbIcl was analyzed by western blotting. The proteins were fractionated by one-dimensional gel electrophoresis. The proteins were blotted onto a nitrocellulose membrane and the ~60 kDa protein species was detected by using the rabbit polyclonal antibody anti-PbIcl [43]. Densitometric analysis of immunoblotting bands was performed using the software AphaEaseFC.

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released for each sample was determined by comparing to a standard curve. One unit (U) of formamidase specific activity was defined as the amount of enzyme required to hydrolyze 1 µmol of formamide (corresponding to the formation of 1 µmol of ammonia) per min per mg of total protein.
Figure 2. Growth of *Paracoccidioides* under carbon starvation. A total of 5.10^7 cells/50 mL of *Paracoccidioides* yeast cells were incubated in minimal medium (MVM) with carbon (4% glucose) or not (0% glucose) up to 72 h. At time points 0, 24, 48 and 72 h, cells were collected, killed by heat, and lyophilized to determine the cell dry weight. Data are expressed as the mean ± standard deviation of the triplicates of independent experiments.*, significantly different from the carbon condition, at a p-value of ≤0.05.

Figure 3. Overview of metabolic responses of *Paracoccidioides* to carbon starvation. The figure summarizes the data from transcriptome and proteomic analyses and suggests the mechanism and the first flow of carbon used by this fungus to overcome the carbon starvation stress. HXK: hexokinase; PGM: phosphoglucomutase; PFK: 6-phosphofructokinase; FBPase: fructose-1,6-bisphatase; ALD: fructose-bisphosphate aldolase; TPI: triosephosphate isomerase; PGK: phosphoglycerate kinase; ENO: enolase; PEPC: phosphoenolpyruvate carboxykinase; PYC: pyruvate carboxylase; PDC: pyruvate dehydrogenase; ADH: alcohol dehydrogenase; ALDH: aldehyde dehydrogenase; ICL: isocitrate lyase; MLS: malate synthase; IDH: isocitrate dehydrogenase; OGD: 2-oxoglutarate dehydrogenase E1; SUCLA: succinyl-CoA ligase; FUM: fumarate hydratase (fumarase) and MDH: malate dehydrogenase. Enzymes were colored according to their differences in expression and labeled to indicate whether the data were obtained from transcriptome or proteomics. Italic, bold, and underlined labels indicate that the data were obtained from transcriptome, proteome, or both, respectively. Green or red indicate up- or down-regulated proteins, respectively. The numbers 1, 2, 3, and 4 indicate up-regulated amino acids involved in pyruvate, oxaloacetate, succinate, and acetyl-CoA production, respectively. 1) pyruvate production: *tryptophan* and *cysteine*. 2) oxaloacetate production: phenylalanine, *glutamate* and tyrosine. 3) succinate production: threonine. 4) acetyl-CoA production: threonine, *tryptophan*, *tyrosine* and *leucine*. Italic, bold, and underlined labels indicate that the amino acids accumulations were obtained from transcriptome, proteome, or both analyzes, respectively. OXA: oxaloacetate; e⁻: released electrons from enzymatic reaction.

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D,L-isocitrate (Sigma Aldrich, Co.), 2 mM MgCl₂, 10 mM phenylhydrazine HCl (Sigma Aldrich, Co.), 2 mM dithiothreitol and 50 mM potassium phosphate at pH 7.0. Specific activity was determined as the amount of enzyme required to form 1 μmol of glyoxylate-phenylhydrazone per min per mg of total protein. For both assays, the statistical comparisons were performed using the Student’s t test and p-values ≤0.05 were considered statistically significant.

**Macrophage infection assays**

To evaluate whether carbon starvation influenced the internalization and survival of *Paracoccidioides*, the survival rate (viable fungi after co-cultivation) was determined by the number of colony-forming units (CFUs) recovered from macrophage infection. Macrophages, cell line J774 A.1 (Rio de Janeiro Cell Bank – BCRJ/UFRJ, accession number: 0121), maintained in RPMI medium (RPMI 1640, Vitrocell, Brazil), with 10% FBS (fetal bovine serum, [v/v]) were used in assays. A total of 10⁶ macrophages were seeded into each well of a 24-well tissue culture plate and 100 U. mL⁻¹ of IFN-gamma (murine IFN-γ, PeproTech, Rocky Hill, New Jersey, USA) was used for 24 h at 36°C with 5% CO₂ for activation of macrophages [56]. Prior to co-cultivation, *Paracoccidioides* yeast cells were grown in BHI liquid medium (4% [w/v] glucose, 3.7% [w/v] brain heart infusion, pH 7.2) for 72 h and subjected to both conditions, a carbon source (4% [w/v] glucose) or carbon starvation (no glucose) at 36°C, in McVeigh/Morton medium (MMcM) for 48 h. The same ratio of 1:2.5 macrophage:yeast was used for infection of both, carbon and carbon-starved yeast cells. The cells were co-cultivated for 24 h with 5% CO₂ at 36°C to allow fungal internalization. Prior to macrophages lysis, dilutions of the supernatant (culture from co-cultivation) removed by aspiration, were plated in BHI medium supplemented with 5% FBS (fetal bovine serum, [v/v]) incubated in 5% CO₂ at 36°C for 10 days. The number of viable cells was determined based on the number of CFUs. Infected macrophages were lysed with distilled water and dilutions of the lysates were plated in BHI medium supplemented with 5% FBS (fetal bovine serum, [v/v]). Colony-forming units (CFU) were determined after growth at 36°C, in 5% CO₂, for 10 days. For both experiments, the CFUs data were expressed as the mean value ± standard deviation from triplicates and the statistical analyses were performed using Student’s t test.

To analyze the expression of genes from *Paracoccidioides* yeast cells infecting macrophages, the same cell line described above was used. *Paracoccidioides* yeast cells were cultivated in BHI liquid medium (4% [w/v] glucose, 3.7% [w/v] brain heart infusion, pH 7.2) for 72 h and incubated with macrophages for 24 h, followed by washing with distilled water to promote macrophages lysis. RNAs and cDNAs were obtained as previously described. Specific oligonucleotides were used to amplify the fructose-1,6-biphosphatase, isocitrate lyase and 3-ketoacyl-CoA thiolase genes from *Pb*01 yeast cells. The negative amplification was obtained when cDNAs were used only from macrophages.

To determine the number of adhered/internalized fungi cells by macrophages, the same macrophage cell line described above was used. A total of 10⁶ macrophages were plated on glass coverslips per
Table 1. The most abundant up-regulated proteins of Paracoccidioides (Pb01) yeast cells under carbon starvation detected using NanoUPLC-MS.

| IDa | Annotationb | Fold change (log2)c | Biological processd |
|-----|-------------|---------------------|---------------------|
| **METABOLISM** | | | |
| **Amino acid metabolism** | | | |
| PAAG_08166 | 4-hydroxyphenylpyruvate dioxygenase | 4 | glycine biosynthesis |
| PAAG_03138 | alanine-glyoxylate aminotransferase | 4 | glycine biosynthesis |
| PAAG_01969 | arginase | 2 | glutamyl biosynthesis |
| PAAG_01144 | aspartate aminotransferase | 2 | arginine biosynthesis |
| PAAG_01365 | choline dehydrogenase | 2 | glycine biosynthesis |
| PAAG_08649 | cysteine dioxygenase | 2 | cysteine degradation |
| PAAG_07954 | gamma-glutamyl phosphate reductase | 2 | proline biosynthesis |
| PAAG_03506 | glutamate decarboxylase | 2 | glutamate biosynthesis |
| PAAG_07998 | glutamate synthase small chain | 2 | glutamate biosynthesis |
| PAAG_07317 | PENR2 protein | 2 | methionine biosynthesis |
| PAAG_08162 | maleylacetocetate isomerase | 2 | phenylalanine degradation |
| PAAG_04099 | methylcrotonyl-CoA carboxylase subunit alpha | 2 | leucine degradation |
| PAAG_02693 | saccharopine dehydrogenase | 2 |lysine biosynthesis |
| PAAG_02901 | S-adenosylmethionine synthetase | 2 | L-methionine biosynthesis |
| **Nitrogen and sulfur metabolism** | | | |
| PAAG_07811 | sulfite oxidase | 2 | nitrogen, sulfur and selenium metabolism |
| **C-compound and carbohydrate metabolism** | | | |
| PAAG_06953 | short chain dehydrogenase/reductase family | 2 | carbohydrate metabolism_sugar epimerase family |
| PAAG_04181 | sorbitol utilization protein SOU2 | 2 | C-compound and carbohydrate metabolism |
| PAAG_02653 | acetyl-coenzyme A synthetase | 2 | C-compound and carbohydrate metabolism |
| PAAG_00685 | alpha-mannosidase | 2 | polysaccharide |
| PAAG_01935 | formyl-coenzyme A transferase | 2 | oxalate catabolic process |
| PAAG_05984 | glutaryl-CoA dehydrogenase | 2 | aromatic hydrocarbons catabolism |
| PAAG_03765 | NADP-dependent glycerol dehydrogenase | 2 | Sugar, glucoside, polyol and carboxylate catabolism |
| PAAG_05416 | NADP-dependent leukotriene B4 12-hydroxydehydrogenase | 2 | C-compound and carbohydrate metabolism |
| PAAG_06057 | aldose 1-epimerase | 2 | hexose metabolic process |
| PAAG_02162 | lactam utilization protein LamB | 2 | C-compound and carbohydrate metabolism |
| | short-chain dehydrogenase/reductase SDR | 2 | Carbohydrate metabolism_sugar epimerase family |
| PAAG_03316 | polysaccharide export protein | 2 | surface polysaccharide biosynthesis process |
| **Lipid, fatty acid and isoprenoid metabolism** | | | |
| PAAG_02163 | acetyl-/propionyl-coenzyme A carboxylase alpha chain | 3 | lipid metabolism |
| PAAG_05249 | aldehyde dehydrogenase | 2 | lipid and fatty acid metabolism oxidation |
| PAAG_01928 | peroxisomal dehydratase | 2 | lipid, fatty acid and isoprenoid metabolism |
| PAAG_06099 | glycerol-3-phosphate dehydrogenase | 2 | lipid metabolism |
| PAAG_01833 | 2-succinylbenzoate-CoA ligase | 2 | fatty acid metabolism |
| PAAG_06224 | carnitine O-acetyltransferase | 2 | Lipid, fatty acid and isoprenoid metabolism |
| PAAG_07279 | farnesyl pyrophosphate synthetase | 2 | lipid metabolism |
| PAAG_04142 | NAD-dependent 15-hydroxyprostaglandin dehydrogenase | 2 | lipid metabolism |
| PAAG_04030 | short-chain-fatty-acid-CoA ligase | 2 | lipid and fatty acid metabolism |
| **Purin nucleotide/nucleoside/nucleobase metabolism** | | | |
| PAAG_09072 | mitochondrial nuclease | 2 | polynucleotide degradation |
| PAAG_04974 | adenylosuccinate lyase | 2 | purine biosynthesis |
| ID* | Annotation | Fold change (log2) | Biological process |
|-----|------------|-------------------|--------------------|
| PAAG_05803 | inosine-5’-monophosphate dehydrogenase IMD2 | **a** | purine nucleotide/nucleoside/nucleobase anabolism |
| PAAG_08856 | nicotinate-nucleotide pyrophosphorylase | **a** | biosynthesis of the pyridine nucleotides NAD and NADP |
| PAAG_02633 | ribose-phosphate pyrophosphokinase | **a** | nucleotide biosynthesis |
| PAAG_01751 | cytidine deaminase | **a** | UMP synthesis |
| **Secondary metabolism** | | | |
| PAAG_00851 | 6,7-dimethyl-8-ribityllumazine synthase | **a** | biosynthesis of riboflavin |
| PAAG_01324 | folic acid synthesis protein | **a** | folic acid-containing compound |
| PAAG_01934 | riboflavin synthase alpha chain | **a** | biosynthesis of riboflavin |
| PAAG_04443 | spermidine synthase | **a** | B complex vitamins |
| PAAG_02656 | NADH-ubiquinone oxidoreductase 51 kDa subunit | **a** | ubiquinone |
| **ENERGY** | | | |
| **Glycolysis and gluconeogenesis** | | | |
| PAAG_01583 | 6-phosphofructokinase subunit beta | **a** | glycolysis |
| PAAG_06172 | glucokinase | **a** | glycolysis |
| **Tricarboxylic-acid pathway** | | | |
| PAAG_00588 | fumarate hydratase | **a** | TCA cycle |
| PAAG_04597 | malate dehydrogenase | **a** | TCA cycle |
| **Electron transport and membrane-associated energy conservation** | | | |
| PAAG_02656 | NADH-ubiquinone oxidoreductase 51 kDa subunit | **a** | electron transport |
| PAAG_04570 | ATP synthase D chain, mitochondrial | **a** | respiration |
| PAAG_05605 | ATP synthase delta chain | **a** | respiration |
| PAAG_08088 | cytochrome b-c1 complex subunit 2 | **a** | respiration |
| PAAG_06268 | cytochrome c | **a** | electron transport |
| PAAG_00173 | electron transfer flavoprotein subunit alpha | **a** | electron transport |
| PAAG_03599 | formate dehydrogenase | **a** | electron transport |
| PAAG_01265 | cytochrome b5 | **a** | electron transport |
| PAAG_06796 | cytochrome c oxidase polypeptide IV | **a** | electron transport |
| **Ethanol production** | | | |
| PAAG_02512 | pyruvate decarboxylase | **a** | alcohol fermentation |
| PAAG_08248 | alcohol dehydrogenase | **a** | alcohol fermentation |
| **Pentose Phosphate pathway** | | | |
| PAAG_05621 | 6-phosphogluconolactonase | **a** | pentose-phosphate shunt |
| PAAG_05146 | ribose 5-phosphate isomerase A | **a** | pentose-phosphate shunt |
| **CELL CYCLE and DNA PROCESSING** | | | |
| PAAG_07608 | DNA helicase | **a** | DNA repair |
| PAAG_08917 | histone H2a | **a** | DNA processing |
| PAAG_07098 | histone H4,1 | **a** | DNA processing |
| PAAG_00126 | histone H4,2 | **a** | DNA processing |
| PAAG_08918 | late histone H2B,L4 | **a** | DNA processing |
| PAAG_05147 | mitotic checkpoint protein BUB3 | **a** | cell cycle control |
| PAAG_03054 | G2/M phase checkpoint control protein Sum2 | **a** | cell cycle control |
| **TRANSCRIPTION** | | | |
| PAAG_02467 | Transcription initiation factor TFIID subunit 14 | 3.22 | transcription regulation |
| PAAG_06250 | nuclear cap-binding protein | **a** | mRNA processing |
| PAAG_05509 | C2H2 type zinc finger domain-containing protein | **a** | transcriptional control |
| ID*        | Annotation                                      | Fold change (log2) | Biological process* |
|------------|------------------------------------------------|-------------------|---------------------|
| PAAG_02268 | DNA-directed RNA polymerase I subunit RPA12    | 2                 | rRNA synthesis      |
| PAAG_05397 | DNA-directed RNA polymerase II subunit RPB1    | 2                 | rRNA synthesis      |
| PAAG_02255 | mRNA decapping hydrolase                       | 2                 | mRNA processing     |
| PAAG_02437 | U2 small nuclear ribonucleoprotein B           | 2                 | splicing            |
|            | **TRANSLATION**                                |                   |                     |
| PAAG_06886 | zinc finger protein GIS2                       | 5.54              | positive regulation of translation |
| PAAG_02865 | translation initiation factor RLI1             | 3.92              | translation         |
| PAAG_05882 | translation factor SUI1                        | 2                 | translation         |
| PAAG_01834 | 60S ribosomal protein L16                      | 2                 | ribosome biogenesis  |
| PAAG_04425 | 60S ribosomal protein L22                      | 2                 | ribosome biogenesis  |
| PAAG_05233 | 60S ribosomal protein L26                      | 2                 | ribosome biogenesis  |
| PAAG_01939 | 60S ribosomal protein L27-A                    | 2                 | ribosome biogenesis  |
| PAAG_08847 | 60S ribosomal protein L28                      | 2                 | ribosome biogenesis  |
| PAAG_06627 | 60S ribosomal protein L32                      | 2                 | ribosome biogenesis  |
| PAAG_00648 | 60S ribosomal protein L33-B                    | 2                 | ribosome biogenesis  |
| PAAG_06569 | 60S ribosomal protein L43                      | 2                 | ribosome biogenesis  |
| PAAG_03019 | 60S ribosomal protein L6-B                     | 2                 | ribosome biogenesis  |
| PAAG_04998 | 60S ribosomal protein L8-B                     | 2                 | ribosome biogenesis  |
| PAAG_08285 | 50S ribosomal protein L12                      | 2                 | ribosome biogenesis  |
| PAAG_01435 | 40S ribosomal protein S16                      | 2                 | ribosome biogenesis  |
| PAAG_03322 | 40S ribosomal protein S20                      | 2                 | ribosome biogenesis  |
| PAAG_00385 | 40S ribosomal protein S23                      | 2                 | ribosome biogenesis  |
| PAAG_03816 | 40S ribosomal protein S4                       | 2                 | ribosome biogenesis  |
| PAAG_01050 | cytosolic large ribosomal subunit protein L30  | 2                 | ribosome biogenesis  |
| PAAG_07028 | histidyl-tRNA synthetase                       | 2                 | translation         |
| PAAG_08172 | lysyl-tRNA synthetase                          | 2                 | translation         |
| PAAG_03951 | prolyl-tRNA synthetase                        | 2                 | translation         |
| PAAG_08702 | seryl-tRNA synthetase                          | 2                 | translation         |
|            | **PROTEIN FATE**                                |                   |                     |
| PAAG_07802 | proteasome component PRE6                     | 2.8               | protein degradation |
| PAAG_07500 | xaa-Pro aminopeptidase                        | 2                 | proteolysis         |
| PAAG_07319 | xaa-Pro aminopeptidase                        | 2                 | proteolysis         |
| PAAG_03719 | Thimet oligopeptidase                         | 2                 | proteolysis         |
| PAAG_01472 | ubiquitin-conjugating enzyme                   | 2                 | protein degradation |
| PAAG_00852 | proteasome component C1                       | 2                 | proteasomal degradation (ubiquitin/proteasomal pathway) |
| PAAG_00868 | proteasome component PRE4                     | 2                 | proteasomal degradation (ubiquitin/proteasomal pathway) |
| PAAG_03536 | proteasome component PRE5                     | 2                 | proteasomal degradation (ubiquitin/proteasomal pathway) |
| PAAG_02720 | proteasome-activating nucleotidase            | 2                 | proteasomal degradation (ubiquitin/proteasomal pathway) |
| PAAG_03279 | aminopeptidase                                | 2                 | proteolysis         |
| PAAG_00664 | aspartyl aminopeptidase                       | 2                 | protein/peptide degradation |
| PAAG_03464 | bleomycin hydrolase                           | 2                 | proteolysis         |
| PAAG_05583 | cysteine protease PalB                        | 2                 | proteolysis         |
| PAAG_00768 | peptidase family protein                      | 2                 | proteolysis         |
| PAAG_05417 | mitochondrial-processing peptidase            | 2                 | proteolysis         |
| PAAG_00739 | peptidyl-prolyl cis-trans isomerase B          | 2                 | protein folding     |
| ID       | Annotation                                      | Fold change (log2) | Biological process   |
|----------|-------------------------------------------------|--------------------|----------------------|
| PAAG_02155 | peroxisomal targeting signal 2 receptor r(PTS2) | ^α                 | protein fate         |
| PAAG_04555 | sarcosine oxidase                              | ^α                 | protein modification |
| PAAG_00472 | serine/threonine-protein phosphatase           | ^α                 | protein dephosphorilation |
| PAAG_03573 | vacuolar protein sorting-associated protein     | ^α                 | protein fate         |

**BINDING**

| ID       | Annotation                                      | Fold change (log2) | Biological process   |
|----------|-------------------------------------------------|--------------------|----------------------|
| PAAG_07038 | APAF1-interacting protein                      | ^α                 | metal binding        |
| PAAG_08026 | MYB DNA-binding domain-containing protein       | ^α                 | DNA binding          |
| PAAG_07753 | RNA-binding La domain-containing protein        | ^α                 | RNA binding          |

**TRANSPORT**

| ID       | Annotation                                      | Fold change (log2) | Biological process   |
|----------|-------------------------------------------------|--------------------|----------------------|
| PAAG_03577 | ABC drug exporter AtF                          | ^α                 | drug/toxin transport |
| PAAG_05425 | Golgi membrane protein (Coy1)                  | ^α                 | Golgi vesicle transport |
| PAAG_04571 | nascent polypeptide-associated complex subunit alpha | ^α                  | protein transport |
| PAAG_08082 | plasma membrane ATPase                         | ^α                 | hydrogen ion transport |

**SIGNAL TRANSDUCTION**

| ID       | Annotation                                      | Fold change (log2) | Biological process   |
|----------|-------------------------------------------------|--------------------|----------------------|
| PAAG_02973 | diploid state maintenance protein chpA         | ^α                 | cellular signalling |
| PAAG_04261 | signal recognition particle 54 kDa protein     | ^α                 | GTP catabolic process |
| PAAG_02377 | rho GDP-dissociation inhibitor                  | ^α                 | regulator of G-protein signaling |

**CELL RESCUE, DEFENSE and VIRULENCE**

| ID       | Annotation                                      | Fold change (log2) | Biological process   |
|----------|-------------------------------------------------|--------------------|----------------------|
| PAAG_07020 | thioredoxin reductase                          | 3.78               | cell redox homeostase |
| PAAG_02926 | superoxide dismutase                          | ^α                 | detoxification       |
| PAAG_03502 | cytochrome c peroxidase                        | ^α                 | oxidative stress response |
| PAAG_00566 | aflatoxin B1 reductase member 2                | ^α                 | detoxification       |
| PAAG_06606 | cyanate hydratase                              | ^α                 | stress response/cyanate metabolic process |
| PAAG_06947 | gamma-glutamyltranseptidase                    | ^α                 | cell redox homeostase |
| PAAG_02548 | hydroxyacylglutathione hydrolase               | ^α                 | glutathione biosynthetic process/stress response |
| PAAG_08277 | nitroreductase family protein                  | ^α                 | detoxification       |

**CELL GROWTH/MORPHOGENESIS**

| ID       | Annotation                                      | Fold change (log2) | Biological process   |
|----------|-------------------------------------------------|--------------------|----------------------|
| PAAG_00997 | actin-interacting protein                      | ^α                 | actin depolymerization |
| PAAG_00875 | Arp2/3 actin-organizing complex subunit Sop2   | ^α                 | actin filament organization |
| PAAG_03624 | Arp2/3 complex subunit Arc16                   | ^α                 | actin filament organization |
| PAAG_04602 | mannosyl-oligosaccharide glucosidase           | ^α                 | cell wall biogenesis |
| PAAG_02186 | nuclear segregation protein Bfr1               | ^α                 | meiosis               |
| PAAG_01931 | phosphoacetylglucosamine mutase                | ^α                 | cell wall processing |

**MISCELLANEOUS**

| ID       | Annotation                                      | Fold change (log2) | Biological process   |
|----------|-------------------------------------------------|--------------------|----------------------|
| PAAG_02139 | methyltransferase family                        | ^α                 | methyltransferase    |
| PAAG_01302 | phosphorylase family protein                    | ^α                 | phosphoprotein       |
| PAAG_03233 | oxidoreductase                                  | ^α                 | oxidation-reduction process |
| PAAG_00712 | N-acetyltransferase                             | ^α                 | acetyltransferase    |
| PAAG_06955 | thiol methyltransferase                         | ^α                 | thiol methyltransferase activity |

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*aIdentification of differentially regulated proteins from *Paracoccidioides* genome database ([http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html](http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html)) using the ProteinLynx Global Server (PLGS) version 3.0 (Waters Corporation. Manchester. UK);

*bProteins annotation from *Paracoccidioides* genome database or by homology from NCBI database ([http://www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/));

*cProtein expression profiles in log*2*-fold change (S^-threshold);* obtained from ProteinLynx Global Server (PLGS) analysis normalized with internal standard.

**Biological process of differentially expressed proteins from MIPS ([http://pedant.helmholtz-muenchen.de/pedant3htmlview/pedant3view?Method=analysis&Db=p3_r48325_Par_bra_Pb01](http://pedant.helmholtz-muenchen.de/pedant3htmlview/pedant3view?Method=analysis&Db=p3_r48325_Par_bra_Pb01)) and Uniprot database ([http://www.uniprot.org/](http://www.uniprot.org/)).

#^: identified only in carbon starvation condition.

doi:10.1371/journal.pntd.0002855.t001
Table 2. The most abundant down-regulated proteins of *Paracoccidioides* (Pb01) yeast cells under carbon starvation detected using NanoUPLC-MS.

| ID* | Annotation* | Fold change (log2)* | Biological process* |
|-----|-------------|---------------------|---------------------|
| METABOLISM | | | |
| **Amino acid metabolism** | | | |
| PAAG_03569 | 1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase | * | methionine biosynthesis |
| PAAG_05328 | 3-isopropylmalate dehydrogenase A | * | leucine biosynthesis |
| PAAG_07605 | acetalactate synthase small subunit | * | L-isoleucine biosynthesis |
| PAAG_03043 | adenylyl-sulfate kinase | * | methionine biosynthesis |
| PAAG_07563 | asparagine synthetase | * | asparagine biosynthesis |
| PAAG_05198 | chorismate mutase | * | cysteine biosynthesis and aromatic groups |
| PAAG_07813 | cysteine synthase | * | cysteine biosynthesis |
| PAAG_07089 | homocitrate synthase | * | lysine biosynthesis |
| PAAG_04348 | homoserine kinase | * | threonine biosynthesis |
| PAAG_07102 | pentafuctional AROM polypeptide | * | aromatic group biosynthesis |
| PAAG_05929 | sulfate adenylytransferase | * | cysteine and methionine biosynthesis |
| **C-compound and carbohydrate metabolism** | | | |
| PAAG_02975 | 2,4-dihydroxyhept-2-ene-1,7-dioic acid aldolase | * | phenylacetate catabolic process |
| PAAG_02769 | pyruvate dehydrogenase protein X component | * | carbohydrate metabolism |
| PAAG_03774 | S-(hydroxymethyl)glutathione dehydrogenase | * | C-compound and carbohydrate metabolism/metanabolism |
| PAAG_00799 | uroporphyrinogen decarboxylase | * | porphyrin biosynthesis |
| **Lipid, fatty acid and isoprenoid metabolism** | | | |
| PAAG_02211 | GDSL Lipase/Acylhydrolase | * | lipid metabolism |
| PAAG_07013 | enoyl-CoA hydratase/carnithine racemase | * | lipid, fatty acid and isoprenoid metabolism |
| PAAG_01525 | fatty acid synthase subunit alpha reductase | * | lipid and fatty acid biosynthesis |
| PAAG_01524 | fatty acid synthase subunit beta dehydratase | * | lipid and fatty acid biosynthesis |
| PAAG_00869 | fumarylacetoacetate hydrolase domain-containing protein | * | lipid, fatty acid and isoprenoid metabolism |
| PAAG_06215 | hydroxymethylglutaryl-CoA lyase | * | lipid, fatty acid and isoprenoid metabolism |
| **Purin nucleotide/nucleoside/nucleobase metabolism** | | | |
| PAAG_06906 | adenine phosphoribosyltransferase | * | purin nucleotide/nucleoside/nucleobase metabolism |
| PAAG_07529 | orotidine 5'-phosphate decarboxylase | * | pyrimidine biosynthesis |
| PAAG_01437 | uricase | * | purine metabolism |
| **Secondary metabolism** | | | |
| PAAG_00047 | siroheme synthase | * | cobalamin (vitamin B12) biosynthesis |
| PAAG_01519 | inositol monophosphatase | * | secondary metabolism |
| **ENERGY** | | | |
| PAAG_01015 | hexokinase | * | glycolysis |
| **Electron transport and membrane-associated energy conservation** | | | |
| PAAG_07285 | vacuolar ATP synthase catalytic subunit A | * | ATP hydrolysis coupled proton transport |
| PAAG_06288 | vacuolar ATP synthase subunit B | * | ATP hydrolysis coupled proton transport |
| PAAG_06155 | vacuolar ATP synthase subunit E | * | ATP hydrolysis coupled proton transport |
| **Pentose Phosphate pathway** | | | |
| PAAG_00633 | glucose-6-phosphate 1-dehydrogenase | * | glucose metabolism |
| **CELL CYCLE and DNA PROCESSING** | | | |
| PAAG_03834 | vacuolar sorting-associated protein | * | protein sorting in cell division |
| PAAG_07773 | cyclin-dependent kinases regulatory subunit | * | mitotic cell cycle and cell cycle control |
| PAAG_00106 | histone acetyltransferase type B catalytic subunit | * | DNA repair |
| ID*       | Annotationb | Fold change (log2)c | Biological processd |
|-----------|-------------|---------------------|---------------------|
| PAAG_02055 | histone chaperone asf1 * | DNA replication     |
| PAAG_01943 | spindle pole body component alp6 * | cell cycle          |
| **TRANSCRIPTION** | | | |
| PAAG_01695 | arsenite resistance protein Ars2 * | transcription       |
| PAAG_04161 | transcription factor * | transcription control |
| PAAG_08234 | transcription factor RfeF * | transcription control |
| PAAG_01733 | 28 kDa ribonucleoprotein * | transcriptional control |
| PAAG_00101 | small nuclear ribonucleoprotein * | splicing            |
| PAAG_01630 | small nuclear ribonucleoprotein LSM2 * | splicing            |
| PAAG_02329 | U2 small nuclear ribonucleoprotein A * | splicing            |
| PAAG_07983 | ribonuclease H * | RNA processing      |
| PAAG_06966 | RNA methyltransferase * | RNA processing      |
| **TRANSLATION** | | | |
| PAAG_00765 | 60S ribosomal protein L36 * | ribosome biogenesis  |
| PAAG_00689 | ATP-dependent RNA helicase eIF4A * | protein biosynthesis |
| PAAG_07283 | ATP-dependent RNA helicase FAL1 * | ribosome biogenesis  |
| PAAG_06140 | eukaryotic translation initiation factor 1A * | translation         |
| PAAG_00747 | eukaryotic translation initiation factor 2 subunit gamma * | translation         |
| PAAG_01330 | eukaryotic translation initiation factor 3 RNA-binding subunit * | translation         |
| PAAG_00815 | eukaryotic translation initiation factor 3 subunit A * | translation         |
| PAAG_02837 | eukaryotic translation initiation factor 3 subunit H * | translation         |
| PAAG_04958 | eukaryotic translation initiation factor 6 * | translation         |
| PAAG_02071 | glutamyl-tRNA synthetase * | aminoacyl-tRNA-synthetases |
| PAAG_01786 | phenylalanyl-tRNA synthetase beta chain * | translation         |
| PAAG_08025 | tRNA (uracil-5-)methyltransferase TRM9 * | tRNA modification   |
| **PROTEIN FATE** | | | |
| PAAG_02497 | WD repeat domain 5B * | Ubq conjugation pathway |
| PAAG_01926 | 26S protease regulatory subunit 6A * | ubiquitin-dependent protein proteolysis |
| PAAG_05943 | 26S proteasome non-ATPase regulatory subunit 12 * | assembly of proteasome |
| PAAG_01706 | 26S proteasome regulatory subunit RPN10 * | protein/peptide degradation |
| PAAG_08200 | 26S proteasome regulatory subunit rpn-8 * | protein/peptide degradation |
| PAAG_07037 | calnexin * | protein folding and stabilization |
| PAAG_01962 | proteasome 26S subunit * | protein/peptide degradation |
| PAAG_08184 | T-complex protein 1 epsilon subunit * | protein folding |
| PAAG_07165 | T-complex protein 1 subunit gamma * | protein folding |
| PAAG_01588 | SNARE Ykt6 * | protein targeting, sorting and translocation |
| PAAG_04327 | ubiquitin carboxyl-terminal hydrolase * | protein deubiquitination |
| PAAG_03932 | ubiquitin-activating enzyme E1 Y * | protein ubiquitination |
| **BINDING** | | | |
| PAAG_03941 | G4 quadruplex nucleic acid binding protein * | RNA binding         |
| **TRANSPORT** | | | |
| PAAG_08487 | MIT family metal ion transporter * | ion transport (cobalt) |
| PAAG_05135 | tetratricopeptide repeat protein 1 * | potassium transport  |
| PAAG_04904 | ATP-binding cassette sub-family F member 2 * | ABC transport       |
| PAAG_03644 | mitochondrial import receptor subunit tom-40 * | protein transport   |
were performed using Student’s t test.

fungal cells was shown in percentage of the total as the mean value
number of adhered/internalized fungal cells, as described before
acquired using the software AxioVision (Carl Zeiss AG, Germany).
using the Axio Scope A1 microscope and digital images were
methanol and stained with Giemsa (Sigma). The cells were observed
processed for light microscopy. The glass coverslips were fixed with
the monolayer was gently washed twice with PBS 1
adhesion/internalization. The supernatants were then aspirated,
non-adhered/internalized yeast cells, and the samples were
co-cultivated for 6 and 24 h with 5% CO2 at 36
was used for infection in both conditions. The cells were co-
cultivated for 48 h, at 36
McVeigh/Morton medium (MMcM), containing or not a carbon
source, for 48 h, at 36
for infection in both conditions. The cells were co-
cultivated for 6 and 24 h with 5% CO2 at 36°C, to allow fungal
adhesion/internalization. The supernatants were then aspirated,
the monolayer was gently washed twice with PBS 1 x to remove any
non-adhered/internalized yeast cells, and the samples were
processed for light microscopy. The glass coverslips were fixed with
methanol and stained with Giemsa (Sigma). The cells were observed
using the Axio Scope A1 microscope and digital images were acquired using the software AxioVision (Carl Zeiss AG, Germany).
A total of 300 macrophages were counted to determine the average number of adhered/internalized fungal cells, as described before
[37,37]. For both experiments, the number of adhered/internalized fungal cells was shown in percentage of the total as the mean value ±
the standard deviation from triplicates. The statistical analyses were performed using Student’s t test.

Results

Effect of carbon starvation on transcripts and proteins of
Paracoccidioides

We first sought to set up a time-point to analyze the fungus
response to carbon starvation at transcriptional and proteomic
levels. Hence, we analyzed gene and protein expression of genes
known to be regulated under carbon-limited microenvironments
in other fungi [9,12], by using qRT-PCR and western blot assays,
in Pb01 (Fig. 1A). Changes in the expression of genes encoding
fructose-1,6-biphosphatase, isocitrate lyase, and 3-ketoacyl-CoA
thioase, which are representatives of gluconeogenesis, the
glyoxylate cycle, and β-oxidation, respectively, were analyzed in
the Paracoccidioides, Pb01, yeast cells subjected to carbon starvation.

Transcriptomic analysis

The transcriptome analysis was performed using next genera-
 sequencing and the Paracoccidioides, isolate Pb01, genome
database [http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html] using the Proteinflynx Global Server (PLGS) version 3.0 (Waters Corporation, Manchester, UK);

Table 2. Cont.

| ID*  | Annotation*  | Fold change (log2)* | Biological process* |
|------|--------------|---------------------|--------------------|
| PAAG_02306 | vascular H+/Ca2+ exchanger | * | ion transport |
| PAAG_07900 | phosphatidylinositol-phosphatidylcholine transfer protein (SEC14) | * | protein transport |
| SIGNAL TRANSDUCTION |
| PAAG_08028 | GTP-binding protein ypt1 | * | protein transport/signal transduction process |
| PAAG_02458 | GTP-binding protein ypt7 | * | protein transport/signal transduction process |
| PAAG_08992 | type 2A phosphatase activator tip41 | * | signal transduction |
| CELL RESCUE, DEFENSE AND VIRULENCE |
| PAAG_05679 | heat shock protein | –3.45 | stress response_protein folding |
| PAAG_07990 | tetracycline transporter | * | antibiotic resistance |
| PAAG_01465 | carbonic anhydrase | * | stress oxidative response/carbon utilization |
| PAAG_03216 | mitochondrial peroxiredoxin PRX1 | * | oxidative stress response |
| CELL GROWTH/MORPHOGENESIS |
| PAAG_06370 | sphingolipid long chain base-responsive protein LSP1 | * | endocytosis |
| MISCELLANEOUS |
| PAAG_04908 | NAD binding Rossmann fold oxidoreductase | * | oxidoreductase |
| PAAG_02354 | serine 3-dehydrogenase | * | serine 3-dehydrogenase activity |

*Identified by differentially regulated proteins from Paracoccidioides genome database [http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html] using the Proteinflynx Global Server (PLGS) version 3.0 (Waters Corporation, Manchester, UK);

*Protein expression profiles in log2-fold change (5 < threshold) obtained from ProteinLynx Global Server (PLGS) analysis normalized with internal standard.

*Biological process of differentially expressed proteins from MIPS [http://pedant.helmholtz-muenchen.de/pedant3htmlview/pedant3view?Method = analysis&Db = p3_r48325_Par_brasi_Pb01] and Uniprot database [http://www.uniprot.org/].

*Identified only in the presence of glucose (carbon condition).

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Figure 6. Number of transcripts and proteins related to metabolism and energy subcategories regulated in *Paracoccidioides*, Pb01, under carbon starvation. The number of transcripts and proteins, in percentage (%), regulated in *Paracoccidioides*, Pb01, under carbon starvation was calculated based on number of transcripts/proteins in each category shown in Figures S3 and S9, panels A. (A) Metabolism. The subcategories were represented by: amino acid; nitrogen/sulfur; C-compound and carbohydrate; lipid/fatty acid and isoprenoid; purin nucleotide/nucleoside/nucleobase; secondary and phosphate metabolism. (B) Energy. The subcategories were represented by glycolysis/gluconeogenesis; TCA cycle; electron transport and membrane associated energy; ethanol production; pentose phosphate pathway and glyoxylate cycle. Black and gray bars indicate genes and proteins, respectively.

doi:10.1371/journal.pntd.0002855.g006
A significant number of genes were regulated during carbon starvation. Although 1.5-fold change can be statistically significant [58], a cut-off of 2-fold change was applied to determine the up- and down-regulated transcripts (Tables S3 and S4, respectively) totaling 1,063 differentially expressed transcripts in Pb01 yeast cells under carbon starvation. A biological process classification was performed to gain a general understanding of the functional categories affected by carbon starvation. A total of 64.6% (687 transcripts) were represented by miscellaneous and unclassified categories, and the other 35.4% (376 transcripts) were represented by classified biological categories. The functional classifications and the percentage of up- and down-regulated transcripts in each classified category are shown in Fig. S3.

The transcriptome analysis showed that transcripts associated with metabolism were the most represented during 6 h of carbon starvation. Although 1.5-fold change can be statistically significant [58], a cut-off of 2-fold change was applied to determine the up- and down-regulated transcripts (Tables S3 and S4, respectively) totaling 1,063 differentially expressed transcripts in Pb01 yeast cells under carbon starvation. A biological process classification was performed to gain a general understanding of the functional categories affected by carbon starvation. A total of 64.6% (687 transcripts) were represented by miscellaneous and unclassified categories, and the other 35.4% (376 transcripts) were represented by classified biological categories. The functional classifications and the percentage of up- and down-regulated transcripts in each classified category are shown in Fig. S3.

The transcriptome analysis showed that transcripts associated with metabolism were the most represented during 6 h of carbon starvation in Pb01 (Fig. S3A). From these, approximately 27% were represented by up-regulated and 17% by down-regulated transcripts (Fig. S3B). Subcategories of metabolism related to amino acid, nitrogen/sulfur, C-compound/carbohydrate, lipid/fatty acid, purines, secondary, and phosphate metabolisms were regulated under carbon starvation stress, and all of them showed a higher number of up- than down-regulated transcripts (Tables S3 and S4). Other categories were also regulated in Pb01 under carbon starvation. The categories associated with protein fate, cell cycle/DNA processing, transcription, and cellular transport were largely represented in the transcriptome (Fig. S3A). The number of transcripts with increased or reduced expression was also investigated for these categories and the results show that, in contrast to metabolism, the number of down-regulated transcripts was generally higher than that of up-regulated transcripts for each category (Fig. S3B). Down-regulated transcripts associated with cell cycle, transcription, cell growth morphogenesis, and signal transduction could reflect the reduced growth of this fungus subjected to carbon starvation, as demonstrated in Fig. 2. Although the reduced growth of Paracoccidioides during carbon starvation, the cells viability, assayed using propidium iodide, was not significantly different from those cultivated with a carbon source (Fig. S4). In the same way, the cells are metabolically active as demonstrated by the high activity of the enzyme formamidase in Paracoccidioides grown under glucose deprivation (Fig. S5).

The cellular transport process was also representative in our transcriptome analysis. The abundance of specific transporters was elevated such as those of copper, hexoses, and monosaccharides.

![Figure 7. Expression of Paracoccidioides fbp, icl and thio genes and susceptibility of yeast cells to macrophages killing during infection. (A) Pb01 yeast cells were grown without (yeast cells) and with macrophages (yeast cells-macrophages) for 24 h in RPMI medium, and the relative expression of genes fbp (fructose-1,6-biphosphatase), icl (isocitrate lyase), and thio (3-ketoacyl-CoA thiolase) was determined. The data were normalized using the constitutive gene encoding the 60S ribosomal L34 gene as the endogenous control and are presented as relative expression in comparison to the experimental control cells value set at 1. (B) Pb01 yeast cells were previously grown in MMCM medium with carbon (4% of glucose) or absence of glucose (carbon starvation) up to 48 h and then were incubated with macrophages at a 1:2.5 macrophages: yeast ratio, for both conditions. As demonstrated, the number of viable cells was determined by quantifying the number of colony forming units/mL (CFUs/mL) during infection from culture supernatant (non-internalized cells removed by aspiration prior to macrophages lysis) and after internalization. Data are expressed as the mean ± standard deviation of the biological triplicates of independent experiments. Student’s t-test was used. *, significantly different from the control, at a p-value of ≤0.05. doi:10.1371/journal.pntd.0002855.g007](#)
Carbon Starvation in *Paracoccidioides*

In order to perform additional analysis, we applied a highly stringent criterion ($\geq 5\times$-fold) to analyze the most induced or repressed proteins in yeast cells upon carbon starvation (Tables 1 and 2). Proteins which were detected only in carbon or carbon-starved conditions were considered as down and up-regulated proteins at a high level (Table 1 and 2). Even at this high cut-off, up-regulated proteins involved in amino acids degradation, in β-oxidation, in ethanol production, and others are present using this high stringent criterion (Table 1), in agreement with the metabolic overview presented in Fig. 3. In the same way, down regulated proteins such as pyruvate dehydrogenase and enzymes involved in fatty acids biosynthesis were detected using this highly stringent fold change criteria (Table 2). Proteins related to cell defense such as thioredoxin reductase, superoxide dismutase and cytochrome c oxidase were also detected among the up-regulated proteins.

A comparative analysis of the transcriptome and proteome of *Paracoccidioides* under carbon starvation

To compare similar aspects between transcriptome and proteome data, we sought the same transcripts and proteins detected by both analyses. The transcripts and proteins identities (IDs), from the *Paracoccidioides* P001, database, were shown including their levels of abundance (Tables S7, S8, S9 and S10). Fifty seven identities (IDs) were matched of which 32 and 17 of them presented the same abundance profile, up- or down-regulated in both data, respectively (Tables S7 and S8). In this way, approximately 86% of the matches

summarized in Fig. 3, which depicts the metabolic and energy adaptation of the fungus to this stress. Pathways associated with ethanol, acetyl-CoA, oxaloacetate, and consequently glucose production were induced. Moreover, amino acid degradation supply precursors such as pyruvate, oxaloacetate, succinate and also acetyl-CoA for glucose production pathways (Fig. 3).

Specific enzymes related to ethanol production were up-regulated in the absence of carbon sources. The ethanol molecule is derived from pyruvate that, in turn, is not involved directly in oxaloacetate production because the pyruvate carboxylase (Pyc) enzyme is down-regulated (Fig. 3). Ethanol measurement was performed, and the results showed that after up to 48 h under carbon starvation, a significantly higher level of ethanol was produced compared with glucose-rich cells (Fig. 4). Regarding the acetyl-CoA molecule, several enzymes associated with its production from pyruvate, via the acetaldehyde precursor, and β-oxidation were also up-regulated. Once produced, acetyl-CoA may be used by the glyoxylate shunt to generate glyoxylate and succinate molecules. This is reinforced by fact that the TCA cycle enzyme isocitrate dehydrogenase is repressed, so the acetyl-CoA pool should be consumed by the glyoxylate cycle. Additionally, succinate can be converted in oxaloacetate by enzymes from the tricarboxylic acid cycle (Fig. 3). The activity of isocitrate lyase, a representative enzyme of the glyoxylate cycle, was also determined confirming our proteomic data and reinforcing our suggested carbon flow. The analysis revealed that a significant higher specific isocitrate lyase activity was obtained after *Paracoccidioides* yeast cells were subjected to carbon starvation for 48 h (Fig. 5).

The oxaloacetate molecule is a key intermediate of gluconeogenesis. Once produced, gluconeogenic enzymes convert it into glucose. We detected up-regulated specific enzymes that support this suggestion in P001 under carbon starvation, such as phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase (FBPase), and phosphoglucosamutase (PGM) (Fig. 3).

The most up-regulated proteins in *Paracoccidioides* upon carbon starvation

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showed the same pattern of transcript and protein levels. On the other hand, the minority of IDs showed discrepancy in their abundance. Several of the transcripts in these groups were decreased in abundance, while the protein levels were increased and vice versa (Tables S9 and S10).

A comparative analysis including all transcripts and proteins for metabolism and energy categories from RNAseq and NanoUPLC-MS analysis was also performed (Fig. 6). Metabolism, which was the most regulated category in our data and energy are considered essential categories for understanding the carbon flow used by P601 during carbon starvation. The results show that the amino acid, carbohydrate/C-compound, and lipid metabolism were similarly regulated in both approaches, showing the consistency with the suggested carbon flow in Paracoccidioides, P601, under carbon starvation (Figs. 3 and 6A). Amino acids and lipids are supposed to be intensively degraded (Tables S3 and S5) suggesting the production of precursors during carbon starvation, which include acetyl-CoA, pyruvate, oxaloacetate and succinate. Furthermore, the percentage of transcripts and proteins related to energy categories such as glycolysis/gluconeogenesis, electron transport/membrane associated energy conservation, and TCA cycle are also similar, in accordance with suggested responses of P601 to carbon starvation (Fig. 6B). Thus, the induction of gluconeogenesis, \( \beta \)-oxidation, part of TCA, and glyoxylate cycles was required to compensate for the absence of glucose and depicts the rearrangement of pathways when a carbon source condition is changed.

**Fungus-macrophage interaction**

We investigated the response to macrophages in Paracoccidioides, P601, under carbon starvation. We analyzed whether the fungus differentially expresses genes involved in gluconeogenesis, glyoxylate cycle, and \( \beta \)-oxidation pathways after internalization by the J744 A.1 macrophages. The relative expression analysis of transcripts encoding fructose-1,6-biphosphatase, isocitrate lyase and 3-ketoacyl CoA thiolase was performed using qRT-PCR. The Fig. 7A demonstrates that genes encoding isocitrate lyase and 3-ketoacyl CoA thiolase were induced (\( p \leq 0.05 \)), suggesting a response of Paracoccidioides to carbon starvation in phagosomes. Furthermore, whether yeast cells under carbon starvation were more susceptible to macrophage killing than cells growing in plentiful glucose was analyzed. Firstly, plating of recovered yeast cells by aspiration of culture supernatant (non-internalized yeast cells) and from lysis of macrophages (internalized yeast cells) was performed (Fig. 7B). The result showed that the number of yeast cells recovered from culture supernatant was not significantly different between carbon and carbon starved yeast cells (Fig. 7B, on the left). On the contrary, the Paracoccidioides, P601, yeast cells pre-exposed to carbon starvation were recovered in a lower number than those grown under carbon source (Fig. 7B, on the right). In addition, to verify if the yeast cells were, in fact, more susceptible to macrophage killing we evaluated the average number of adhered/internalized Paracoccidioides, P601, using the light microscopy after 6 and 24 h of infection (Fig. S10 and S11). After 24 h of infection, it was observed, in carbon-starved condition, a significant decrease in the number of yeast cells adhered/internalized by macrophages (Fig. S10). Overall, the data suggest that yeast cells pre-exposed to carbon starvation were more susceptible to macrophage killing, reinforcing our suggestion that the carbon starvation can affect the survival of the P601 yeast cells inside of the macrophages.

**Discussion**

The major focus of this work is directed towards a global view on the responses of Paracoccidioides, P601, to carbon starvation using both, high-throughput transcriptome and proteomic analysis. P601 yeast cells were able to adapt to carbon starving conditions. The data presented in this study reflected how carbon-starved cells modulate the metabolism by induction or repression of cellular activities. We show that the fungus regulates pathways that lead to glucose production to compensate the effect of stress. The fungus regulates transcripts and proteins that are mainly associated with gluconeogenesis and ethanol production via precursors from \( \beta \)-oxidation, glyoxylate and tricarboxylic acid cycles. Our study presents a detailed response of Paracoccidioides spp. facing carbon starvation and contributes to investigations of the importance of alternative carbon adaptation during fungus pathogenesis.

Changes in the kinetics of expression of representatives of gluconeogenesis, the glyoxylate cycle, and \( \beta \)-oxidation as well as the differential expression of isocitrate lyase at the protein level could establish a better time-point for our transcriptional (6 h) and proteomic (48 h) analysis, using RNAseq and NanoUPLC-MS\(^E\), respectively (Fig. 1). The transcriptome and proteomic analysis demonstrated that general metabolism and energy were the most represented regulated categories. Transcripts/proteins classified in energy and cell rescue, defense, and virulence categories were also induced in both approaches although less representative than metabolism (Fig. S3 and S9). Categories involved in the cell cycle, transcription, cellular transport, growth/morphogenesis, and signal transduction were predominantly down-regulated at transcription and protein levels. Although P601 yeast cells displayed no significant difference in cell viability until 72 h under carbon starvation (Fig. S4), the yeast biomass was significantly reduced in this condition (Fig. 2). In addition, the abundance of specific transporters was elevated such as those related to copper, hexose, and monosaccharide uptake, suggesting a nutrient limitation and a hostile environment as detected in other fungi [60].

Paracoccidioides, P601, presented a complex mechanism to respond to nutrient deprivation. The suggestion is that the fungus uses a carbon flow (Fig. 3) through classical biochemical pathways such as glyoxylate cycle, \( \beta \)-oxidation, and gluconeogenesis which is in accordance with previous data on other fungi facing nutrient deprivation [8,9,12]. Fungi such as *C. albicans* and *C. neoformans* appear to experience a nutrient limited and stressful environment in the context of interaction with host cells. The elevated expression of the glyoxylate pathway and gluconeogenesis genes during *Cryptococcus* interactions with host tissue and phagocytic cells is similar to the regulation observed in *C. albicans*. These pathogenic fungi present a niche dependent metabolism, with activation of an alternative carbon source consuming process and the up-regulation of transcripts for enzymes of the glyoxylate cycle, \( \beta \)-oxidation, and gluconeogenesis [9,12] which was also detected in Paracoccidioides, P601. Our data suggest that P601 yeast cells facing carbon starvation use the oxaloacetate molecule as a key intermediate of gluconeogenesis. It is possibly supported by \( \beta \)-oxidation and, in part, by glyoxylate and TCA cycles activation. The glyoxylate cycle can allow the fungus to assimilate two-carbon compounds, a relevant aspect to the viability and growth inside macrophages [61,62]. Studies involving the isocitrate lyase gene, representative of glyoxylate cycle, displayed that this gene is important as a marker for gluconeogenic carbon source utilization and starvation rather than a marker for lipid metabolism [63,64]. Despite induction of isocitrate lyase and genes required for fatty acid utilization especially after phagocytosis by macrophages [9], this induction may derive from a general stress response due to nutrient or glucose limitation rather than a specific induction from fatty acid utilization [64]. In fact, null mutants to isocitrate lyase in *C. albicans*, *A. fumigatus* and *C. neoformans* and for \( \beta \)-oxidation genes in *C. albicans* revealed no virulence defects, showing that fatty acids
do not provide an essential nutrient source during infection [2,64–67]. In addition, isocitrate lyase activity increased when C. albicans was subjected to carbon starvation or other carbon sources such as acetate, glutamate and peptone as solely carbon source [63] reinforcing its importance as a marker for gluconeogenic carbon source utilization and starvation. Moreover, the isocitrate dehydrogenase enzyme was detected as down-regulated in our proteomic data (Table S6). The activities of isocitrate dehydrogenase and isocitrate lyase enzymes, regulate the flow of isocitrate into either the tricarboxylic acid cycle or the glyoxylate cycle [68,69]. Here, while the isocitrate dehydrogenase is down, the isocitrate lyase is up-regulated, in accordance with suggested carbon flow in Pb01 through glyoxylate shunt. As the same importance, the increased expression of protein phosphoenolpyruvate carboxykinase confirms that gluconeogenesis process is ongoing (Table S5). Then, the global characterization of the responses of Pb01 to carbon starvation becomes relevant in this context especially by flow of carbon used by the fungus during this stress.

The same transcripts and proteins detected by both analyses were identified (Tables S7, S8, S9 and S10). Approximately 86% of the matches (a total of 57) showed the same pattern of transcript and protein levels. In this way, we believe that the transcriptional and proteome time-points were enough to characterize the global responses of the Paracoccidioides to carbon starvation conditions. Similarly to previous study in A. fumigatus, few times, transcripts and proteins do not follow the same trend of expression that could be explained, for example, by mRNA stabilization process or by active post-transcriptional and translational regulatory mechanisms [70]. In our data, in many metabolic aspects, transcripts and protein levels were correlated. The identities to amino acid degradation, β-oxidation and ethanol synthesis were increased in expression while processes involving the pyruvate molecule were down-regulated (Table S7 and S8) corroborating our hypothesis of a well-established process especially by flow of carbon used by the fungus during this stress.

In addition, comparison of regulated molecules in transcriptome and proteome data with focus in metabolism and energy categories can support the use of alternative carbon sources by Pb01 under carbon starvation (Fig. 6). Metabolism of amino acids, lipids, and carbohydrate/C-compound was the most regulated in both used approaches (Fig. 6A). The amino acids and lipids likely are been used as precursors to important molecules involved in alternative carbon metabolism in Pb01 as depicted in Fig. 3. In fact, Paracoccidioides spp. can use a relatively wide range of amino acids and peptides rather than carbohydrates [21]. In addition to the structural importance of lipids, these molecules provide an energy-rich nutrient source. β-oxidation is a common pathway for the utilization of fatty acids [71] in which of the 3-ketoacyl-CoA thiolases enzymes are important [64]. Recent studies have highlighted the relevance of the β-oxidation in response to nutrient or glucose limitation rather than a specific induction from fatty acid utilization. In fact, fatty acids do not provide an essential nutrient source during infection in C. albicans but is important for coupling the glyoxylate cycle and fatty acid β-oxidation during host-pathogen interactions, regulating responses related to carbon starvation [63,64]. Here, the induction of β-oxidation pathway in Pb01 likely reflects the requirement of this metabolic pathway for carbon starvation adaptation, which is consistent with previous data in C. albicans subjected to a poor-nutrition environment [9]. Regarding energy producing pathways, the gluconeogenesis, tricarboxylic acid and glyoxylate cycles are well represented, which reinforces our model of adaptation to carbon starvation conditions (Fig. 6B). Moreover, the electron transport and ethanol subcategories were also shown. Ethanol metabolism was previously described in Pb01 showing evidence for a more anaerobic metabolism of this fungus compared with other isolates of Paracoccidioides [35,36,72], and this metabolite has also been described as relevant to pathogenic fungi such as A. fumigatus [70,73,74].

The general response to oxidative stress mediated by enzymes provides multiple resistance strategies to Paracoccidioides yeast cells [34]. There was a high increase in production of antioxidant proteins such as thioredoxin reductase, superoxide dismutase and cytochrome c peroxidase that are possibly involved in defense against reactive oxygen species (ROS) during carbon starvation (Tables S5 and 1). In low amounts, ROS are generated continuously as side products of aerobic respiration in the mitochondria and, although potentially cytotoxic, function as signal molecules in cellular processes [75,76]. In this way, the production of ROS during carbon starvation could be related to the increase in electron transport activity in respiratory chain (Table S3 and 1) as well as to the production of endogenous free radicals in β-oxidation.

The response of Pb01 to macrophage infection shows that the fungus most likely faces carbon starvation in macrophages, because a significant higher expression of genes encoding isocitrate lyase and 3-ketoacyl-CoA thiolase was detected. It is important to note the similarities in the transcriptional profile in inducing alternate carbon metabolism between C. albicans phagocytosed cells and those submitted to carbon starvation (39). In fact, in terms of usable nutrients, the phagosome has been reported to not have a rich environment evidenced by the unsubstantial quantities of glucose, other sugars and amino acids [9,11,12,27,77]. Here, we showed that Pb01 yeast cells were more susceptible to macrophage killing when were previously starved of carbon (Fig. 7, S10 and S11). This conclusion is based on the fact that the multiplication of Paracoccidioides inside activated macrophages is inhibited [23]. In this sense, we suggested that Paracoccidioides, Pb01, yeast cells pre-exposed to carbon starvation, were more susceptible to macrophage killing, reinforcing that carbon deprivation affects the survival of the Pb01 yeast cells inside of the macrophages.

Taken together, our data suggest that Pb01 changes its metabolism under carbon starvation reprogramming several biological processes to facilitate its maintenance under this condition. These programs are mainly related to gluconeogenesis, β-oxidation and the glyoxylate cycle to compensate for the starved carbon environment. Considering a new perspective, the transcriptome and proteome data could reinforce the responses of this fungus, which is able to survive in the hostile environment during macrophage infection. This study may elucidate potential molecules involved in host-fungus interactions, an important factor related to pathogenic organisms.

Supporting Information

Figure 81 Initial effects of carbon starvation in Paracoccidioides yeast cells protein expression. A. Proteins (50 μg) of Pb01 yeast cells were incubated at 36°C in MMcM medium with (4%) or without (0%) of glucose for 0, 12, 24, 30 and 48 h. The abundance of PbMcl was analyzed by western blotting. The proteins were fractionated by one-dimensional gel electrophoresis. The proteins were blotted onto a nitrocellulose membrane and the ~60 kDa protein species was detected using the rabbit polyclonal antibody anti-PbMcl [43]. B. Densito-
metric analysis of immunoblotting bands was performed using the software AphaEaseFC. The difference in PbEl expression was just observed in 48 h under carbon starvation.

(TIF)

**Figure S2** Global analysis of RNAseq data. Mapped reads data were analyzed by DEGseq package and plotting graphs were obtained. The transcripts are represented by dots. (A) Scatter plot shows the number of reads (log2) for each transcript in CS (carbon-starved) and C (carbon) conditions. (B) MA-plot of CS versus C conditions shows the intensity of expression of identified transcripts (log2 of fold change) in the y axe [M] and the read counts (log2) for each transcript in the x axe [A]. In addition, the graph shows the number of differentially expressed transcripts obtained from FET (Fisher Exact Test) using a p-value of 0.001 in red color.

(TIF)

**Figure S3** Functional classification and abundance levels of transcripts regulated in Paracoccidioides under carbon starvation obtained by RNAseq. (A) Biological processes of differentially expressed transcripts in Paracoccidioides, Pb01, under carbon starvation are shown. The biological processes were obtained using the Pedant on MIPS (http://pedant.helmholtz-muenchen.de/pedant3htmlview/pedant3view?Method = analysis&Db = p3_r48325_Par_brasi_Pb01) and Uniprot database (http://www.uniprot.org/). A total of 1,063 transcripts are shown represented by the percentage (%) of regulated unclassified (64.6%) and classified (35.4%) transcripts for each category. (B) The number of classified up- and down-regulated transcripts in Paracoccidioides, Pb01, under carbon starvation stress is shown for each category depicted in (A). A total of 190 and 186 transcripts were up- and down-regulated and are depicted by light and dark gray colors bars, respectively. The percentages (%) show the number of up- and down-regulated transcripts for each category based on a total of them.

(TIF)

**Figure S4** Cell viability of Paracoccidioides subjected to carbon and carbon starvation conditions. The effect of carbon (4% glucose) and carbon starvation (0% glucose) conditions in viability of Pb01 yeast cells was investigated using flow cytometry. The viability was determined by a membrane integrity analysis using propidium iodide (1 μg/mL) as a dead cell marker in a C6 Accuri flow cytometer (Accuri Cytometers, Ann Arbor, MI, USA). The experiments were performed in triplicate. Statistical analyses was performed using Student’s t-test; all of the samples showed p-values<0.05 (*) and were considered statistically significant. The errors bars represent the standard deviation of three biological replicates.

(TIF)

**Figure S5** Formamidase activity assay. The activity was determined by the measuring the amount of ammonia formation at 37°C. A total of 1 μg of each total protein extract of Paracoccidioides under carbon (4% glucose) and carbon starvation (0% glucose) conditions for 6, 24, 48 and 72 h in MMcM medium was used. One unit (U) of formamidase specific activity was defined as the amount of enzyme required to hydrolyze 1 μmol of formamide (corresponding to the formation of 1 μmol of ammonia) per min per mg of total protein and are represented as U mg⁻¹. Errors bars represent standard deviation from three biological replicates while * represents p≤0.05.

(TIF)

**Figure S6** Peptide detection type to carbon and carbon-starved samples. The pie graph show the percentage of peptides matched against the Paracoccidioides (Pb01) database by PLGS (PepFrag 1 and PepFrag 2), variables modifications (VarMod), fragmentation that occurred on ionization source (InSource), missed cleavage performed by trypsin (Missed Cleavage) and Neutral loss H₂O and NH₃ correspondent to water and ammonia precursor losses to carbon (A) and carbon starvation (B) conditions. The SpotFire Decision Site 8.0 v program was used. The PepFrag parameters should be predominant, in contrast to insource and missed cleavage which should not reach 20%.

(TIF)

**Figure S7** Peptide mass accuracy analyzes. Peptides data were used to make the bar graph showing the accuracy of mass for peptides in carbon and carbon starvation samples. A total of 94.9 and 95.7% of identified peptides were detected in a 15 ppm error range in both samples, carbon (A) and carbon starvation (B), respectively.

(TIF)

**Figure S8** Detection of dynamic range of proteomic analyzes. The dynamic range the proteomic experiments for each condition was determined. Graphs for carbon (A) and carbon-starved (B) are shown. Regular, reverse, and standard proteins were indicated by gray/square, blue/circle and yellow/triangle colors/shape, respectively. The regular and reverse proteins indicate identified proteins using regular and reverse genomic database from Paracoccidioides, Pb01, respectively. The standard protein was used to normalize the expression data and compare the carbon and carbon-starved proteins. Our data showed an acceptable quantification to standard protein between the both conditions.

(TIF)

**Figure S9** Functional classification and abundance levels of proteins regulated in Paracoccidioides under carbon starvation obtained by NanoUPLC-MS² data. (A) Biological processes of differentially expressed proteins in Paracoccidioides, Pb01, under carbon starvation are shown. The biological processes were obtained using the Pedant on MIPS (http://pedant.helmholtz-muenchen.de/pedant3htmlview/pedant3view?Method = analysis&Db = p3_r48325_Par_brasi_Pb01) and Uniprot databases (http://www.uniprot.org/). Three hundred and thirty-five proteins are represented by the percentage (%) in each category. (B) The number of up- and down-regulated proteins in Paracoccidioides, Pb01, under carbon starvation is shown for each category depicted in (A). Two hundred and three proteins were up-regulated (dark gray) and one hundred and thirty-two proteins were down-regulated and are depicted by the light gray color bar.

(TIF)

**Figure S10** Number of internalized/adhered Paracoccidioides yeast cells. The average number of adhered/ internalized Paracoccidioides cells by macrophages was determined. Macrophages were infected with Paracoccidioides yeast cells which were pre-cultivated under carbon and carbon starvation conditions by 6 and 24 h. A total of 300 macrophages were counted for each time and condition and the number of adhered/internalized fungal cells was shown in percentage of the total as the mean value ± the standard deviation from triplicates. The statistical analyses were performed using Student’s t test.

(TIF)

**Figure S11** Microscopy of adhered/internalized Paracoccidioides yeast cells by macrophages. The Paracoccidioides yeast cells pre-cultivated in the presence of carbon and under carbon starvation were co-incubated with macrophages for 6 and...
24 h. The arrows and double arrows indicate internalized and adhered yeast cells, respectively. The cells were fixed with methanol, stained by Giemsa and visualized via light microscopy (magnification 100×, oil immersion), as detailed in the Materials and Methods section.

(TIF)

Table S1 Candidate reference genes for normalization according to expression stability calculated by Normfinder.

(DOC)

Table S2 Specifics primers used in qRT-PCR.

(DOC)

Table S3 Up-regulated transcripts of Paracoccidioides (Pb01) yeast cells under carbon starvation detected by RNAseq analysis.

(DOC)

Table S4 Down-regulated transcripts of Paracoccidioides (Pb01) yeast cells under carbon starvation detected by RNAseq analysis.

(DOC)

Table S5 Up-regulated proteins of Paracoccidioides (Pb01) yeast cells under carbon starvation detected using NanoUPLC-MSE.

(DOC)

Table S6 Down-regulated proteins of Paracoccidioides (Pb01) yeast cells under carbon starvation detected using NanoUPLC-MSE.

(DOC)

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Table S7 Up-regulated proteins and transcripts of Paracoccidioides (Pb01) yeast cells under carbon starvation detected by NanoUPLC-MS and RNAseq analysis.

(DOC)

Table S8 Down-regulated proteins and transcripts of Paracoccidioides (Pb01) yeast cells under carbon starvation detected by NanoUPLC-MS and RNAseq analysis.

(DOC)

Table S9 Up- and down-regulated proteins and transcripts, respectively, of Paracoccidioides (Pb01) yeast cells under carbon starvation detected by NanoUPLC-MS and RNAseq analysis.

(DOC)

Table S10 Down- and up-regulated proteins and transcripts, respectively, of Paracoccidioides (Pb01) yeast cells under carbon starvation detected by NanoUPLC-MS and RNAseq analysis.

(DOC)

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Author Contributions

Conceived and designed the experiments: CMDAS. Performed the experiments: PdSL LC. Analyzed the data: PdSL AMB ATRdV GdRF CmDAS. Contributed reagents/materials/analysis tools: CmDAS. Wrote the paper: PdSL CmDAS.

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