Upregulation of glyoxylate cycle genes upon *Paracoccidioides brasiliensis* internalization by murine macrophages and *in vitro* nutritional stress condition

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*Paracoccidioides brasiliensis*, the etiologic agent of paracoccidioidomycosis, is a facultative intracellular human pathogen that can persist within macrophage phagolysosomes, indicating that the fungus has evolved defense mechanisms in order to survive under nutritionally poor environments. The analysis of *P. brasiliensis* transcriptome revealed several virulence factor orthologs of other microorganisms, including the glyoxylate cycle genes. This cycle allows the utilization of two-carbon (C2) compounds as carbon source in gluconeogenesis. Semiquantitative RT-PCR analyses revealed that these genes were upregulated when *P. brasiliensis* was recovered from murine macrophages, without any additional *in vitro* growth. The induction of this cycle, in response to macrophage microenvironments, was shown to be coordinated with the upregulation of the gluconeogenic phosphoenolpyruvate carboxykinase gene. In addition, assays employing RNA extracted from *P. brasiliensis* grown in a medium with acetate instead of glucose also showed increased levels of glyoxylate cycle transcripts. Our main results suggest that *P. brasiliensis* uses the glyoxylate cycle as an important adaptive metabolic pathway.

**Keywords** glyoxylate cycle, *Paracoccidioides brasiliensis*, virulence genes, semiquantitative RT-PCR, murine macrophages infection

**Introduction**

The dimorphic fungus *Paracoccidioides brasiliensis* is the etiologic agent of paracoccidioidomycosis (PCM), the most prevalent human systemic mycosis in Latin America [1,2]. Although the ecology of this microorganism still remains unclear, it is believed that *P. brasiliensis* is found in nature in a mycelial saprophytic form. Infection is probably acquired by inhalation of airborne mycelial structures which can differentiate into the yeast form within the lungs [3]. The shift from environmental temperature (26°C) to host body temperature (37°C) triggers the transition from mycelium to yeast and seems to be essential to the establishment of the infective process [4,5]. The different outcomes of this interaction can be expressed as: (i) pathogen eradication, (ii) pathogen encasement in a granuloma, or (iii) pathogen dissemination throughout the body [6,7].

The establishment of infection is associated with the virulence of the pathogen and the host immunological response. The main host defense mechanism against PCM is the cell-mediated immune response, characterized by the production of TNF-α, IL-12 and IFN-γ that activate macrophages [8–10]. However, in the absence of such cytokines, or in susceptible hosts, this fungus is able to survive and replicate within the phagolysosome of nonactivated murine and human macrophages [11,12]. Thus, the facultative intracellular
life of \textit{P. brasiliensis} must be compatible with the inhospitable microenvironment imposed by phagocytic cells. Although little is known about the factors required for intracellular persistence of this fungus, the analysis of the \textit{P. brasiliensis} transcriptome \cite{13,14} allowed Tavares et al. \cite{15} to identify in \textit{P. brasiliensis} orthologs of genes described in other microorganisms as virulence factors that are important to survival inside host cells. Among these, ortholog genes of glyoxylate cycle isocitrate lyase (\textit{icl}) and malate synthase (\textit{mls}), which encode the key enzymes of this cycle \cite{16,17}, were identified.

The role of the glyoxylate cycle in pathogenesis has been demonstrated for several microorganisms. The activity of this cycle allows two-carbon (C2) compounds to be used as carbon sources in gluconeogenesis. In fact, several lines of evidence indicate that intracellular pathogens may preferentially utilize C2 compounds, such as products of fatty-acid degradation, for energy production \cite{18,19}. In this sense, upon phagocytosis, \textit{Mycobacterium tuberculosis} and \textit{Candida albicans}, as other microorganisms, respond by inducing glyoxylate cycle genes, suggesting that its activity is required for persistence and survival within macrophages \cite{20–23}. This shift from fermentative to non-fermentative metabolism during the infective process probably includes a co-activation of glyoxylate cycle and gluconeogenic genes, and a downregulation of glycolytic genes. However, glyoxylate cycle gene expression is not always associated with gluconeogenesis, as observed in \textit{C. albicans} following macrophage infection \cite{23}. Alternatively, the glyoxylate anaplerotic reactions can generate intermediates of other metabolic pathways.

Here we investigated the expression of glyoxylate cycle genes after co-culture of \textit{P. brasiliensis} yeast cells with murine macrophages and during \textit{in vitro} cultivation in a nutritional stress condition. The analyses were done using semiquantitative reverse transcription PCR (sqRT-PCR) and the amplified DNA fragments were quantified by densitometry. The results showed that \textit{P. brasiliensis icl} and \textit{mls} transcript levels were increased following phagocytosis, as well as in response to \textit{in vitro} cultivation under glucose deprivation. Furthermore, in order to evaluate the transcriptional status of genes related to other central carbon metabolism, we have also analyzed the expression of pyruvate kinase (\textit{pky}) and phosphoenolpyruvate carboxykinase (\textit{pck}) genes, which encode key enzymes of the glycolytic and gluconeogenesis pathways, respectively.

**Material and methods**

**Paracoccidioides brasiliensis strain and growth conditions**

The clinical isolate of \textit{P. brasiliensis}, strain Pb01 (ATCC-MYA-826), was maintained as yeast cells at 36°C on the semi-solid Fava Neto’s medium that contains; 0.3% protease peptone, 1% peptone, 0.5% beef extract, 0.5% yeast extract, 4% glucose, 0.5% NaCl, 1.6% agar, pH 7.2 \cite{24}. For \textit{ex vitro} assays, the yeast form was grown in Fava-Neto semi-solid medium for 7 days before infection of murine macrophages or being used as a control \cite{25}. For \textit{in vitro} assays employing different carbon sources, $2.5 \times 10^7$ \textit{P. brasiliensis} yeast cells were first inoculated into the complex medium YPD (yeast-peptone-dextrose) and grown to exponential phase (36°C/130 rpm). The glucose level, as determined by DNS assay (dinitrosalicylic acid), was monitored in order to assure that the fungal cells were not submitted to nutritional stress. Following 6 days, cells were harvested by centrifugation, washed and incubated for different time intervals (0.5 h, 1 h, 5 h) in defined McVeigh Morton (MVM) medium \cite{26} supplemented with 2% glucose or 2% acetate as sole carbon sources. The cell viability was observed after all treatments using vital Janus green stain \cite{27}. These treatments were carried out in triplicate in order to perform a statistical analysis.

**icl gene sequence and deduced protein analysis**

The \textit{P. brasiliensis icl} complete coding sequence was obtained by the sequencing of cDNA clones from mycelium and yeast cDNA libraries of \textit{P. brasiliensis} \cite{13,14}. The nucleotide sequence was submitted to the pipeline of analysis as described by Felipe et al. \cite{13}. Alignments were performed using BLAST (http://www.ncbi.nlm.nih.gov) and Clustal (http://www.ebi.ac.uk/clustaw) programs. The deduced protein sequence was analyzed using the ExPASy proteomics server (http://www.expasy.org).

**Southern blot analysis**

About 15 μg of \textit{P. brasiliensis} genomic DNA were digested with \textit{BamH}I, \textit{BglII}, \textit{EcoRI}, \textit{XbaI} and \textit{XhoI} restriction endonucleases, separated on 1% agarose gel and transferred to a Hybond N+ membrane, as specified by the manufacturer (Amersham Biosciences). The \textit{P. brasiliensis icl}-related cDNA fragments were labelled using (α-32P)-dATP by random priming procedure (MegaPrime DNA labelling System, Amersham Biosciences), purified and used in overnight hybridization at 42°C \cite{28}. The membranes were then washed to
a final stringency of 0.1 X SSPE-0.1% SDS at 65°C for 20 min, and exposed either to an X-ray film at −80°C for 48 h or to a Phosphor Screen (Molecular Dynamics – Amersham Biosciences) for 2 h.

**Total RNA extraction of *P. brasiliensis***

The *P. brasiliensis* total RNA was extracted using Trizol reagent (Gibco-BRL), according to suppliers’ recommendations, as previously described [29,30]. To remove any genomic DNA contamination, RNA was treated with RNase-free-DNaseI (Promega), followed by enzyme inactivation (EDTA 2.5 mM; 65°C /10 min) and ethanol precipitation.

**Infection of J774 macrophage cell line with *P. brasiliensis* yeast cells**

The macrophage-like cell line J774.1 (ATCC TIB-67) was cultured as an adherent monolayer in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated fetal calf serum (Gibco), 3.024 g l⁻¹ NaHCO₃ and 2 mM L-glutamine. *In vitro* cultured yeast cells of *P. brasiliensis* were suspended in RPMI medium containing 20% fresh BALB/c mouse serum at 37°C for 30 min in order to achieve opsonization. Macrophage monolayers (2×10⁶ cells/150 cm² culture flask) were then infected with 2×10⁶ opsonized yeast cells, which gave a yeast-to-macrophage ratio of 1:5 and incubated for 9 h at 37°C in an atmosphere containing 5% CO₂ [25].

**Total RNA extraction of *P. brasiliensis* yeast cells internalized by J774 macrophage cell line**

At 9 h after infection, extracellular fungi were removed by exhaustive washing with RPMI pre-warmed to 37°C. Macrophages were then lysed with GTC solution (guanidine thiocyanate 4M, sodium N-lauryl sarcosine 0.5%, tri-sodium citrate 50mM and 2-mercaptopethanol 0.1M) and intact fungi were harvested by centrifugation (8000 x g for 10 min) followed by RNA extraction and amplification using Trizol (Gibco) and MessageAmp aRNA kit (Ambion), respectively, according to the manufacturer’s instructions. Due to the limited quantity of total RNA extracted in a single macrophage infection experiment (~3 µg), gene expression analyzed here was obtained from three independent sets of pooled RNA. Each pool consisted of 3–4 macrophage infection experiments [25], assuring adequate experimental biological repetitions.

Total RNA from *P. brasiliensis* yeast cells cultured in Fava-Neto’s medium for 7 days was extracted and amplified exactly as described above, and used as control.

**Semi-quantitative RT-PCR**

The transcriptional analysis of *P. brasiliensis* cultured *in vitro* in response to different carbon sources was carried out employing sqRT-PCR methodology. The cDNA first strand was synthesized from 2 µg of DNaseI-treated total RNA in a 25 µl reaction containing: 0.5 µg primer dT₁₂⁻₁₈, first strand buffer 1X, 8 mM DTT, 0.4 mM dNTPs, 200 U SuperScript II Reverse Transcriptase (Invitrogen).

The cDNA synthesis using RNA obtained from *P. brasiliensis* yeast cells recovered from macrophages was accomplished as described below. One µl of RNA amplification reaction (equivalent to 250 ng of mRNA) was employed in cDNA first strand synthesis in a reaction exactly as described above, except for replacing the primer dT₁₂⁻₁₈ by random primers. All cDNA synthesis reactions were carried out at 42°C for 1 h, followed by 20 min at 70°C to inactivate the enzyme, according to suppliers’ recommendations. In all RT-PCR experiments, we always included as a negative control, i.e., a reaction where reverse transcriptase was omitted.

Five µl of cDNA reaction were amplified in a final reaction volume of 25 µl containing; Taq DNA Polymerase buffer 1X, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.2 µM specific primers (icl, mls, pyk or pck) and 0.2 µM α-tubulin primers (used as internal control), 2U of Taq DNA Polymerase (Cenbiot-RS/Brasil). After a first step of denaturation (94°C for 2 min), the amplification was done for 24 cycles consisting of; 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, followed by a final extension at 72°C for 10 min. The conditions were chosen so that all amplifications were in the exponential phase, as previously described by Marone et al. [31]. The appropriate number of cycles in which the amplification is in the exponential range was defined by testing a number of cycles ranging from 24 to 36. The optimal number of cycles should be in the same range for the specific gene of interest and for the housekeeping α-tubulin (α-tub) gene. The latter was used as an internal control [32] in all experiments carried out in this work. Since the intensity of specific amplification products reached a plateau at around 30 cycles (data not shown), all semiquantitative analysis employed 25 cycles. The RT-PCR products were analyzed by 1.5% agarose gel electrophoresis. Each set of reactions always included a negative control containing RNA instead of cDNA, to rule out genomic DNA contamination.

The primers used in sqRT-PCR experiments were determined using the software Primer 3, available online (http://www-genome.wi.mit.edu). Primers were always chosen according to the following parameters;
optimal length 20–22 bases, optimal Tm of 60 °C, length of amplification product between 200 and 600 bp, as described in Table 1.

Quantitative analysis

The quantitative analysis of gene expression levels was performed by densitometry employing the Scion Image software, available on-line (http://www.scioncorp.com). Amplified product intensity was expressed as relative absorbance units (AU). The ratio between the relative AU determined for the amplified gene of interest and the internal control \(\alpha\)-tubulin was calculated to normalize variations in sample concentration and as a control for reaction efficiency. Mean and standard error of all performed experiments were calculated after this normalization in relation to \(\alpha\)-tubulin gene amplification.

The statistical analyses were performed using the software ‘Mynova’, version 1.3 (S. Brooks, Copyright 1993). The statistical test applied was Student’s \(t\) test. A \(P\) value \(\leq 0.05\) was considered significant.

Nucleotide sequence accession number

The GenBank accession numbers of the nucleotide sequence(s) used in this work are cited in Table 1.

Results

The \(P.\) brasiliensis glyoxylate cycle-related genes

The analysis of \(P.\) brasiliensis transcriptome revealed orthologs of the glyoxylate cycle genes (\(icl\) and \(mls\)) of other human facultative intracellular pathogenic fungi [14,15]. The BLAST comparative analyses of the sequences described in the \(P.\) brasiliensis transcriptome project [13,14] enabled us to identify two putative \(P.\) brasiliensis isocitrate lyase genes, with 46% identity at the amino acid level. Southern blot analysis confirmed that these sequences indeed correspond to two different \(icl\)-related genes (Fig. 1). In addition, subsequent analyses revealed that the expression pattern of one of these genes was not significantly altered when this fungus was cultivated in media containing glucose or acetate as the sole carbon source (data not shown).

Altogether, these observations allowed us to suggest that these genes probably correspond to isocitrate lyase (\(icl\)) and methylisocitrate lyase (\(mcl\)), which encode key enzymes of glyoxylate and methylcitrate cycles, respectively. Although these enzymes catalyze analogous reactions, the methylcitrate and glyoxylate cycles differ with respect to the preferential utilization of three-carbon (C3) instead of C2 compounds, respectively [33,34]. In the present work, we describe the regulation pattern of \(icl\) and \(mls\) genes, which encode the major regulatory enzymes of the glyoxylate cycle.

The entire coding sequence of \(P.\) brasiliensis \(icl\) gene was determined, revealing a deduced primary sequence of 537 amino acid residues with a predicted molecular mass of 60 kDa, \(pI\) of 6.79 and a conserved isocitrate lyase signature motif (K-[KR]-C-G-H-[LMQR]) (data not shown).

Table 1 Oligonucleotides employed in the semiquantitative RT-PCR analysis.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Gene and Accession Number*</th>
<th>Sequence</th>
<th>Length of amplification product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'TUB</td>
<td>(\alpha)-tubulin CN242437</td>
<td>5'-TTCTGTTGATCTGGACCCCTTC-3'</td>
<td>199</td>
</tr>
<tr>
<td>3'TUB</td>
<td>malate synthase CN253867</td>
<td>5'-GGAGGGACGGAGGGATTTAAC-3'</td>
<td>414</td>
</tr>
<tr>
<td>5'MLS</td>
<td>isocitrate lyase EF032483</td>
<td>5'-GCTAACCAGATGGTCAAAT-3'</td>
<td>265</td>
</tr>
<tr>
<td>3'ICL</td>
<td>methylisocitrate lyase CN246564</td>
<td>5'-TTTGGTACGAATCCGCTCAGTC-3'</td>
<td>304</td>
</tr>
<tr>
<td>5'ICL-rel</td>
<td>isocitrate lyase-related CA580673</td>
<td>5'-GCCTGCTAGATGCTCTTTC-3'</td>
<td>311</td>
</tr>
<tr>
<td>3'ICL-rel</td>
<td>phosphoenolpyruvate CA580673</td>
<td>5'-ATGGCTGACAGGGATTTTC-3'</td>
<td>391</td>
</tr>
</tbody>
</table>

*These nucleotide sequences were submitted to the GenBank™/EBI Data Bank.
cycle *icl* and *mls* genes, as well as the *pck* and *pyk* genes was undertaken employing sqRT-PCR methodology.

The RT-PCR experiments were performed using RNA extracted from *P. brasiliensis* yeast cells recovered from murine J774 macrophages after 9 hours of co-culture, without any additional *in vitro* growth after host cells infection (Fig. 2). These experiments were carried out employing specific primers directed to the internal control (*α*-tub), and to the experimental genes (*icl, mls, pck* and *pyk*). Following the RT-PCR product quantification by densitometry, appropriate statistical analysis were performed, revealing that after macrophage internalization both glyoxylate cycle genes (*icl* and *mls*) showed higher expression levels when compared to cells grown *in vitro* in a conventional medium as control. Thus, the significant induction of *icl* and *mls* observed following phagocytosis suggests a role of this metabolic pathway in *P. brasiliensis* adaptation inside macrophages, strongly reinforcing nutritional deprivation inside the phagosome. In addition to the induction of glyoxylate cycle genes in the macrophage inhospitable environment, the up-regulation of *pck* gene expression was also observed (Fig. 2). The co-regulation of glyoxylate cycle and gluconeogenesis is consistent with the importance of these pathways in providing energy from C2 compounds and fatty acid metabolism, as may occur inside macrophages phagolysosome [21,23,35]. In contrast, following phagocytosis, no significant repression of the glycolytic gene *pyk* was observed in our sqRT-PCR analyses (Fig. 2).

**Fig. 1** Two different *Paracoccidioides brasiliensis* putative *icl*-related genes. Equal amounts of *P. brasiliensis* genomic DNA (15 µg) were digested with several restriction enzymes and separated onto 1% agarose gel (lane 1 – BglII, lane 2 – HinIII, lane 3 – PstI, lane 4 – XhoI and lane 5 – BamHI). The results after gel blotting to a nylon membrane and hybridization with the two radiolabeled cDNA probes corresponding to PbAEST 1688 (P1 – *pbiicl*) and PbAEST 1381 (P2 – *pbiicl*-related) are shown in panels (A) and (B), respectively. The size of some restriction DNA fragments, *λ*-DNA/EcoRI/HinIII (A) or *λ*-DNA/HinIII (B), used as molecular markers, are shown.

**Fig. 2** Expression analysis of *Paracoccidioides brasiliensis* genes encoding key enzymes of the glyoxylate cycle, gluconeogenesis and glycolysis, in response to fungal internalization by murine macrophages. After co-culture of *P. brasiliensis* yeast cells with murine macrophage cells J774 for 9 h, the fungal cells were harvested by centrifugation, after macrophages lysis. The *P. brasiliensis* total RNA was extracted and used in RT-PCR experiments, as described in material and methods section. As a control condition, total RNA from *P. brasiliensis* yeast cells cultivated *in vitro*, in conventional Fava Neto’s medium for 7 days, was employed. Panel (A) shows the results of agarose gel electrophoresis analysis, where the amplified RT-PCR products resulting from the control and macrophage internalization experiments are shown in lines C and M, respectively. Panel (B) shows the semiquantitative analysis, performed exactly as described in Fig. 2, of *P. brasiliensis* *icl, mls, pck* and *pyk* genes expression, when *P. brasiliensis* were cultivated *in vitro* (■) or upon murine macrophages internalization (□). Bars represent standard errors of RT-PCR duplicate experiments, *P* < 0.05.

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Upregulation of P. brasiliensis glyoxylate cycle genes under in vitro nutritional stress condition

Aiming to investigate the expression profile of the genes analyzed upon fungal internalization by macrophages, we evaluated the transcriptional response of P. brasiliensis yeast cells to an in vitro nutritional condition thought to simulate a feature of the phagosome. The gene expression analysis was carried out by sqRT-PCR as described above. At first, P. brasiliensis yeast cells were grown in a complex medium for 6 days, a time at which the fungal cells were not submitted to a nutritional stress, as shown by the determination of reducing sugars by DNS assay (data not shown). After 6 days, the cells were harvested, washed and incubated for three different periods of time (0.5 h, 1 h and 5 h) in defined media containing glucose 2% (MVM-glucose) or acetate 2% (MVM-acetate), as the sole carbon sources. Following all treatments, carried out in triplicates, we observed no differences between the viability of cells cultivated in MVM-glucose or MVM-acetate as accessed by vital Janus green staining (data not shown). The fungal cells were harvested by centrifugation and total RNA was extracted and used in sqRT-PCR experiments as described above. Fig. 3 shows the levels of the target amplification product relative to the internal control (α-tub) when P. brasiliensis was grown for different periods of time in the presence of glucose (G) or acetate (A) as the sole carbon sources (Fig. 3B). Following appropriate statistical analysis, we observed a significant increase in the expression levels of icl and mls genes when the cells were grown in a medium containing acetate as the sole carbon source. This result strongly suggests that the expression levels of these genes are dependent on the primary carbon source, as observed with other pathogenic fungi, e.g., C. albicans [23] and Cryptococcus neoformans [35]. However, as opposed to other fungi, a considerable level of these glyoxylate cycle transcripts was consistently detected when P. brasiliensis was grown in 2% glucose (Fig. 3).

Analysis of gluconeogenic and glycolytic genes expression when P. brasiliensis was cultivated under nutritional stress in vitro

According to the results described above, the growth of P. brasiliensis yeast cells on C2 compounds as the sole carbon source activate the glyoxylate cycle in order to produce biosynthetic metabolic precursors. In conjunction with the phosphoenolpyruvate carboxykinase (PCK) or malic enzyme, the glyoxylate cycle can also provide phosphoenolpyruvate (PEP) and pyruvate, respectively, required to direct the carbon flux into the gluconeogenic pathway. The involvement of PCK in the modulation of the carbon flux in response to nutrient availability suggests that this metabolic gene expression may be also regulated by different carbon sources. In this context, the pck mRNA level of P. brasiliensis yeast cells was quantified by sqRT-PCR assay following in vitro treatment in defined media, supplemented with only glucose or acetate. In contrast to the glyoxylate cycle genes expression results, the pck gene was expressed at equivalent levels during the growth of P. brasiliensis in the presence of glucose as well as acetate (Fig. 3). In addition, the pyruvate kinase (pyk) mRNA level, that encodes an enzyme of glycolysis, was also quantified when P. brasiliensis yeast cells were cultivated in glucose or acetate as carbon sources.

Similar to pck gene expression data, we observed that the P. brasiliensis pyk gene was expressed at roughly equivalent levels during growth for 5 hours in the presence of both glucose and acetate (Fig. 3).

Discussion

The ability of P. brasiliensis to survive and successfully adapt within phagocytic cells depends on its mechanisms to respond to the metabolic constraints imposed by macrophages. Since it is believed that the microenvironment inside host cells is a hostile habitat, differential regulation of specific genes is probably involved in the establishment and adaptation of this pathogen in the context of the host. In accordance with this scenario, the analysis of P. brasiliensis transcriptome by Tavares et al. [15] revealed several putative orthologs to virulence genes of other human facultative intracellular pathogens, such as metabolism-, cell wall-, detoxification-related genes and secreted factors. Among these virulence factors, we identified other fungi icl and mls ortholog genes which encode isocitrate lyase and malate synthase, respectively.

It is often assumed that the modulation of metabolic-related genes can be associated with the microbiome’s response to a changing environment. In this context, we verified the carbon source influence in P. brasiliensis glyoxylate cycle genes expression. As expected, our studies showed that P. brasiliensis icl and mls genes are upregulated in response to acetate as the sole carbon source (Fig. 3). However, a basal level of glyoxylate cycle genes expression was observed when glucose was the sole carbon source, suggesting some carbon fluxes through this pathway. This result is consistent with icl regulation in M. tuberculosis [36,37]. On the other hand, in the pathogenic fungus C. neoformans, northern blot analyses showed a repression of icl gene expression by as little as 0.2% of glucose [35]. However, previous results from our
Expression analysis of *Paracoccidioides brasiliensis* genes encoding key enzymes of the central C-metabolism, in response to fungal *in vitro* incubation in the presence of glucose or acetate as the sole carbon source. After *P. brasiliensis* incubation in defined media containing 2% glucose (MVM-glucose) or 2% acetate (MVM-acetate) for different periods of time, total RNA was extracted and used in RT-PCR experiments, employing specific primers directed to the internal control (α-tub) and the experimental icl, mls, pck or pyk genes. Panel (A) presents the results of one of the triplicate RT-PCR experiments, showing the amplified *P. brasiliensis* cDNAs obtained in response to fungal cultivation in MVM-glucose (line G) or MVM-acetate (line A) medium, for 0.5 h, 1 h or 5 h. Panel (B) shows the semiquantitative analysis of *P. brasiliensis* icl, mls, pck and pyk genes expression, performed by densitometry, employing the Scion Image software (http://www.scioncorp.com). The icl, mls, pck or pyk transcript levels were calculated in relation to the internal control α-tub for each experimental condition, glucose (■) or acetate (□), as described in panel (A). Bars represent standard errors of triplicate experiments, *P < 0.05.*
group, also using northern blot assay, revealed a relatively high level of icl transcripts when P. brasiliensis yeast cells were grown in a rich medium [14]. In the present work, using RT-PCR methodology, we confirm these data, strongly suggesting that different from other fungi, high glucose levels do not repress P. brasiliensis glyoxylate cycle genes. This basal expression could be responsible for the apparently weak induction in the experiments described in this manuscript. In accordance with our results, Cánovas and Andrianopoulos [38] recently described in Penicillium marneffei, also a thermally dimorphic opportunistic human pathogen, that the acuD gene, encoding ICL enzyme, is regulated by both carbon source and temperature. Northern blot analyses revealed that the acuD gene was strongly induced at 37°C, with relatively small differences in the transcript levels when the fungus was incubated at 37°C with glucose or acetate as carbon sources [38].

The transcriptional modulation of the glyoxylate cycle genes in response to nutrient availability is probably connected with the regulation of other metabolic pathways. In fact, a constitutive carbon source-responsive promoter element necessary for activation of glyoxylate cycle genes was also identified in upstream regions of genes involved in gluconeogenesis in S. cerevisiae [39–41]. Accordingly, it was verified in other microorganisms, like C. albicans and M. bovis, that the gluconeogenic gene pck is induced after growth on non-fermentative media [23,42]. However, differences in the control of gluconeogenesis in Aspergillus nidulans have been reported where the transcriptional activator FacB, which mediates acetate induction of icl gene, is not directly involved in pck induction [43]. In P. brasiliensis, we verified that the regulation of gluconeogenic and glyoxylate cycle genes was not completely linked. We noted that the pck gene was expressed at the same levels when this fungus was cultured for 5 h in either acetate or glucose (Fig. 3). Interestingly, Barell et al. [23] also described that during C. albicans internalization by macrophages and neutrophils the glyoxylate anaplerotic reactions can produce intermediates to other metabolic pathways. In this sense, the analysis of P. brasiliensis icl, mls and pck promoters, in progress by our group, should provide more information to elucidate the role of the transcriptional regulation of these genes in the central carbon metabolism.

On the other hand, in a nutritional stress condition, the pyk and pfk genes are supposed to be repressed, as observed in C. albicans and S. cerevisiae yeast cells [23,44]. However, in P. brasiliensis, we observed an equivalent expression level of the pyk gene during growth for 5 h in glucose or acetate (Fig. 3). It is possible that during this initial growth of P. brasiliensis yeast cells on acetate, part of the phosphoenolpyruvate generated from oxaloacetate could be metabolized in gluconeogenesis, while another part is converted by pyruvate kinase in the glycolytic direction to sustain proper pyruvate availability for biomass synthesis [45]. At this incubation period, the growth rate is similar when P. brasiliensis is cultivated in both carbon sources. However, an overnight culture shows a significant reduction of cells growth in MVM-acetate when compared to MVM-glucose medium (data not shown). This result may be also explained by the pre-culture conditions since even when fungal cells were grown in YPD for 6 days more than 1.5% of reducing sugars were still observed (data not shown). Under this condition, P. brasiliensis cells may have synthesized reserve carbohydrates, such as glycogen, which may be used in carbon metabolism. Finally, it is probable that the central carbon metabolism is regulated to a large extent by post-transcriptional mechanisms, mainly in the early response to environmental changes [46].

The induction of glyoxylate cycle genes in media containing C2 compounds can play an important role in P. brasiliensis persistence during infection. Since this fungus is a facultative intracellular pathogen and glycolytic substrates are supposed to be absent or sparse in the phagolysosome environment [22], glyoxylate cycle may be required for the utilization of C2 compounds derived from fatty acids in energy production. The role of this pathway in the survival of microorganisms within macrophages has been shown in pathogens, such as M. tuberculosis and C. albicans [20–23,37]. In P. brasiliensis, glyoxylate cycle genes were also induced following phagocytosis (Fig. 2), suggesting that this pathway could be used by this intracellular pathogen to subsist on C2 (acetyl-CoA) compounds within host cells. The switches to this anaplerotic pathway, in conjunction with the sequential action of PCK enzyme, are required for the production of phosphoenolpyruvate (PEP), an essential biosynthetic precursor of glucose synthesis. Here, we show that pck gene is also upregulated after phagocytosis (Fig. 2) indicating that the respective gene product probably plays a role in the successfully P. brasiliensis adaptation within macrophages. Although the in vitro results when P. brasiliensis was incubated for 5 h with acetate as carbon source do not show a significant increase of pck transcript (Fig. 3), it is important to emphasize that the phagolysosome corresponds to a complex habitat, with probably other factors being also required for pck gene regulation. While the nutritional composition of this organelle is essentially unknown, it is recognized that the phagolysosome milieu is replete of antimicrobial compounds and has an acidic pH environment [19]. In
fact, consistent with our proposition, studies showed that *Agrobacterium tumefaciens* *pek* gene is induced by acidic pH conditions [47].

The activation of gluconeogenic pathway is normally coordinated with the repression of the glycolytic pathway. Although our analysis shows that the expression levels of *P. brasiliensis* *pyk* gene was not significantly altered after phagocytosis (Fig. 2), DNA microarray assays performed by our group showed downregulation of the glycolytic phosphofructokinase gene (*pfk*) when *P. brasiliensis* infected murine peritoneal macrophages [25]. The *pfk* gene encodes another controlling protein of the glycolytic pathway that also catalyzes an essential irreversible reaction. We believe that the apparently conflicting results found in glycolytic genes expression upon *P. brasiliensis* yeast cells internalization by macrophages could be explained by the observation that a small percent of cells are non-phagocytosed at the end of the time course of our experiment. A similar result was previously described by Fradin et al. [48], comparing the transcriptional response of *C. albicans* in human blood and in a mouse infection model. Furthermore, since the sqRT-PCR methodology is less sensitive when the gene expression levels among samples show small differences, the presence of a heterogeneous cell population would also influence this apparently conflicting result.

In conclusion, we observed that the transcriptional regulation of *P. brasiliensis* glyoxylate cycle genes is carbon source dependent, as demonstrated by in vitro assay. The upregulation of these glyoxylate cycle genes, coordinated with the increase of the gluconeogenic transcript levels, was also verified following co-culture of *P. brasiliensis* with murine macrophages, indicating a shift to a non-fermentative metabolism when *P. brasiliensis* is phagocytosed by host cells. The differential expression of *icl*, *mls* and *pek* genes suggests a role of glyoxylate cycle and gluconeogenic pathways in the adaptation of this fungus to the internal harsh milieu of macrophages, as well as to other hostile environments. Although the real importance of these pathways in the disease progression needs to be confirmed by virulence assays, our results reinforce the importance of the central carbon metabolism regulation to pathogen survival and adaptation inside host cells.

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