

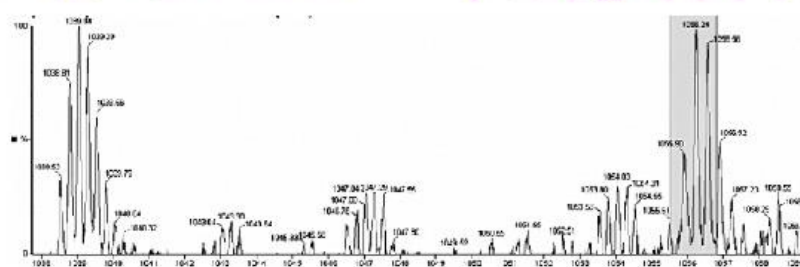
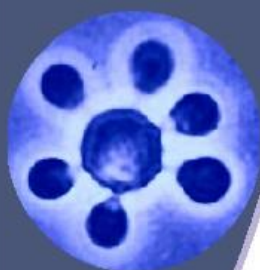


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Dissertação de Mestrado

**Mapa proteômico de
espécies filogenéticas do
complexo *Paracoccidioides***



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Orientador (a):	Célia Maria de Almeida Soares
E-mail:	cmasoares@gmail.com
Co-orientador (a):	Ana Flávia Alves Parente
E-mail:	afparente@gmail.com CPF:87232413149

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**UNIVERSIDADE FEDERAL DE GOIÁS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA**

Laurine Lacerda Pigosso

**Mapa proteômico de espécies filogenéticas do complexo
Paracoccidioides.**

Orientadora: Prof^a. Dr^a. Célia Maria de Almeida Soares

**Dissertação apresentada ao Programa
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BANCA EXAMINADORA

TITULARES:

Profa. Dra. Célia Maria de Almeida Soares

Instituto de Ciências Biológicas, Universidade Federal de Goiás.

Profa. Dra. Juliana Alves Parente

Instituto de Ciências Biológicas, Universidade Federal de Goiás.

Profa. Dra. Rosely Maria Zancopé-Oliveira

Instituto de Pesquisa Clínica Evandro Chagas, Fundação Oswaldo Cruz - RJ.

SUPLENTE:

Prof. Dr. Alexandre Melo Bailão

Instituto de Ciências Biológicas, Universidade Federal de Goiás.

Profa. Dr. Clayton Luiz Borges

Instituto de Ciências Biológicas, Universidade Federal de Goiás.

"Possuímos em nós mesmos pelo pensamento e a vontade, um poder de ação que se estende muito além dos limites de nossa esfera corpórea."

Allan Kardec

"A vida é como sorvete de casquinha... você precisa aprender equilibrá-la!"

Charlie Brown

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"Aqueles que passam por nós, não vão sós, não nos deixam sós. Deixam um pouco de si, levam um pouco de nós."

ANTOINE DE SAINT- EXUPÉRY

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RESUMO

O gênero *Paracoccidioides* compreende um complexo de espécies filogenéticas do fungo patogênico dimórfico, agente etiológico da paracoccidioidomicose (PCM), uma doença restrita à América Latina e de relevância acentuada em suas áreas endêmicas, devido à sua alta frequência e gravidade. Os membros do gênero *Paracoccidioides* são distribuídos em espécies filogenéticas distintas (*S1*, *PS2*, *PS3* e *01-like*) que diferem potencialmente nas suas características bioquímicas e moleculares. Neste trabalho, foi realizada a caracterização proteômica de diferentes membros do gênero *Paracoccidioides*. Foram comparados os perfis proteômicos de *Pb01* (*01-like*), *Pb2* (*PS2*), *Pb339* (*S1*) e *PbEPM83* (*PS3*) utilizando eletroforese 2D e espectrometria de massa. As proteínas / isoformas foram selecionados com base na intensidade de coloração dos spots conforme determinado por análise de imagem. As proteínas / isoformas foram excisadas do gel, digeridas e identificadas por PMF (*Peptide mass fingerprinting*) e fragmentação iônica. Um total de 714 spots foi detectado, 343 foram analisados. A partir destes spots, 301 apresentaram-se diferencialmente expressos entre os quatro isolados analisados, determinado por ANOVA. Depois de aplicar a correção FDR, um total de 267 spots foram diferencialmente expressos. Do total, 193 / isoformas proteínas foram identificadas por PMF e confirmadas por fragmentação iônica. Comparando-se os perfis de expressão dos isolados, as proteínas / isoformas que foram relacionados para a glicólise / gliconeogênese e fermentação alcoólica foram mais abundantes em *Pb01* do que em outros representantes do gênero *Paracoccidioides*, indicando uma maior utilização das vias anaeróbias para a produção de energia. Enzimas relacionadas com a resposta ao estresse oxidativo foram mais abundantes em *Pb01*, *Pb2* e *Pb339*, indicando uma melhor resposta às ROS nestes membros do complexo *Paracoccidioides*. As enzimas da via das pentoses foram abundantes em *Pb2*. Proteínas antigênicas, tal como GP43 e uma proteína antigênica de 27 kDa, foram menos abundantes em *Pb01* e *Pb2*. O perfil proteômico indica diferenças metabólicas entre os membros analisados do gênero *Paracoccidioides*.

ABSTRACT

The genus *Paracoccidioides* comprises a complex of phylogenetic species of dimorphic pathogenic fungi, the etiologic agents of paracoccidioidomycosis (PCM), a disease confined to Latin America and of marked relevance in its endemic areas due to its high frequency and severity. The members of the *Paracoccidioides* genus are distributed in distinct phylogenetic species (S1, PS2, PS3 and 01-like) that potentially differ in their biochemical and molecular characteristics. In this work, we performed the proteomic characterization of different members of the genus *Paracoccidioides*. We compared the proteomic profiles of *Pb01* (01-like), *Pb2* (PS2), *Pb339* (S1) and *PbEPM83* (PS3) using 2D electrophoresis and mass spectrometry. The proteins/isoforms were selected based on the staining intensity of the spots as determined by image analysis. The proteins/isoforms were in-gel digested and identified by peptide mass fingerprinting and ion fragmentation. A total of 714 spots were detected, of which 343 were analyzed. From these spots, 301 represented differentially expressed proteins/isoforms among the four analyzed isolates, as determined by ANOVA. After applying the FDR correction, a total of 267 spots were determined to be differentially expressed. From the total, 193 proteins/isoforms were identified by PMF and confirmed by ion fragmentation. Comparing the expression profiles of the isolates, the proteins/isoforms that were related to glycolysis/gluconeogenesis and to alcohol fermentation were more abundant in *Pb01* than in other representatives of the genus *Paracoccidioides*, indicating a higher use of anaerobic pathways for energy production. Those enzymes related to the oxidative stress response were more abundant in *Pb01*, *Pb2* and *Pb339*, indicating a better response to ROS in these members of the *Paracoccidioides* complex. The enzymes of the pentose phosphate pathway were abundant in *Pb2*. Antigenic proteins, such as GP43 and a 27-kDa antigenic protein, were less abundant in *Pb01* and *Pb2*. The proteomic profile indicates metabolic differences among the analyzed members of the *Paracoccidioides* genus.



Introdução



1- INTRODUÇÃO

1.1- Aspectos Gerais e biologia do fungo

Paracoccidioides spp, um complexo de, no mínimo, quatro espécies filogenéticas, comumente designado *Paracoccidioides brasiliensis* é um fungo termodimórfico, agente etiológico da micose sistêmica denominada paracoccidioidomicose (PCM) (Brummer et al. 1993). O fungo cresce como levedura à 36 °C nos tecidos do hospedeiro ou quando cultivado *in vitro* sob essa temperatura. A forma infectiva, miceliana, é encontrada em condição saprobiótica no ambiente, ou quando cultivada em temperaturas inferiores a 28°C (San-Blas et al. 2002).

Morfologicamente, as leveduras de *P. brasiliensis* apresentam múltiplos brotamentos formados por evaginações da célula-mãe, revelando uma célula central circundada por várias células periféricas, microscopicamente similar à roda de leme de navio, o que caracteriza a presença do fungo em materiais biológicos. A forma miceliana pode ser identificada por filamentos septados com conídios terminais ou intercalares (Queiroz-Telles, 1994, Restrepo-Moreno, 2003). Esse dimorfismo característico, bem como a tolerância térmica, são considerados fatores de virulência importantes para a adaptação do patógeno às condições ambientais do hospedeiro, como alta temperatura, influência hormonal e a resposta imune (Bagagli et al. 2008).

O habitat natural do fungo ainda é desconhecido, porém, estudos relatam que *P. brasiliensis*, adapta-se bem em solos úmidos, com pH ácido e ricos em vegetação e matéria orgânica, em regiões de temperatura moderada com curto período de seca, onde pode crescer sob a forma miceliana e produzir conídios. Considera-se, portanto que esse

seja o nicho ecológico mais provável desse microrganismo (Restrepo et al. 1985). Além do seu isolamento de solo do Brasil (Silva-Vergara et al. 1998), da Argentina (Negroni et al. 1966) e da Venezuela (Albornoz et al. 1971), o fungo tem sido freqüentemente isolado de tatus (*Dasypus novem cinctus* no Brasil e *Cabassous centralis* na Colômbia) em áreas endêmicas e esses animais são reconhecidos como reservatórios naturais do fungo (Corredor et al. 2005, Bagagli et al. 2008). O patógeno também tem sido relatado em cachorros (Farias et al. 2011), pingüins (Garcia et al. 1993) e morcegos frugívoros (Grose & Tamsit 1965). Testes sorológicos e intradérmicos também sugeriram a presença do fungo em animais domésticos e primatas (Corte et al. 2007).

1.2- A Paracoccidioidomicose

A PCM é uma micose humana sistêmica granulomatosa caracterizada pela formação de aglomerados de células epiteliais com áreas centrais de necrose e agregados de leucócitos polimorfonucleares. Evidências indicam que o granuloma está intimamente relacionado à resposta imune do hospedeiro e deve representar uma resposta tecido-específica ao fungo na tentativa de destruí-lo e prevenir a sua multiplicação (De Brito et al. 1994).

A infecção ocorre, usualmente, pela inalação de propágulos do micélio, como artroconídeos, que atingem os pulmões onde transitam para a forma leveduriforme, podendo disseminar-se para outros órgãos (Restrepo et al. 2001). extrapulmonares como fígado, baço e sistema nervoso central (San-Blas 1993, Camargo & Franco 2000, Valera et al. 2008).

A PCM é endêmica com alta prevalência na América Latina (Franco et al. 1987) onde se estima que cerca de dez milhões de pessoas estejam infectadas, embora somente cerca de 2% apresentam predisposição para desenvolver a doença (McEwen et

al. 1995). Dados epidemiológicos indicam uma distribuição que se estende desde o México até a Argentina (San-Blas et al. 2002). Sua distribuição é heterogênea e os países que se destacam por apresentar o maior número de casos relatados são o Brasil, Colômbia e a Venezuela. No Brasil, as regiões Sul, Sudeste e Centro-Oeste apresentam maior incidência, apresentando o estado de Goiás os maiores números de casos (Wanke & Londero 1994). Acometendo principalmente a população rural, devido possivelmente ao contato contínuo com o habitat do fungo, a PCM é a décima causa de mortalidade por doença crônica, entre as infecciosas e parasitárias, e a primeira entre as micoses sistêmicas no Brasil (Prado et al. 2009), revelando ser um importante problema de saúde pública.

A doença acomete principalmente adultos do sexo masculino em uma razão de 48 homens para cada mulher (Restrepo 1978). Estudos relatam que esta predominância, provavelmente, está ligada tanto à atuação do hormônio feminino β -estradiol na inibição da transição (Restrepo 1985, Sano et al. 1999). Acredita-se que a interação do hormônio com uma EBP (Estradiol Binding Protein), identificada em *P. brasiliensis*, iniba a transição morfológica do fungo, explicando a baixa incidência da PCM em mulheres (Felipe et al. 2005, Shankar et al. 2011).

A apresentação clínica e o curso da infecção dependem do paciente e do isolado e o aparecimento de lesões secundárias nas mucosas, pele, linfonodos e glândulas adrenais é frequente (Restrepo-Moreno 1993). A PCM pode evoluir sob duas formas principais. A forma aguda é a evolução mais grave da doença, que representa de 3 a 5% dos casos, acomete ambos os sexos, geralmente pacientes jovens, e origina-se a partir de uma lesão primária não detectada que progride rapidamente com disseminação para o baço, fígado, linfonodos e medula óssea. A forma crônica tem sua origem em um foco primário ou em uma lesão antiga contendo formas viáveis de *P. brasiliensis*,

podendo estar restrita ou atingir múltiplos órgãos, apresenta progressão mais lenta e representa mais de 90% dos casos (Montenegro 1986, Franco 1987, Tristano et al. 2004) . A maioria dos indivíduos infectados, no entanto, desenvolve a forma assintomática ou subclínica, a qual pode evoluir para doença com uma diversidade de formas clínicas em função de fatores do hospedeiro, níveis de virulência das linhagens e condições ambientais (San-Blas et al. 2002).

1.3- Classificação Taxonômica

A paracoccidioidomicose foi primeiramente descrita por Lutz, em 1908, que isolou o microrganismo a partir de granulomas ganglionares e lesões na mucosa oral de pacientes. O fungo foi inicialmente caracterizado por Almeida (1930) como sendo do gênero *Paracoccidioides* e da espécie *brasiliensis*. Desde então, devido à dificuldade de classificação taxonômica, já que não se conhece a forma sexuada do fungo, diversas denominações foram atribuídas. A classificação aceita hoje é baseada em comparações filogenéticas de sequências de nucleotídeos da subunidade maior do RNA ribossomal realizada por Leclerc e colaboradores (1994), associada a análises morfológicas, classificando-o como pertencente ao reino Fungi, filo Ascomycota, subdivisão Euascomycotina, classe Plectomyceto, subclasse Euascomycetidae, ordem Onygenales, família Onygenaceae, subfamília Onygenaceae Anamórficos, gênero *Paracoccidioides*, espécie *Paracoccidioides brasiliensis* (San-Blas et al. 2002). *P. brasiliensis* está classificado no mesmo táxon que outros fungos dimórficos e patogênicos como *Blastomices dermatitides*, *Emmonsia* spp. e *Histoplasma capsulatum* (Peterson & Singler 1998).

Desde a sua descrição em 1908 por Adolfo Lutz, o fungo *P. brasiliensis* vem sendo considerado como uma espécie única, no entanto, sua variabilidade genética

ainda não é totalmente conhecida. Análises por marcadores RAPD (Randomly Amplified Polymorphic DNA) demonstraram fortes evidências de alta variabilidade entre isolados de *P. brasiliensis*, mostrando uma possível correlação com a virulência, distribuição geográfica e resistência a drogas (Soares et al. 1995, Calcagno et al. 1998; Molinari-Madlum et al. 1999, Motta et al. 2002, Hahn et al. 2003).

Para caracterizar a variação genética natural deste fungo, Matute e colaboradores (2006) descreveram com base em estudos filogenéticos por MLST (*Multi Locus Sequencing Type*) de 65 isolados de *P. brasiliensis*, descreveram a existência de três diferentes espécies filogenéticas de *P. brasiliensis*: S1 (espécie 1), PS2 (espécie filogenética 2) e PS3 (espécie filogenética 3). A espécie filogenética PS3 (espécie filogeneticamente distinta contendo 21 isolados) está geograficamente restrita à Colômbia, enquanto S1 (38 isolados em uma única espécie) está distribuída no Brasil, Argentina, Paraguai, Peru e Venezuela. Alguns isolados da espécie filogenética PS2 (espécie filogeneticamente distinta constituída de 5 isolados) foram encontrados na Venezuela e no Brasil, nos Estados de São Paulo e Minas Gerais. A presença de recombinação nas regiões gênicas analisadas constituiu uma forte evidência de uma possível reprodução sexuada neste fungo (Matute et al. 2006).

Carrero et al. (2008), no intuito de analisar as relações filogenéticas entre os isolados de *P. brasiliensis*, realizaram comparações de sequências de regiões codantes, não codantes e ITS (sequência espaçadora interna - “internally transcribed sequence”) de 7 novos isolados e 14 isolados já estudados de *P. brasiliensis* oriundos do Brasil, Colômbia e Venezuela. Nesse estudo realizado pela técnica de concordância genealógica para reconhecimento de espécies filogenéticas analisaram 14 genes de 21 isolados de *P. brasiliensis*. Com exceção das análises filogenéticas realizadas para os genes codificantes para quitina sintase 1 e catalase A, o isolado Pb01 apresentou-se

filogeneticamente distinto dos outros isolados analisados. Vinte isolados se agruparam nos três grupos filogenéticos descritos anteriormente, com exceção do isolado *Pb01*. Este isolado apresentou uma alta variabilidade genética quando comparado com os outros isolados de *P.brasiliensis*. Sugeriu-se então, que o gênero *Paracoccidioides* seria composto por quatro linhagens filogenéticas distintas (*PS2*, *PS3*, *S1* e *Pb01-like*), que variam na virulência, na adaptação ambiental e nas diferentes respostas imunológicas induzidas no hospedeiro. (Carrero et al. 2008, Theodoro et al. 2008).

Na continuidade dos estudos filogenéticos e na tentativa de elucidarem o status taxonômico do isolado *Pb01*, Teixeira e colaboradores (2009), prosseguiram os estudos iniciados por Matute e colaboradores (2006), via concordância genealógica pelo Método de Reconhecimento de Espécies Filogenéticas (GCPSR) investigaram a possível existência de novas espécies incluindo *Pb01* e outros isolados Latino-Americanos e sugeriram que o isolado *Pb01* poderia ser classificado como um novo grupo filogenético. Foram identificados 17 isolados similares genotipicamente, incluindo *Pb01*, que se distanciavam do clado das três espécies filogenéticas *S1/PS2/PS3*, quando analisado o grau de divergência entre eles. Isso indica que os dois grupos são altamente divergentes um do outro. Análises morfológicas de um membro escolhido de cada grupo filogenético do gênero *Paracoccidioides* revelaram características que são exclusivas de cada grupo corroborando com os dados filogenéticos. (Teixeira et al. 2009). Assim, *P. brasiliensis* atualmente consiste em um complexo de espécies (Richini-Pereira et al. 2009).

Adicionalmente, a organização genômica de *P. brasiliensis* foi também investigada. De acordo com o sequenciamento dos genomas estruturais dos isolados *Pb01*, *Pb03* e *Pb18*, foi possível identificar a presença de cinco cromossomos em cada isolado. As análises permitiram verificar que o isolado *Pb01* apresenta 9132 genes e

32,94 Mb, apresentando um maior número de genes quando comparado aos isolados *Pb03* e *Pb18*, que apresentaram 7875 e 8741 genes e 29,06 e 29,95Mb, respectivamente(<http://www.broad.mit.edu/science/projects/msc/data-release-summary>). Recentemente, *Pb01*, o isolado clínico mais filogeneticamente distinto das outras linhagens, foi sugerido como uma nova espécie, *Paracoccidioides lutzii* (Desjardins et al. 2011).

1.4- Análises Proteômicas

O termo proteoma foi utilizado pela primeira vez por Wilkins e Williams, em 1995, e atualmente tem sido definido como a análise em grande escala de proteínas expressas por uma determinada célula, tecido ou organismo numa condição específica (Wilkins et al. 1997).

A análise proteômica ganhou considerável atenção desde que foi revelado que o genoma humano consiste de poucos genes (Venter 2001) e constatou-se que, embora importante, a análise das sequências de nucleotídeos nem sempre reflete uma relação direta com os níveis de proteínas expressas e, consequentemente, de atividade biológica (Gygi et al., 1999). O perfil de expressão de proteínas altera-se em situações distintas ou diferentes tipos celulares em um mesmo organismo, constituindo-se um fluxo dinâmico respondendo a estímulos internos e externos. As proteínas envolvidas em processos biológicos são detectadas devido a alterações quantitativas pouco ou muito reguladas, aparecimento ou desaparecimento e modificações pós-traducionais (Westermeyer et al. 2002).

O objetivo inicial dos estudos proteômicos foi a identificação em larga escala de todas as proteínas presentes em uma célula ou tecido. Atualmente, consistem na análise simultânea de misturas complexas de proteínas como as provenientes de lisados

celulares e extratos de tecidos com o intuito de detectar diferenças quantitativas e qualitativas na expressão proteica (Westermeyer & Naven 2002). Utiliza-se de diferentes metodologias, no contexto da proteômica comparativa, onde o objetivo é identificar diferenças quantitativas e qualitativas entre amostras de proteínas, a técnica de eletroforese bidimensional (2-DE) é geralmente o método escolhido, gerando dados em formato que possibilita uma boa avaliação visual e fornece comparações quantitativas (Rabilloud, 2000; 2002). Recentemente, um progresso importante pode ser visto no campo da proteômica, com o desenvolvimento de métodos alternativos para separação e identificação de proteínas, como tecnologias baseadas em chips, nos chamadas “*arrays*” de proteínas (Ramachandran et al., 1987), a análise direta de complexos proteicos por espectrometria de massas (Link et al., 1999; Washburn et al., 2003), o uso de “*tags*” de afinidade (Gygi et al., 1999), são exemplos. Entre todas as técnicas de proteoma de uso atual, a mais utilizada é a eletroforese bidimensional (2D-PAGE), acoplada a espectrometria de massas do tipo MALDI-TOF.

Em fungos patogênicos humanos, como *Cândida albicans*, as análises proteômicas têm contribuído para um melhor conhecimento dos processos envolvidos na morfogênese, virulência, resposta ao hospedeiro, bem como para o desenvolvimento de alvos antifúngicos potenciais e abordagens terapêuticas (Pitarch et al. 2003). Para membros do complexo *Paracoccidioides*, a abordagem proteômica foi utilizada para estudos de transição morfológica micélio-levedura (Rezende et al. 2011), de comportamento mediante a privação de ferro (Parente et al. 2011), dentre outros. Estudos têm demonstrado que a utilização de técnicas de proteômica pode auxiliar na identificação de proteínas envolvidas no processo de interação parasito-hospedeiro (Pastorelli et al. 2006).

Os diferentes isolados de *Paracoccidioides spp*, podem diferir em suas características bioquímicas, o que justifica a necessidade de se caracterizar os isolados (Soares, 1995). Estudos realizados por Pinel e colaboradores (2011) utilizando a metodologia de espectrometria de massas SELDI-TOF-MS para analisar os perfis de proteínas de diferentes isolados *Aspergillus fumigatus* e *Aspergillus lentulus*, mostraram que a análise proteômica permite a distinção tanto de espécies estreitamente relacionadas quanto de mutantes naturais dentro das espécies. Além disso, poderia ser uma ferramenta analítica na investigação dos mecanismos moleculares envolvidos na fisiopatologia do *A. fumigatus*.

2- JUSTIFICATIVA

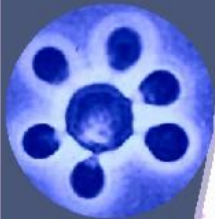
Através de vários estudos utilizando diferentes técnicas de biologia molecular, o fungo dimórfico *Paracoccidioides* spp encontra-se atualmente descrito como um complexo de, no mínimo, quatro espécies filogenéticas (*S1*, *PS2*, *PS3* e “*01-like*”). Apesar de o isolado *Pb01*, obtido de um paciente com PCM crônica na cidade de Goiânia, seja o mais bem estudado dentre os demais, sua classificação taxonômica ainda não está bem definida. Estudos recentes têm apresentado o *Pb01* como uma nova espécie denominada *P. lutzii*. Adicionalmente, pouco se conhece sobre as diferenças, estruturais e funcionais, dos demais isolados pertencentes ao complexo *Paracoccidioides* e a comparação entre diferentes isolados está restrita, principalmente, à estudos genômicos.

Nesse sentido, a utilização de técnicas de proteômica pode contribuir para elucidar e caracterizar diferencialmente os isolados de cada clado filogenético do fungo. Além disso, mapas proteômicos detalhados contendo o maior número possível de proteínas expressas e identificadas entre estes diferentes isolados, podem ser uma ferramenta para estudos relacionados com vias metabólicas preferenciais, virulência e sinalização celular, dentre outros.

3- OBJETIVOS

Considerando o exposto, observa-se a relevância do conhecimento da importância das proteínas na estrutura e na função da célula de patógenos humanos, como o fungo *Paracoccidioides*. O presente trabalho tem como objetivo estabelecer uma comparação entre os subproteomas das proteínas solúveis do citoplasma e identificar amplamente as proteínas expressas, diferenciais ou não, nos diferentes isolados correspondente a cada espécie filogenética (S1, PS2, PS3 e “01-like”) do complexo *Paracoccidioides*.

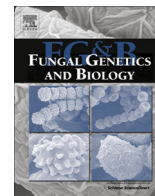
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journal homepage: www.elsevier.com/locate/yfgbiComparative proteomics in the genus *Paracoccidioides*

Laurine Lacerda Pigosso^a, Ana Flávia Alves Parente^a, Alexandre Siqueira Guedes Coelho^b,
Luciano Paulino Silva^c, Clayton Luiz Borges^a, Alexandre Melo Bailão^a, Célia Maria de Almeida Soares^{a,*}

^a Laboratório de Biologia Molecular, Instituto de Ciências Biológicas, Universidade Federal de Goiás, Goiânia, GO, Brazil

^b Laboratório de Genética e Genômica de Plantas, Escola de Agronomia, Universidade Federal de Goiás, Goiânia, GO, Brazil

^c Laboratório de Espectrometria Massas, Centro Nacional de Pesquisa de Recursos Genéticos e Biotecnologia, Empresa Brasileira de Pesquisa Agropecuária, Brasília, DF, Brazil

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ABSTRACT

The genus *Paracoccidioides* comprises a complex of phylogenetic species of dimorphic pathogenic fungi, the etiologic agents of paracoccidioidomycosis (PCM), a disease confined to Latin America and of marked relevance in its endemic areas due to its high frequency and severity. The members of the *Paracoccidioides* genus are distributed in distinct phylogenetic species (*S1*, *PS2*, *PS3* and *O1-like*) that potentially differ in their biochemical and molecular characteristics. In this work, we performed the proteomic characterization of different members of the genus *Paracoccidioides*. We compared the proteomic profiles of *Pb01* (*O1-like*), *Pb2* (*PS2*), *Pb339* (*S1*) and *PbEPM83* (*PS3*) using 2D electrophoresis and mass spectrometry. The proteins/isoforms were selected based on the staining intensity of the spots as determined by image analysis. The proteins/isoforms were in-gel digested and identified by peptide mass fingerprinting and ion fragmentation. A total of 714 spots were detected, of which 343 were analyzed. From these spots, 301 represented differentially expressed proteins/isoforms among the four analyzed isolates, as determined by ANOVA. After applying the FDR correction, a total of 267 spots were determined to be differentially expressed. From the total, 193 proteins/isoforms were identified by PMF and confirmed by ion fragmentation. Comparing the expression profiles of the isolates, the proteins/isoforms that were related to glycolysis/gluconeogenesis and to alcohol fermentation were more abundant in *Pb01* than in other representatives of the genus *Paracoccidioides*, indicating a higher use of anaerobic pathways for energy production. Those enzymes related to the oxidative stress response were more abundant in *Pb01*, *Pb2* and *Pb339*, indicating a better response to ROS in these members of the *Paracoccidioides* complex. The enzymes of the pentose phosphate pathway were abundant in *Pb2*. Antigenic proteins, such as GP43 and a 27-kDa antigenic protein, were less abundant in *Pb01* and *Pb2*. The proteomic profile indicates metabolic differences among the analyzed members of the *Paracoccidioides* genus.

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1. Introduction

The genus *Paracoccidioides* comprises thermally dimorphic pathogens from the family Ajellomycetaceae, order *Onygenales* (Untereiner et al., 2004). Those human pathogens are the etiologic agents of paracoccidioidomycosis (PCM), one of the most frequent systemic mycoses that affect rural populations in Latin America (Restrepo and Tobon, 2005). The fungus is found in nature as mycelium and in the human host in the form of yeast. The mycelia-to-yeast morphological transition is triggered by temperature, and this event is essential to the establishment of the infection (San-Blas et al., 2002). The infection begins with the inhalation of fungal propagules, which reach the epithelium of the alveoli,

where they differentiate into the pathogenic yeast form. Although most clinical forms of the disease are asymptomatic, severe and progressive infections occur, involving pulmonary and extrapulmonary tissues, such as the skin, lymph nodes, oropharyngeal mucosa, adrenal gland and central nervous system (Brunner et al., 1993; Franco, 1987; Restrepo et al., 2001). A Multi-Locus Sequencing Typing (MLST) analysis revealed the existence of four cryptic species in the *Paracoccidioides* genus: *S1*, *PS2* and *PS3* from the *Paracoccidioides brasiliensis* complex and *P. lutzii* harboring *Pb01-like* isolates (Teixeira et al., 2009; Matute et al., 2006).

The availability of sequenced and annotated genomes of members of the *Paracoccidioides* genus (isolates *Pb01*, *Pb18* and *Pb3*) in the Dimorphic Fungal Database of the Broad Institute site (http://www.broadinstitute.org/annotation/genome/dimorph_collab/MultiHome.html) provided a good start to compare the members of this genus and to better understand the biology of these medically significant fungi. Due to subtle or inexistent morphological differences among cryptic species, molecular techniques have

* Corresponding author. Address: Laboratório de Biologia Molecular, Instituto de Ciências Biológicas, ICBII, Campus II, Universidade Federal de Goiás, 74001-970, Goiânia, Goiás, Brazil. Fax: +55 62 35211110.

E-mail address: cmasoares@gmail.com (C.M.A. Soares).

become the tools of choice in distinguishing between such members (Teixeira et al., 2009; Theodoro et al., 2008). Although no differences in disease manifestation have been described, the phylogenetic species differ in genome size (29.1–32.9 Mb) and in the number of genes (7610–8130) (Desjardins et al., 2011).

With plenty of genomic information for *Paracoccidioides*, further functional research can provide more information at protein level. So far, few studies have been published regarding the proteomics of *Paracoccidioides*. Attempts have been made by our group in characterizing the proteome of *Paracoccidioides* (Pb01) which have helped in elucidating the proteins that are associated with the fungus phase transition (Rezende et al., 2011), the fungal response to iron and zinc starvation (Parente et al., 2011, 2013) and the secretomes of yeast and mycelia (Weber et al., 2012), as well as the fungal response to oxidative stress (Grossklau et al., 2013). Those studies revealed the numerous and complex composition of proteins and identified molecules that are potentially relevant to the transition of the fungus to the yeast pathogenic phase and to the fungus adaptation to the host milieu, as well as to the *Paracoccidioides* interaction with the host. Proteomic studies have also focused on the proteomic composition of extracellular vesicles and the exoproteome of Pb18; these studies provided an overall view of the extracellular vesicle protein of this fungus (Vallejo et al., 2011).

There is still a lack of quantitative comparisons between the proteome maps of members of the *Paracoccidioides* complex. In this work, we used two-dimensional protein fractionation coupled with mass spectrometry to visualize the potential biological differences among members of the *Paracoccidioides* complex. Proteomic profiles were obtained for members of the following phylogenetic species: *P. lutzii* (Pb01) and *P. brasiliensis*: Pb2 (PS2), Pb339 (S1) and PbEpm83 (PS3). A total of 714 protein spots were detected and 343 were analyzed. From these spots, 267 proteins/isoforms were differentially expressed greater than 1.5-fold. From the differentially expressed proteins spots, 193 were identified. A comparative analysis of the proteome revealed potential differential metabolic aspects among the members of the four phylogenetic lineages in the genus *Paracoccidioides*. To our knowledge, this is the first study using proteomics to investigate the potential biochemical differences among members of the *Paracoccidioides* genus.

2. Materials and methods

2.1. Strains and culture conditions

Paracoccidioides Pb01 (ATCC-MYA-826) (Parente et al., 2011; Weber et al., 2012), Pb2 (Matute et al., 2006), Pb339 (Matute et al., 2006) and PbEpm83 (Theodoro et al., 2008) were used in this study. The yeast phase was maintained *in vitro* by subculturing at 36 °C in Fava Netto's semisolid medium [1% (w/v) peptone, 0.5% (w/v) yeast extract, 0.3% (w/v) proteose peptone, 0.5% (w/v) beef extract, 0.5% (w/v) NaCl, 4% (w/v) glucose, 1.2% (w/v) agar, pH 7.2] every 7 days.

2.2. Preparation of protein extracts

To obtain protein extracts, yeast cultures were prepared by inoculating 250 mL Fava Netto's liquid medium with 10^6 cells/mL incubated under agitation at 36 °C for 72 h. The cells were collected by centrifugation at 10,000g for 5 min, followed by the addition of extraction buffer (20 mM Tris–HCl pH 8.8; 2 mM CaCl₂) containing a mixture of nuclease and protease inhibitors (serine, cysteine and calpain) (GE Healthcare, Uppsala, Sweden). This suspension was distributed in tubes, glass beads were added, and the suspension was processed on ice in the bead beater (BioSpec,

Oklahoma, USA) apparatus for 5 cycles of 30 s, followed by centrifugation at 10,000g for 15 min at the same temperature. The supernatant was collected, the protein concentrations were determined using the Bradford reagent (Sigma Aldrich, Co., St. Louis, MO), and bovine serum albumin (BSA) was used as a standard.

2.3. Two-dimensional gel electrophoresis

Equal amounts of proteins (300 µg of protein extract *per gel*) were loaded. The interfering molecules were removed by cleaning with the 2-D Clean-up Kit (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. The proteins were solubilized in 250 µL of buffer containing 7 M urea, 2 M thiourea, 130 mM 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.002% (w/v) dithiothreitol (DTT), ampholyte-containing buffer (IPG buffer, GE Healthcare) and traces of bromophenol blue (Shaw and Riederer, 2003). The samples were applied to 13-cm Immobiline™ DryStrip Gels (GE Healthcare) with a linear separation range of pH 3–11. Isoelectric focusing was successively conducted in a Ettan IPGphor 3 Electrophoresis system (GE Healthcare) at 30 V for 14 h, 500 V for 500 Vh (step), 1 kV for 800 Vh (gradient), 8 kV for 11.3 kVh (gradient) and 8 kV for 2.9 kVh (step) at a maximum setting of 50 µA per strip. As previously described (Herbert et al., 2001), after isoelectric focusing, the strips were kept in equilibration buffer [50 mM Tris–HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS), and 0.002% (w/v) bromophenol blue] containing 18 mM DTT for 40 min. This step was followed by the incubation in equilibration buffer with 135 mM iodoacetamide for 40 min prior to polyacrylamide gel electrophoresis under denaturing conditions (SDS–PAGE) (Laemmli, 1970). The IPG strips were placed over the polyacrylamide gels and covered with agarose [0.5% (w/v) in running buffer containing 25 mM Tris–HCl, 192 mM glycine, 0.1% (w/v) SDS]. The SDS–PAGE of each experimental biological triplicate was simultaneously performed at 10 °C for 1 h at 150 V and 250 V until the front reached the gel end. The proteins were visualized by Coomassie brilliant blue staining (PlusOne Coomassie Tablets PhastGel Blue R-350, GE Healthcare) according to manufacturer's instructions.

2.4. Image analyses

The images of the Coomassie blue 2-D gels were scanned using Image Scanner III (GE Healthcare) using LabScan software (GE Healthcare) in transparent mode. The image analysis was performed with Image Master 2D Platinum v 6.0 (GE Healthcare). The gels were aligned, and the intensity of the spots was normalized and matched between different gels, procedures that were performed automatically using the Image Master software. The automated matching of the gels was checked manually and corrected if necessary. The spots were quantified and normalized using the volume criterion. For each spot, the volume percentage was calculated. The intensities of the Coomassie staining were normalized considering the standardized relative intensity volume of spots; this value is the volume of each spot divided by the total volume of all of the spots. The volume ratio values of the matched spots were used for statistical analyses.

2.5. Statistical analyses

The differences in protein expression levels among members of the phylogenetic groups were tested using the two-way ANOVA test. The FDR (False Discovery Rate) criterion was used for multiple comparisons for the correction of type I errors (less than 0.05). When declared statistically significant by ANOVA, the differences among the means were evaluated using Tukey's test. The statistical

analyses were performed using R software (<http://www.r-project.org/>). A *p*-value equal to or less than 0.05 was considered statistically significant.

The MultiExperiment Viewer software V.4.8 (www.tm4.org/mev/) was used to group the comparative proteomic data. To analyze the isolates, the up- and down-regulated proteins were combined and the expression levels that were estimated for each protein were used to build a heat map. In this way, the differences in the proteomic profiles were examined by hierarchical clustering using the Pearson correlation as a measure of similarity.

To analyze the data from enzymatic activities, statistical comparisons were performed using a two-way ANOVA, and, when declared statistically significant, the differences among the means were evaluated using Tukey's test.

To analyze the data regarding the oxidative stress and heat shock response, statistical comparisons were performed using Student's *t*-test. Samples with *p*-values <0.05 were considered statistically significant.

2.6. In-gel enzymatic digestion

The Coomassie blue-stained spots were manually excised from the gels and diced into small pieces. The gel pieces were washed twice with MilliQ H₂O and then dehydrated in 100 μ L acetonitrile (ACN) and dried in a speed vacuum. The gel pieces were then reduced with 10 mM DTT and alkylated with 55 mM iodoacetamide. The supernatant was removed, and the gels were washed with 100 μ L ammonium bicarbonate by vortexing for 10 min. The supernatant was removed, and the gel pieces were dehydrated in 100 μ L of a solution containing 25 mM ammonium bicarbonate/50% ACN (v/v), vortexed for 5 min, and centrifuged. This step was repeated. The gel pieces were dried and a 12.5-ng/mL trypsin solution was added (Sequencing Grade Modified Trypsin Promega, Madison, WI, USA), followed by rehydration on ice at 4 °C for 10 min. The supernatant was removed, and to the gel pieces, 25 μ L of a 25-mM solution of ammonium bicarbonate was added. This step was followed by incubation at 37 °C for 16 h. Following digestion, the supernatant was placed into a clean tube. To the gel pieces, 50 μ L of a solution containing 50% (v/v) ACN, 5% (v/v) trifluoroacetic acid (TFA) were added. The samples were mixed for 10 min and sonicated for three cycles of 3 min, and the solution was then combined with the above aqueous extraction. The samples were dried in a speed vacuum, and the peptides were solubilized in water. Two microliters of each sample were delivered to a target plate and dried at room temperature. Next, the peptide mixtures were covered with 2 μ L of 10 mg/mL alpha cyano-4-hydroxycinnamic acid in 50% (v/v) ACN, 5% (v/v) TFA. A concentration and a purification step using a pipette tip with a bed of chromatographic media (ZipTips® C18 Pipette Tips, Millipore, Bedford, MA, USA) was used to favor the identification of less abundant proteins.

2.7. Mass spectrometry analysis

The peptides that were obtained by the in-gel digestion were analyzed by PMF and MS/MS in a matrix-assisted laser desorption/ionization (MALDI) quadrupole, time-of-flight (Q-TOF) mass spectrometer (MS) (Synapt, Waters, Manchester, UK). The mass spectra were performed using the Mass Lynx (Waters) software. The raw data were processed using the Protein Lynx Global Server software (version 2.1, Micromass/Waters). Both the resulting peptide mass and the associated fragmentation spectra were submitted to MASCOT software (www.matrixscience.com). The searches were performed against an NCBI non-redundant database with a 100-ppm mass tolerance. The searches were performed without a constraining protein molecular weight or isoelectric point, with variable carbamidomethylation of cysteine and oxidation of

methionine residues. The search parameters were restricted to fungi and allowed for one missed cleavage. The proteins were identified by comparing the collected data with the NCBI databank (<http://www.ncbi.nlm.nih.gov/>).

2.8. Formamidase activity assay

The formamidase activity was measured by monitoring ammonia formation, as previously described (Borges et al., 2010). One microgram of the total protein extract was added to 200 μ L of the formamide substrate solution at a 100-mM final concentration in 100 mM phosphate buffer, pH 7.4, and 10 mM EDTA. The reaction mixture was incubated at 37 °C for 30 min, followed by the addition of 400 μ L phenol-nitroprusside and 400 μ L alkaline hypochlorite (Sigma Aldrich, Co.). The samples were incubated for 6 min at 50 °C, and the absorbance was read at 625 nm. The amount of released ammonia was determined from a standard curve. One unit (U) of formamidase-specific activity was defined as the amount of enzyme required to hydrolyze 1 μ mol of formamide (corresponding to the formation of 1 μ mol of ammonia) per min per mg of total protein.

2.9. Measurement of the intracellular NADPH /NADP⁺

The intracellular NADP⁺ and NADPH concentrations were measured using the NADP/NADPH Assay Kit (Abcam, Cambridge, UK) according to the manufacturer's instructions. Briefly, the *Paracoccidioides* yeast cells were collected and washed with PBS. After centrifugation, the cells (10⁸) were resuspended in 400 μ L NADP/NADPH extraction buffer. Glass beads were added, and the suspension was processed on ice in the bead beater (BioSpec, Oklahoma, USA) for 3 cycles of 30 s. After centrifugation, the supernatants were diluted 5 $\times$ and used to perform the NADP⁺ and NADPH quantifications. The quantifications were performed in a 96-well microplate reader at 450 nm. The experiment was performed in three independent replicates.

2.10. Ethanol measurement

The concentrations of ethanol in the cultures were quantified using the enzymatic detection kit, a UV-test for ethanol, (R-Biopharm, Darmstadt, Germany), according to the manufacturer's instructions. 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, the amount of which is determined by means of its absorbance at 340 nm. The yeast cells of the members of the *Paracoccidioides* complex (10⁸ cells) were used in this assay. The cell suspension was distributed in tubes; glass beads were added, and the suspension was processed on ice in the bead beater (BioSpec, Oklahoma, USA) for 5 cycles of 30 s. The cell lysate was centrifuged at 10,000g for 15 min at 4 °C, and the supernatant was used for the enzymatic assay according to the manufacturer's instructions. The concentrations of ethanol were obtained in triplicate.

2.11. Response of *Paracoccidioides* to oxidative stress

The response to oxidative stress was investigated in yeast cells of *Paracoccidioides*. A total of 10⁷ yeast cells/mL were exposed to 1 mM menadione (Sigma Aldrich, Co.) at 36 °C for 2, 4 and 6 h with agitation at 150 rpm as previously described (Grossklau et al., 2013). The controls were obtained, and all of the experiments were performed in triplicate. The viability was determined by a membrane integrity analysis using propidium iodide as a marker for

dead cells. The cell suspension (10^6 yeast cells/mL) was centrifuged, and the supernatant was discarded. The cells were stained with the propidium iodide solution (1 mg/mL) for 20 min in the dark at room temperature. After dye incubation, the stained cell suspension was immediately analyzed in a C6 Accuri flow cytometer (Accuri Cytometers, Ann Arbor, MI, USA). A minimum of 5000 cells per sample was acquired with the FL3-H channel. The data were collected and analyzed using the FCS Express 4 Plus Research Edition software (De Novo Software, Los Angeles, CA, USA).

2.12. Response of *Paracoccidioides* to heat shock

A total of 10^6 yeast cells/mL of the *Paracoccidioides* isolates were subjected to different heat stress conditions (42 °C/1 h and 42 °C/2 h). The equivalent of 10^2 viable cells/mL was plated on BHI semisolid medium. The plates were incubated at 37 °C, and the number of colony forming units (CFUs) was determined after 7 days. The assay was performed in three independent replicates.

2.13. Growth of *Paracoccidioides* in different carbon sources

The isolates that were used in this study were grown at 36 °C in Fava Netto's liquid medium under gentle shaking for 72 h. The exponentially growing cultures of *Paracoccidioides* isolates were washed with sterile 0.9% (w/v) NaCl and resuspended in the same solution, and the number of cells was adjusted to 10^6 /mL. Different

volumes were spotted onto McVeigh/Morton medium (Restrepo and Jiménez, 1980) containing the carbon sources glucose or oleic acid, both at 20 mM. The plates were incubated for 7 to 15 days at 37 °C and photographed.

3. Results

3.1. Proteomic profiles of members of the *Paracoccidioides* complex

In this study, we applied a proteomic strategy to identify proteins that were preferentially expressed by the yeast phase in the following members of the *Paracoccidioides* complex: Pb01 (O1-like), Pb339 (S1), Pb2 (PS2), and PbEPM83 (PS3). The protein expression profiles of *Paracoccidioides* were obtained using 2D electrophoresis. Fig. 1 depicts the representative two-dimensional gel of the analyzed isolates that were performed in biological triplicates. The proteins spots with molecular weights ranging from 11.0 to 84 kDa and pI values between 3 and 11 were detected, as depicted. Nonetheless, these four selected species are a representative sample of the *Paracoccidioides* proteomic diversity. The qualitative and quantitative differences in the spots can be visualized, as depicted in Fig. 1.

A total of 714 spots were detected, and from those, 343 were analyzed, considering the four proteomes that were included for statistical analysis, as depicted in Fig. S1. A total of 343 spots were matched and assessed for statistical significance using an ANOVA

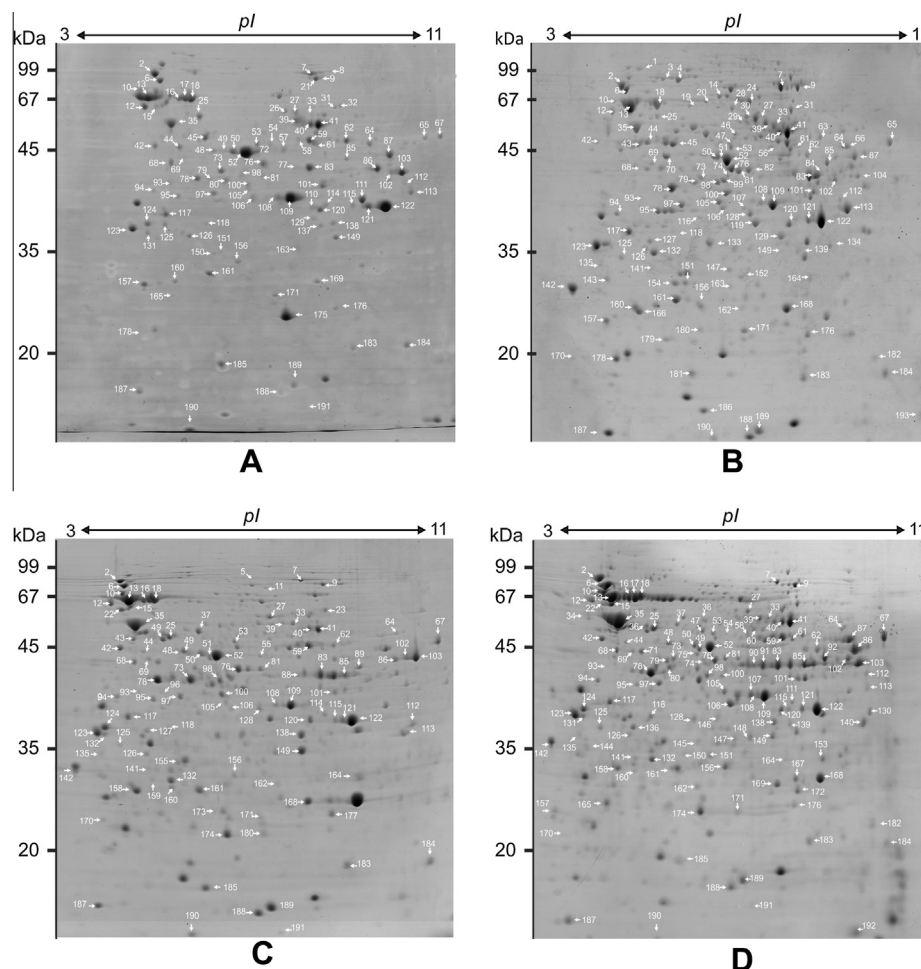


Fig. 1. Analysis of proteins from *Paracoccidioides* phylogenetic groups by two-dimensional gel electrophoresis: (A) Pb01; (B) Pb2; (C) Pb339; and (D) PbEPM83. The proteins were extracted by mechanical cell rupture. Equal amounts of proteins were submitted to two-dimensional electrophoresis using ImmobilineZ DryStrip Gels for isoelectric focusing, followed by SDS-PAGE. The proteins were visualized by using Coomassie brilliant blue staining.

Table 1

Proteins/isoforms with increased abundance in Pb01.

Spot number ^a	Accession number ^b	Protein description	MW (kDa) Theo/Exp ^c	pI Theo/Exp ^d	p-value ^e
<i>Energy</i>					
<i>Glycolysis and gluconeogenesis</i>					
108	gi 29826036	Fructose 1,6-biphosphate aldolase 1	39.76/36.00	6.1/7.1	9.13E–03
109	gi 29826036	Fructose 1,6-biphosphate aldolase 1	39.76/36.10	6.1/7.3	7.96E–07
52	gi 146762537	Enolase	47.41/44.50	5.6/6.5	3.78E–06
50	gi 146762537	Enolase	47.41/45.90	5.6/6.2	8.51E–03
48	gi 146762537	Enolase	47.41/45.50	5.6/5.8	8.54E–06
122	gi 295658119	Glyceraldehyde-3-phosphate dehydrogenase	36.62/34.10	8.2/9.1	2.89E–03
115	gi 295658119	Glyceraldehyde-3-phosphate dehydrogenase	36.62/34.00	8.2/8.6	3.10E–04
121	gi 295658119	Glyceraldehyde-3-phosphate dehydrogenase	36.62/34.20	8.2/8.9	1.25E–02
161	gi 295670663	Triosephosphate isomerase	27.15/23.00	5.3/5.7	1.16E–02
83	gi 295669690	Phosphoglycerate kinase	45.31/42.00	6.4/7.7	5.10E–04
25	gi 295659988	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	57.30/57.00	5.3/5.6	1.06E–04
26	gi 295662174	Pyruvate kinase	59.50/58.60	6.3/7.5	*
<i>Tricarboxylic-acid pathway</i>					
21	gi 225684009	Aconitase	85.06/78.60	7.2/7.9	*
<i>Pentose-phosphate pathway</i>					
110	gi 295666688	Transaldolase	35.75/34.74	6.4/7.7	*
<i>Fermentation</i>					
111	gi 295674635	Alcohol dehydrogenase	38.00/36.00	7.5/8.7	5.23E–03
114	gi 29566616	Alcohol dehydrogenase	44.83/35.00	8.9/8.0	3.16E–02
39	gi 295671230	Pyruvate decarboxylase	63.79/56.00	6.3/7.5	4.07E–02
<i>Glyoxylate cycle</i>					
57	gi 295664927	ATP-citrate-lyase	52.95/45.50	5.9/7.1	*
58	gi 295664927	ATP-citrate-lyase	52.95/47.00	5.9/7.0	1.25E–02
<i>Methylcitrate cycle</i>					
87	gi 295666179	2-methylcitrate synthase	51.51/44.00	9.0/9.3	4.21E–04
<i>Metabolism</i>					
<i>Amino acid metabolism</i>					
64	gi 295659992	Serine hydroxymethyltransferase	52.42/47.00	8.2/8.9	1.30E–04
8	gi 295659538	Methionine Cobalamin-independent synthase	87.30/83.40	6.2/8.2	*
106	gi 295673122	L-threonine 3-dehydrogenase	38.08/36.00	5.8/6.6	7.85E–03
101	gi 225678712	Ketol-acid reductoisomerase	44.86/38.00	9.1/8.0	1.01E–02
181	gi 226294753	1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase	20.90/19.10	5.5/6.1	1.12E–04
137	gi 295663891	2,5-diketo-D-gluconic acid reductase A	32.05/31.88	6.6/7.9	4.34E–09
151	gi 295665021	Chorismate mutase	38.05/28.00	8.4/6.0	1.08E–04
<i>Nitrogen metabolism</i>					
72	gi 295668479	Formamidase	46.15/45.40	6.1/7.2	*
<i>Nucleotide metabolism</i>					
32	gi 295672652	Bifunctional purine biosynthesis protein ADE17	67.21/60.94	6.7/8.5	*
96	gi 295674697	Adenosine kinase	36.61/37.00	5.4/5.8	3.85E–03
45	gi 295666938	Nucleoside diphosphate kinase	16.88/50.40	6.8/5.7	1.58E–04
190	gi 295658312	L-PSP endoribonuclease family protein (Hmf1)	18.72/14.00	8.9/6.5	1.52E–02
<i>Cell rescue, defense and virulence</i>					
<i>Stress response</i>					
18	gi 225681491	Hsp70-like protein	73.80/67.96	5.9/5.2	6.73E–04
13	gi 295659116	Hsp70-like protein	70.91/65.00	5.0/4.8	8.01E–04
79	gi 14538021	Heat shock protein 70	40.97/40.00	5.0/6.0	4.09E–02
2	gi 225679503	Heat shock protein SSB	67.26/82.43	5.3/4.8	4.76E–02
<i>Detoxification</i>					
40	gi 24528587	Peroxisomal Catalase	57.47/54.00	6.5/7.8	2.06E–03
176	gi 295669402	Mn superoxide dismutase	24.81/18.08	8.9/8.2	2.31E–04
175	gi 17980998	Y20 protein	21.64/17.10	6.0/7.2	*
62	gi 295664022	Glutathione reductase	51.95/47.00	6.7/8.5	5.57E–04
12	gi 295673162	Disulfide isomerase Pdi1	59.30/61.00	4.8/4.6	5.99E–03
<i>Cell cycle and DNA processing</i>					
123	gi 295661300	DNA damage checkpoint protein rad24	29.73/32.00	4.6/4.3	1.59E–03
162	gi 295666440	Sporulation-regulated protein	44.15/43.45	5.2/5.1	*
<i>Protein fate</i>					
156	gi 295674837	HAD-superfamily hydrolase	27.32/25.00	5.6/6.3	1.49E–02
120	gi 295661059	G-protein complex beta subunit CpcB	35.46/34.00	6.5/7.9	2.89E–02
184	gi 295672447	Peptidyl-prolyl cis–trans isomerase H	20.02/15.00	8.8/9.7	5.95E–03
183	gi 295672668	Peptidyl-prolyl cis–trans isomerase B	22.81/14.00	7.8/8.5	3.98E–03
<i>Protein synthesis</i>					
65	gi 295671178	Elongation factor 1- α	50.55/48.00	9.2/10.2	4.42E–03

(continued on next page)

Table 1 (continued)

Spot number ^a	Accession number ^b	Protein description	MW (kDa) Theo/Exp ^c	pI Theo/Exp ^d	p-value ^e
124	gi 295667868	Elongation factor 1-beta	25.93/32.00	4.7/4.6	6.90E–03
86	gi 295668925	Elongation factor 1-gamma 1	45.88/41.00	8.2/9.1	1.10E–02
<i>Miscellaneous</i>					
117	gi 225680961	Inorganic pyrophosphatase	51.79/34.00	8.1/4.9	5.69E–04
157	gi 295656848	TCTP family protein	20.28/21.00	4.7/4.5	6.13E–05
126	gi 226293104	Spermidine synthase	33.64/30.58	5.3/5.4	3.06E–02
89	gi 30351156	14–3–3-like protein	33.80/35.40	4.9/4.4	4.66E–02
53	gi 295665123	Aldehyde dehydrogenase	54.55/48.00	5.8/6.7	3.89E–05
<i>Unclassified proteins</i>					
80	gi 295674501	Conserved hypothetical protein	40.91/40.00	5.8/6.1	3.65E–04
125	gi 295660977	Conserved hypothetical protein	32.44/32.00	5.1/4.9	9.34E–03
185	gi 295658863	Conserved hypothetical protein	17.48/13.00	5.1/5.9	8.27E–04
187	gi 295672073	Hypothetical protein PAAG_01591	16.53/11.00	5.1/4.3	4.37E–04

^a Spots were numbered according to Fig. 1.^b GenBank general information identifier. The accession number refers to higher scores on Mascot search.^c Experimental/theoretical molecular weight.^d Experimental/theoretical isoelectric point.^e $p \leq 0.05$ was considered statistically significant.^{*} Spots visualized only in Pb01.

test to compare the differences in protein expression among the four analyzed proteomes. According to ANOVA and Tukey's test, a total of 301 spots presented differential expression. At this step, the FDR correction for protein identification was estimated (values below 0.05%) and indicated the statistical significance of 267 spots, as depicted in Fig. S1. Those spots with over a 1.5-fold difference in their relative abundance among the four samples numbered 267, and those with a p -value equal to or less than 0.05 in ANOVA/Tukey's test were considered differentially expressed. The Coomassie G-250 blue-stained maps were used for the mass spectrometry identification of the individual protein spots. A total of 193 proteins/isoforms that were differentially expressed when comparing Pb01, Pb339, Pb2 and PbEPM83 were unambiguously identified by PMF and confirmed by ion fragmentation. A summation of the analyzed spots is presented in Fig. S1.

Table S1 displays all of the proteins and isoforms, totaling 193, that were identified in the analysis. The list of identified proteins/isoforms and additional information, such as spot identification, GenBank general information identifiers (gi) and MS/MS mascot scores, are presented.

3.2. Abundantly detected proteins in Pb01, Pb2, Pb339 and PbEPM83

In this report, we describe the proteins that were differentially expressed in the cited members of the *Paracoccidioides* complex. The identified proteins were classified based on their main activity according to the Munich Information Center for Protein Sequences (MIPS) categorization, molecular weight and pI , theoretical and calculated, as well as the p -values associated with each spot comparison as depicted in Tables 1–4.

Some of the exclusively expressed proteins are related to basic processes found in virtually all organisms. To exclude the influence of differences in the protein predicted sequences in mass or pI , a sequence comparison was performed using the three genomes that were available for *Paracoccidioides*. The analysis revealed that the sequences are present as high sequence similarity among the investigated isolates and that the predicted pI s and masses are very similar among orthologous sequences.

When comparing the proteomes, from the 193 identified, 59 proteins/isoforms were preferentially expressed in Pb01, compared to Pb2, Pb339, and PbEPM83, as depicted in Table 1. Most of the enzymes of the glycolysis pathway, represented by twelve isoforms corresponding to seven different proteins, were more abundant

in Pb01 than in the other representatives of the genus *Paracoccidioides*, as depicted in Table 1. It is also noteworthy that the member of the phylogenetic species Pb01-like presented a high expression level of alcohol dehydrogenases, compared to the representatives of other phylogenetic species. In addition, pyruvate decarboxylase was over-expressed in this phylogenetic species. Considering nitrogen metabolism, an isoform of formamidase was highly induced in Pb01 compared to the representatives of other phylogenetic species. Additionally, some enzymes of amino acid metabolism, such as serine hydroxymethyltransferase, methionine cobalamin independent synthase, L-threonine-3-dehydrogenase, and chorismate mutase, were up-regulated in Pb01, strongly suggesting that the biosynthesis of some amino acids, such as valine, methionine and tyrosine, could be over-regulated in this isolate (Table 1). Proteins of the detoxification pathways that are represented by peroxisomal catalase, Mn superoxide dismutase, Y20, glutathione reductase and disulfide isomerase presented isoforms with high expression in Pb01 compared to Pb339, Pb2 and PbEPM83 (Table 1).

When comparing the proteome of Pb2 with those of Pb01, Pb339 and PbEPM83, 53 proteins/isoforms were identified that were preferentially expressed by Pb2, as depicted in Table 2. The enzymes of the oxidative and non-oxidative reactions of the pentose-phosphate pathway were preferentially expressed in Pb2 compared to the other analyzed members of the genus *Paracoccidioides* as follows: transaldolase, 6-phosphogluconate dehydrogenase, 6-phosphogluconolactonase and transketolase were increased in Pb2 (Table 2). Notably, also in Pb2, similar to that observed in Pb01, the metabolism of amino acids seems to be over-regulated. The enzymes that are related to the biosynthesis of the branched chain amino acids lysine and methionine were over-accumulated in Pb2. Of special note, the production of fumarate and acetoacetyl CoA seems to be induced, as interpreted from the over-accumulation of the enzymes 4-hydroxyphenylpyruvate dioxygenase and fumarylacetoacetase, both involved in the degradation of tyrosine and phenylalanine (Table 2). Cellular detoxification enzymes, such as peroxisomal catalase, Cu and Zn superoxide dismutase and 2-alkenal reductase, were also induced in Pb2 (Table 2).

When comparing the proteome of Pb339 with the other proteomes, 43 proteins/isoforms were preferentially expressed. β -oxidation enzymes, such as acyl CoA dehydrogenase, 3-hydroxybutyryl CoA dehydrogenase and enoyl-CoA hydratase were up-regulated

Table 2Proteins/isoforms with increased abundance in *Pb2*.

Spot number ^a	Accession number ^b	Protein description	MW (kDa) Theo/Exp ^c	pI Theo/Exp ^d	p-value ^e
<i>Energy</i>					
<i>Glycolysis and gluconeogenesis</i>					
30	gi 295658778	Phosphoenolpyruvate carboxykinase	63.89/58.00	6.1/7.2	1.08E–03
27	gi 295658778	Phosphoenolpyruvate carboxykinase	63.89/59.00	6.1/7.3	1.44E–02
<i>Tricarboxylic-acid pathway</i>					
129	gi 295673937	Malate dehydrogenase	36.02/33.00	8.9/7.8	5.10E–04
9	gi 226293399	Aconitase	82.47/79.00	7.0/8.1	4.69E–04
56	gi 226294995	Dihydrolipoyl dehydrogenase	55.40/47.00	8.0/7.6	3.24E–04
61	gi 295668473	Dihydrolipoyl dehydrogenase	56.06/47.00	8.2/7.8	1.37E–03
85	gi 295658897	Citrate synthase	52.20/43.00	8.7/8.4	4.84E–03
<i>Pentose-phosphate pathway</i>					
119	gi 225683456	Transaldolase	35.86/34.50	6.1/7.3	3.93E–06
51	gi 226292147	6-phosphogluconate dehydrogenase	54.60/44.50	5.7/6.6	9.55E–08
152	gi 225677822	6-phosphogluconolactonase	29.30/27.90	5.8/7.1	9.15E–06
24	gi 295665967	Transketolase	74.94/63.40	5.9/7.3	2.97E–06
<i>Fermentation</i>					
163	gi 295674635	Alcohol dehydrogenase	38.00/35.60	7.5/9.0	6.01E–04
<i>Glyoxylate cycle</i>					
33	gi 295660969	Isocitrate lyase	60.17/56.00	6.7/7.6	2.29E–03
<i>Methylcitrate cycle</i>					
31	gi 295666177	Mitochondrial 2-methylisocitrate lyase	67.25/60.00	8.7/8.3	8.60E–03
<i>Metabolism</i>					
<i>C-compound and carbohydrate metabolism</i>					
29	gi 225678915	1,4- α -glucan-branching enzyme	80.30/66.20	5.7/6.8	5.36E–04
100	gi 295662360	Mannitol-1-phosphate 5-dehydrogenase	43.11/39.00	5.6/6.5	2.00E–03
<i>Amino acid metabolism</i>					
70	gi 226289087	Argininosuccinate synthase	46.80/45.00	5.4/5.7	1.78E–03
44	gi 295660644	Argininosuccinate synthase	46.76/46.00	5.1/5.2	1.25E–03
7	gi 295659538	Methionine Cobalamin-independent synthase	87.30/83.00	6.2/7.9	2.16E–02
28	gi 295663883	Dihydroxy-acid dehydratase	65.40/61.00	6.0/6.9	2.20E–02
105	gi 295658704	4-hydroxyphenylpyruvate dioxygenase	45.67/38.00	5.5/6.5	8.85E–03
74	gi 225682808	Adenosylhomocysteinase	49.00/42.70	5.8/6.9	7.42E–04
76	gi 295669670	Adenosylhomocysteinase	49.02/43.00	5.8/6.8	5.52E–04
99	gi 225685311	Saccharopine dehydrogenase	42.10/41.00	5.8/6.7	2.23E–06
82	gi 226288132	Fumarylacetoacetase	46.70/42.30	6.1/7.2	1.67E–06
<i>Sulfur metabolism</i>					
43	gi 225679471	Sulfite oxidase	48.90/48.40	5.1/5.3	4.18E–06
<i>Nucleotide Metabolism</i>					
164	gi 225678337	Adenosine kinase	39.00/36.80	6.0/5.9	1.59E–03
63	gi 295664573	Adenylosuccinate lyase	54.90/49.00	7.1/8.6	8.99E–04
<i>Cell rescue, defense and virulence</i>					
<i>Detoxification</i>					
41	gi 225681400	Peroxisomal catalase	57.65/53.00	6.4/7.9	6.54E–04
188	gi 295666684	Cu, Zn superoxide dismutase	15.97/11.00	5.9/7.1	4.20E–03
113	gi 295664270	2-alkenal reductase	38.64/37.00	8.7/9.7	1.75E–02
<i>Cell cycle and dna processing</i>					
112	gi 225681947	UDP-galactopyranose mutase	59.60/50.10	6.5/7.6	1.92E–04
<i>Protein fate</i>					
139	gi 225682490	G-protein complex beta subunit CpcB	35.40/34.20	6.5/8.0	1.14E–04
154	gi 226290547	HAD-superfamily hydrolase	27.30/27.20	5.4/5.8	7.94E–03
78	gi 295668481	Peptidyl-prolyl cis-trans isomerase D	41.36/40.00	5.3/5.5	8.59E–04
135	gi 225679389	Ubiquitin-conjugating enzyme	28.50/30.40	4.9/4.7	2.26E–07
143	gi 225684267	Ubiquitin carboxyl-terminal hydrolase	27.20/27.80	4.8/4.4	5.96E–04
3	gi 225679863	Aminopeptidase	109.4/82.00	6.3/5.6	2.13E–03
4	gi 226295047	Puromycin-sensitive aminopeptidase	102.6/80.10	6.7/5.9	5.62E–07
14	gi 225685107	Beta-Ala-His dipeptidase	53.50/48.50	5.3/5.7	1.87E–02
<i>Protein synthesis</i>					
178	gi 295657799	40S ribosomal protein S12	16.87/16.00	5.0/4.6	1.02E–04
84	gi 225678523	Elongation factor 1-gamma 1	45.90/41.00	7.6/8.5	1.03E–04
131	gi 225679636	Translation elongation factor eEF-1 beta chain	26.40/31.90	4.6/4.4	3.07E–05
20	gi 225685037	Glycyl-tRNA synthetase	74.80/64.00	5.7/6.4	8.28E–06
46	gi 225685085	Seryl-tRNA synthetase	55.20/51.40	5.5/7.0	1.03E–02

(continued on next page)

Table 2 (continued)

Spot number ^a	Accession number ^b	Protein description	MW (kDa) Theo/Exp ^c	pI Theo/Exp ^d	p-value ^e
<i>Cellular transport, transport facilities and transport routes</i>					
169	gi 295666381	GTP-binding nuclear protein GSP1/Ran	24.08/22.00	6.9/7.8	5.91E–03
<i>Miscellaneous</i>					
81	gi 295669073	12-oxophytodienoate reductase	43.24/41.90	8.6/6.9	5.72E–04
97	gi 295659136	Coproporphyrinogen III oxidase	51.97/41.00	6.6/6.4	2.51E–03
186	gi 225678115	Peroxisomal matrix protein	18.00/16.80	5.6/6.3	2.14E–02
180	gi 225678502	Decarboxylase family protein	27.40/27.10	5.6/6.0	1.54E–02
<i>Unclassified proteins</i>					
133	gi 225681437	Conserved hypothetical protein	31.80/31.90	5.7/6.5	4.16E–04
193	gi 226294299	Conserved hypothetical protein	14.90/13.60	10.0/10.3	2.25E–04
94	gi 295668298	Conserved hypothetical protein	34.72/37.00	5.3/5.2	2.45E–03

* Spots visualized only in *Pb2*.

^a Spots were numbered according to Fig. 1.

^b GenBank general information identifier. The accession number refers to higher scores on Mascot search.

^c Experimental/theoretical molecular weight.

^d Experimental/theoretical isoelectric point.

^e $p \leq 0.05$ was considered statistically significant.

(Table 3). The proteins that were involved in mitochondrial electron transport, such as ATP synthase, were more abundantly expressed in this isolate. Some isoforms of enzymes that were related to cellular detoxification, such as Mn superoxide dismutase and mitochondrial peroxiredoxin PRX1, were up-regulated in *Pb339* compared to *Pb01*, *Pb2* and *PbEPM83*. The 27 kDa antigenic protein presented a higher level of expression in *Pb339* (Table 3).

When comparing the other proteomes to that of *PbEPM83*, a member of the PS3 clade, 38 proteins were preferentially expressed. The isoforms of proteins that were related to the heat shock response were more abundant in *PbEPM83*, as depicted in Table 4. The isoforms of enzymes of the glycolysis and TCA cycles were also abundantly expressed in this isolate. The antigenic protein GP43 also presented a higher level of expression in *PbEPM83*.

Pie charts representing the distribution of the identified differential spots of each isolate, according to their biological functions, are shown in Fig. S2.

3.3. Proteomic variation among members of the *Paracoccidioides* genus

The proteins/isoforms exhibiting significantly altered expression in the analyzed isolates entered into the Multi Experiment Viewer software V.4.8 (www.tm4.org/mev/) for hierarchical clustering as depicted in Fig. S3. The clustered data demonstrated a clear pattern of proteomic diversity and provided clear and detailed information regarding the cellular processes that were represented more in one *Paracoccidioides* isolate compared to the other three analyzed. As observed, several groups of proteins/isoforms representing different metabolic processes with differences in spots intensities were found. The general pattern evidenced by the hierarchical clustering indicated a differential protein profile among the analyzed members of the *Paracoccidioides* genus.

3.4. A comparative analysis of the most induced metabolic pathways in members of *Paracoccidioides* complex, as revealed by proteomic analysis

The abundance of the different protein categories revealed the plausible metabolic/physiological strategies used by each member of the *Paracoccidioides* genus. Six main strategies were established according to the proteomic data: (i) glycolysis and alcoholic fermentation; (ii) TCA cycle; (iii) pentose phosphate pathway; (iv) β -oxidation; (v) heat shock response; and (vi) reactive oxygen species (ROS) scavenging, as depicted in Fig. 2. Although the experi-

ments were performed in a complex medium, the differential proteomic profile among *Paracoccidioides* cryptic species has evidenced specific metabolic profiles for each species. *Pb01* seems to use glycolysis preferentially via the anaerobic pathway, producing ethanol, as suggested by the presence of seven induced glycolytic enzymes and the enzymes pyruvate decarboxylase and alcohol dehydrogenase. Notably, proteins related to the detoxification response were also up-regulated, possibly to counteract the reactive oxygen species in *Pb01*.

Pb2 presents a high number of enzymes that are related to the metabolism of amino acids such as lysine, tyrosine, cysteine and methionine. In particular, this isolate seems to obtain fumarate and acetoacetyl CoA by the degradation of the amino acids phenylalanine and tyrosine to supply the Krebs cycle, which is up-regulated in this isolate, indicating that the member of the cryptic species PS2 can preferentially degrade amino acids to obtain energy by the TCA aerobically. The enzymes of the pentose phosphate pathway were induced in *Pb2*, putatively producing NADPH for biosynthetic processes and/or to degrade the reactive oxygen species that are produced by aerobic metabolism, as indicated by the up-regulation of the detoxification enzyme isoforms peroxisomal catalase and Cu and Zn superoxide dismutase.

In contrast, the isolate *Pb339* seems to metabolically favor the degradation of lipids by beta-oxidation, as depicted by the increased levels of the enzymes enoyl-CoA hydratase, short chain-specific acyl-CoA dehydrogenase and 3-hydroxyisobutyryl-CoA hydrolase. In addition, the levels of ATP synthase subunits increased in *Pb339*, indicating a preferential aerobic degradation of fatty acids to yield energy.

Regarding *PbEPM83*, the comparative proteomic analysis indicates that this isolate presents a preferential aerobic degradation of glucose-related molecules, as depicted by the up-regulation of some glycolytic and TCA cycle-related enzymes. Interestingly, the induction of several heat shock proteins in *PbEPM83* could be attributed to a pronounced heat shock response in this member of the phylogenetic species PS3.

3.5. Biochemical confirmatory assays

To validate the significance of the proteomic results, biochemical assays were performed. The intracellular ethanol was higher in *Pb01* after 48 h of culture growth, as depicted in Fig. 3A, indicating increased alcoholic fermentation compared to the other isolates. Additionally, the activity of the enzyme formamidase increased in *Pb01* (Fig. 3B), confirming the proteomic data in which an

Table 3

Proteins/isoforms with increased abundance in Pb339.

Spot number ^a	Accession number ^b	Protein description	MW (kDa) Theo/Exp ^c	pI Theo/Exp ^d	p-value ^e
<i>Energy</i>					
<i>Glycolysis and gluconeogenesis</i>					
49	gi 146762537	Enolase	47.41/45.00	5.6/6.0	1.15E–04
47	gi 226290885	Glyceraldehyde-3-phosphate dehydrogenase	36.57/33.30	7.1/8.3	*
<i>Tricarboxylic-acid pathway</i>					
88	gi 295658595	Pyruvate dehydrogenase E1 component subunit alpha	45.31/40.20	8.62/7.7	*
<i>Oxidation of fatty acids</i>					
153	gi 226289575	Enoyl-CoA hydratase	31.57/25.11	8.8/8.7	1.92E–05
158	gi 225677560	Short-chain specific acyl-CoA dehydrogenase	47.81/39.14	8.2/5.6	2.48E–10
38	gi 295670601	3-hydroxyisobutyryl-CoA hydrolase	57.35/50.94	7.0/6.5	1.78E–05
<i>Electron transport and membrane-associated energy conservation</i>					
77	gi 295658821	ATP synthase subunit beta	55.18/40.20	5.2/4.6	*
42	gi 295658821	ATP synthase subunit beta	55.18/47.00	5.2/4.7	5.62E–03
34	gi 226287911	ATP synthase subunit beta	55.24/51.20	5.3/4.4	3.98E–05
<i>Metabolism</i>					
<i>C-compound and carbohydrate metabolism</i>					
149	gi 295666718	Sorbitol utilization protein SOU2	31.48/30.00	7.0/8.2	1.17E–04
<i>Amino acid metabolism</i>					
5	gi 226291641	3-isopropylmalate dehydratase	85.11/79.03	5.8/6.9	*
95	gi 225677671	3-isopropylmalate dehydrogenase	38.91/37.90	5.3/5.4	*
<i>Nitrogen metabolism</i>					
55	gi 225678657	Formamidase	46.08/43.60	6.0/7.0	*
174	gi 295658559	Nitroreductase family protein	25.26/22.60	6.2/7.8	1.48E–04
<i>Nucleotide metabolism</i>					
23	gi 226292595	Bifunctional purine biosynthesis protein ADE17	69.40/58.16	7.2/8.2	*
<i>Cell rescue, defense and virulence</i>					
<i>Stress response</i>					
16	gi 295659837	Heat shock protein SSB1	60.69/64.00	5.4/5.2	7.25E–06
170	gi 225680288	Wos2	23.62/26.61	4.3/3.8	1.04E–02
11	gi 225678945	Clock-controlled gene-9 protein	81.88/69.60	6.0/7.2	*
<i>Detoxification</i>					
172	gi 225682872	Mn superoxide dismutase	23.81/21.95	7.0/8.2	*
177	gi 225682872	Mn superoxide dismutase	23.81/22.31	7.0/8.4	*
166	gi 226291569	Mitochondrial peroxiredoxin PRX1	24.90/24.30	5.2/5.1	5.02E–05
160	gi 295668244	Mitochondrial peroxiredoxin PRX1	24.88/22.00	5.2/5.1	6.42E–04
<i>Cell cycle and DNA processing</i>					
1	gi 295664474	Cell division cycle protein	90.50/91.30	4.9/5.1	6.16E–09
73	gi 295668877	Actin	41.82/41.00	5.6/6.0	2.73E–04
68	gi 295672568	ATP-dependent RNA helicase eIF4A	45.00/42.70	5.1/5.1	1.03E–02
<i>Protein fate</i>					
132	gi 226292478	Proteasome component C1	32.80/31.40	5.1/5.2	7.98E–04
159	gi 225680770	Proteasome subunit beta type-1-A	29.20/27.40	6.1/5.1	*
136	gi 225678501	Proteasome subunit alpha type-1 Pb03	29.00/27.88	5.2/5.1	6.18E–06
189	gi 295662699	Peptidyl-prolyl cis–trans isomerase cypE	17.52/12.00	6.0/7.3	5.85E–04
37	gi 226294989	Peptidyl-prolyl cis–trans isomerase D	41.27/48.15	5.4/5.6	9.90E–07
171	gi 225684870	V Chain V	21.63/21.46	6.0/7.0	*
36	gi 225681422	Kynureninase	54.19/49.24	5.3/5.5	2.07E–07
75	gi 226292231	Prolyl peptidase	51.19/41.40	5.5/6.4	7.68E–05
<i>Protein synthesis</i>					
130	gi 226289773	40S ribosomal protein S3	29.80/40.80	8.9/8.5	1.20E–07
93	gi 295671352	40S ribosomal protein S0	32.07/37.00	4.8/4.7	1.50E–03
<i>Antigenic proteins</i>					
168	gi 1778408	27 kDa antigen, partial	24.71/23.13	8.8/8.6	2.42E–13
167	gi 1778408	27 kDa antigen, partial	24.71/23.13	8.8/8.2	6.43E–06
<i>Miscellaneous</i>					
102	gi 295669073	12-oxophytodienoate reductase	43.24/40.48	8.6/9.3	2.43E–03
103	gi 295669073	12-oxophytodienoate reductase	43.24/40.00	8.6/9.5	1.54E–02
148	gi 226294448	Nicotinate-nucleotide pyrophosphorylase	33.85/27.68	6.5/7.8	1.02E–10
54	gi 225680265	Aldehyde dehydrogenase	54.69/44.80	5.9/6.6	8.31E–06

(continued on next page)

Table 3 (continued)

Spot number ^a	Accession number ^b	Protein description	MW (kDa) Theo/Exp ^c	pI Theo/Exp ^d	p-value ^e
<i>Unclassified proteins</i>					
191	gi 225681129	Predicted protein	13.46/11.64	5.7/6.5	*
182	gi 225678257	Conserved hypothetical protein	19.20/19.70	8.6/9.7	2.35E–02

^a Spots were numbered according to Fig. 1.^b GenBank general information identifier. The accession number refers to higher scores on Mascot search.^c Experimental/theoretical molecular weight.^d Experimental/theoretical isoelectric point.^e $p \leq 0.05$ was considered statistically significant.* Spots visualized only in *Pb339*.**Table 4**Proteins/isoforms with increased abundance in *PbEPM83*.

Spot number ^a	Accession number ^b	Protein description Theo/Exp ^c	MW (kDa)	pI Theo/Exp ^d	p-value ^e
<i>ENERGY</i>					
<i>Glycolysis and gluconeogenesis</i>					
116	gi 225682647	Fructose-1,6-bisphosphatase	38.90/38.00	5.8/6.3	1.02E–04
107	gi 295671120	Fructose-bisphosphate aldolase	39.70/37.50	6.0/7.4	1.78E–05
98	gi 295669690	Phosphoglycerate kinase	45.31/42.10	6.4/7.4	1.32E–04
<i>Tricarboxylic-acid pathway</i>					
140	gi 295673937	Malate dehydrogenase	36.02/29.30	8.9/9.4	6.66E–03
118	gi 239609129	Pyruvate dehydrogenase E1 component subunit beta	41.28/30.20	7.5/5.5	*
138	gi 225683092	Pyruvate dehydrogenase E1 component subunit alpha	40.99/32.65	8.1/5.7	6.09E–04
144	gi 225684846	Fumarate hydratase	60.70/42.45	9.0/8.3	8.39E–06
69	gi 225683829	Citrate synthase	51.60/43.40	9.0/5.4	7.93E–04
92	gi 295658897	Citrate synthase	52.20/40.54	8.7/8.7	6.39E–05
<i>Methylcitrate cycle</i>					
59	gi 295666197	2-methylcitrate dehydratase	62.26/59.00	8.5/7.7	2.79E–07
<i>Metabolism</i>					
<i>C-compound and carbohydrate metabolism</i>					
147	gi 225678580	Polysaccharide deacetylase family protein	36.20/35.90	5.8/7.2	2.51E–3
<i>Amino acid metabolism</i>					
66	gi 225560729	Serine hydroxymethyltransferase	52.30/46.00	8.5/9.2	4.65E–03
146	gi 225683962	Anthranilate synthase component 1	58.09/52.30	5.6/6.1	3.99E–03
<i>Cell rescue, defense and virulence</i>					
<i>Stress response</i>					
6	gi 60656557	Heat shock protein 90	80.32/78.00	4.9/4.9	1.41E–04
17	gi 295671569	Heat shock protein SSC1	73.82/66.00	5.9/5.4	3.82E–04
10	gi 295673716	Hsp70-like protein	68.86/62.85	5.3/4.7	1.88E–04
71	gi 4164594	Heat shock protein 70	65.28/41.94	5.4/5.4	3.08E–10
22	gi 295663681	Heat shock protein 90	78.65/57.44	4.9/4.8	1.19E–02
15	gi 295663681	Heat shock protein 90	78.64/62.00	4.9/4.8	4.06E–04
35	gi 295658865	Heat shock protein 60	62.26/54.00	5.5/5.1	2.42E–04
<i>Cell cycle and DNA processing</i>					
60	gi 225681947	UDP-galactopyranose mutase	59.60/47.90	6.5/7.4	6.76E–06
<i>Protein fate</i>					
127	gi 225680783	Proteasome subunit alpha type-3	29.70/32.40	5.2/5.3	7.17E–04
173	gi 225679093	Proteasome subunit alpha type-4	28.10/24.70	5.7/6.3	9.30E–05
145	gi 225684609	Proteasome subunit alpha type-2	26.97/25.52	5.3/6.6	1.28E–03
150	gi 295672968	Phosphomannomutase	30.57/27.00	5.6/5.8	2.43E–05
141	gi 226289845	Gamma-glutamyltranspeptidase	64.08/40.2	5.9/7.1	1.58E–03
<i>Protein synthesis</i>					
19	gi 225682377	Phenylalanyl-tRNA synthetase beta chain	68.60/62.00	5.6/6.1	1.80E–04
<i>Cellular transport, transport facilities and transport routes</i>					
67	gi 295662102	Rab GDP-dissociation inhibitor	52.53/48.45	5.4/10.4	*
<i>Antigenic proteins</i>					
90	gi 8576322	43 kDa secreted glycoprotein precursor	46.28/39.19	6.8/8.1	6.34E–07
91	gi 8576322	43 kDa secreted glycoprotein precursor	46.28/39.35	6.8/7.3	1.33E–02

Table 4 (continued)

Spot number ^a	Accession number ^b	Protein description Theo/Exp ^c	MW (kDa)	pI Theo/Exp ^d	p-value ^e
Miscellaneous					
104	gi 225678587	12-oxophytodienoate reductase	43.00/40.5	8.5/9.4	2.18E–06
134	gi 225683930	Hydrolase	50.50/31.40	9.7/8.8	2.34E–03
128	gi 225685013	Pyridoxine biosynthesis protein PDX1	34.42/33.47	6.0/6.6	3.89E–04
179	gi 226287294	4-carboxymuconolactone decarboxylase family protein	20.90/21.50	5.3/5.6	2.75E–03
Unclassified proteins					
165	gi 295666522	Conserved hypothetical protein	21.15/20.00	5.1/5.0	2.11E–04
142	gi 226295016	Conserved hypothetical protein	34.78/34.50	4.5/3.5	4.78E–05
192	gi 226294299	Conserved hypothetical protein	14.92/12.60	10.0/9.3	1.98E–03
155	gi 225678961	Predicted protein	24.60/23.30	8.4/8.7	1.23E–07

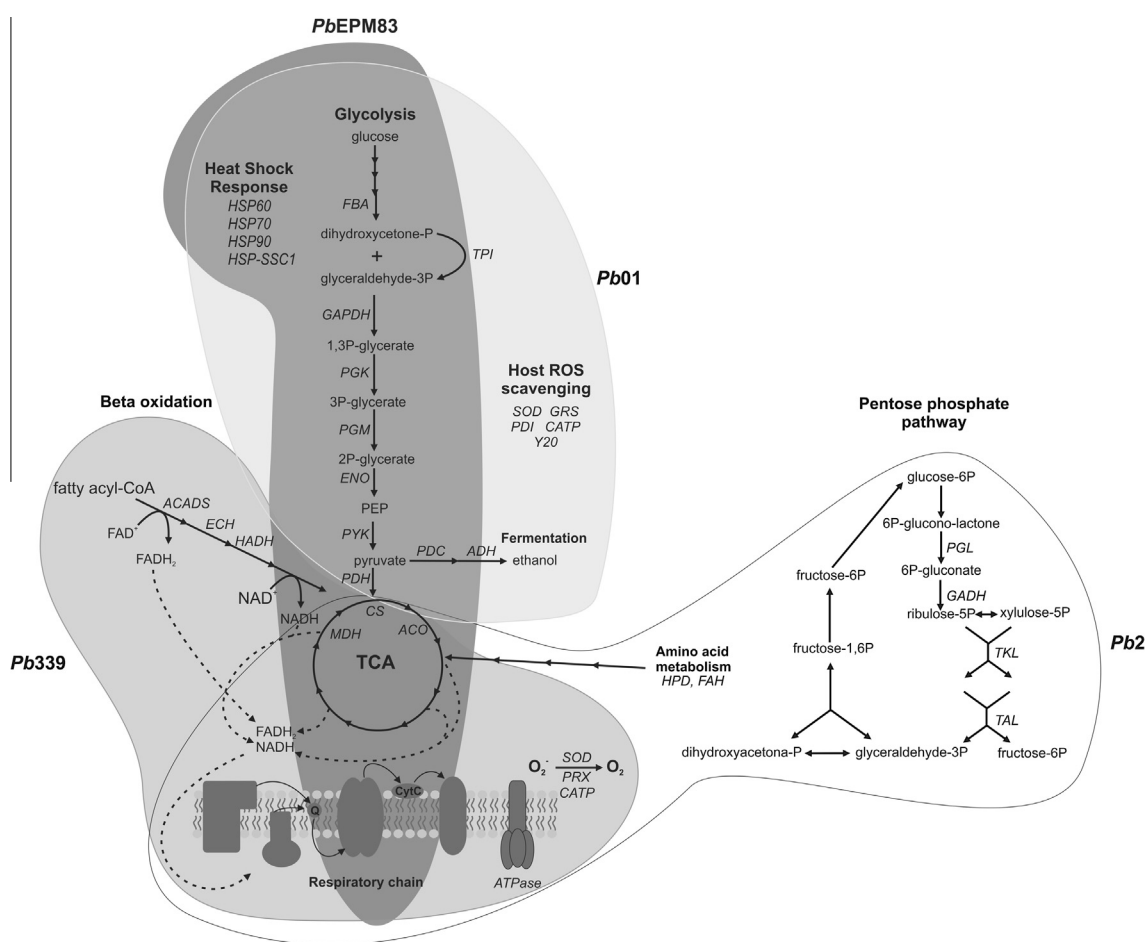
^a Spots were numbered according to Fig. 1.^b GenBank general information identifier. The accession number refers to higher scores on Mascot search.^c Experimental/theoretical molecular weight.^d Experimental/theoretical isoelectric point.^e $p \leq 0.05$ was considered statistically significant.* Spots visualized only in *PbEPM83*.

Fig. 2. Metabolic features of *Paracoccidioides* phylogenetic species identified by proteomic analysis. The pathways were constructed based on the preferential identification of proteins in each *Paracoccidioides* isolate. In the glycolytic pathway, the up-regulated proteins/isoforms were those that were related to the following enzymes: FBA (Fructose 1,6-bisphosphate aldolase 1), TPI (Triosephosphate isomerase), GAPDH (Glyceraldehyde-3-phosphate dehydrogenase), PGK (Phosphoglycerate kinase), PGM (2,3-bisphosphoglycerate-independent phosphoglycerate mutase), ENO (Enolase) and PYK (Pyruvate kinase). The enzymes PDC (Pyruvate decarboxylase) and ADH (Alcohol dehydrogenase) are induced enzymes related to ethanol production through fermentation. The beta-oxidation-induced proteins/isoforms were ACADS (Short chain-specific acyl-CoA dehydrogenase), ECH (Enoyl-CoA hydratase) and 3-hydroxybutyryl-CoA dehydrogenase (HADH). The over-expressed proteins/isoforms of the tricarboxylic acid pathway were PDH (Pyruvate dehydrogenase), CS (Citrate synthase), ACO (Aconitase) and MDH (Malate dehydrogenase). The amino acid metabolism enzymes were 4-hydroxyphenylpyruvate dioxygenase (HPD) and fumarylacetoacetate (FAH). The ATP synthase subunit beta (ATPase) was identified. The over-regulated proteins/isoforms of the pentose phosphate pathway were PGL (6-phosphogluconolactonase), GADH (6-phosphogluconate dehydrogenase), TKL (Transketolase) and TAL (Transaldolase). The detoxification enzymes were SOD (Mn superoxide dismutase), PRX (Mitochondrial peroxiredoxin), CATP (Peroxisomal catalase), GRS (Glutathione reductase), PDI (Disulfide isomerase) and Y20 (flavodoxin-like protein). The over-regulated heat shock response proteins in *PbEPM83* were HSP60 (Heat shock protein 60), HSP70 (Heat shock protein 70), three isoforms of HSP90 (Heat shock protein 90) and HSP-SSC1 (Heat shock protein SSC1).

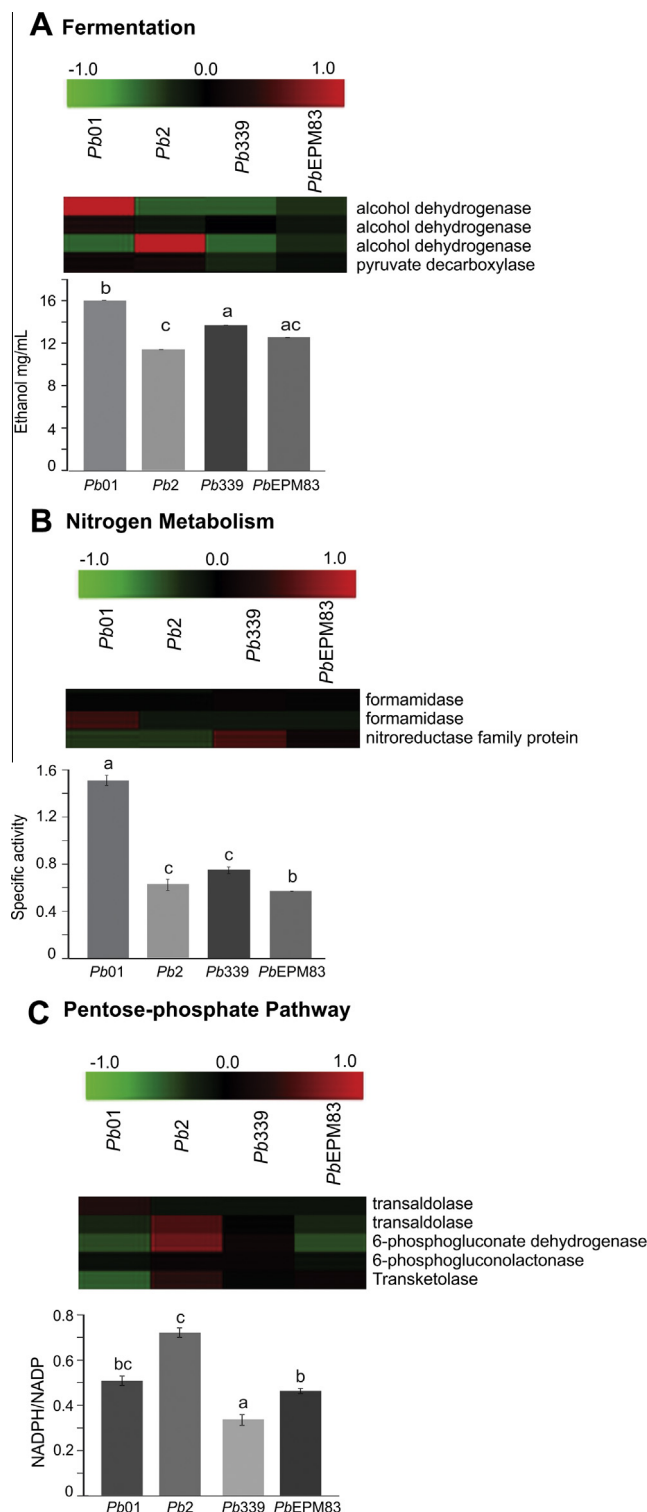


Fig. 3. Validation of proteomic data by enzymatic activity assays. (A) Determination of ethanol in yeast cells. The yeast cells were disrupted in the bead beater to determine the intracellular ethanol concentrations that were quantified using the UV-test Kit for ethanol, (R-Biopharm, Darmstadt, Germany). (B) The formamidase activity was measured by monitoring ammonia formation. The formamidase-specific activity was defined as the amount of enzyme required to hydrolyze 1 μ mol formamide per minute per mg total protein. (C) Measurement of the intracellular NADPH/NADP⁺ ratio in yeast cells. Using the NADP/NADPH Assay Kit (Abcam, Cambridge, UK) according to the manufacturer's instructions, we measured the intracellular NADP⁺ and NADPH concentrations. The error bars indicate the standard deviations from three independent experiments that were performed in triplicate. The data were analyzed by a two-way ANOVA and Tukey's multiple comparison. The letters indicate the significant differences in the expression between samples.

isoform of this enzyme presented a 5.17-fold increase compared to the other isolates. In agreement with the increased expression of the enzymes of the pentose phosphate pathway in *Pb2*, the NADPH/NADP⁺ ratio increased, as depicted in Fig. 3C.

In our proteomic analyses, the enzymes that were related to the inactivation of ROS were more highly expressed in *Pb01*, *Pb2* and *Pb339* than in *PbEPM83*. In tests using menadione as an oxidative stressor, *PbEPM83* depicted a higher susceptibility than the other isolates, corroborating the proteomic data (Fig. 4A).

The proteomic data also indicated that the isolate *Pb2* depicted a low number of over-expressed heat shock proteins compared to the other isolates. Regarding the resistance to the heat shock, the different isolates of *Paracoccidioides* respond to a shift from 36 °C to 42 °C with a decrease in CFU counts. The results obtained for the heat shock analysis are presented in Fig. 4B, depicting a higher sensitivity of the isolate *Pb2* to the temperature increase.

Additionally, the comparative proteomic data suggest that the representatives of the phylogenetic species of *Paracoccidioides* could establish different metabolic pathways by using different sources of carbon. In this sense, *Pb339* seems to metabolically favor the degradation of lipids by beta-oxidation. Growth assays performed in the presence of oleic acid demonstrated that the isolate *Pb339* can use oleic acid as an alternative carbon source (Fig. 4C).

4. Discussion

Metabolic and adaptive strategies comparing members of the *Paracoccidioides* genus have been discussed at the molecular level with genomic analyses (Desjardins et al., 2011). Nevertheless, these strategies have not been discussed in the light of experimental proteomic data until now. In the present study, we show how the proteins that are accumulated by an organism can indicate the physiological state of the cells. The comparative proteome profiles of *Pb01*, *Pb2*, *Pb339* and *PbEPM83* are described here for the first time. Using a proteomic analysis, from a total of 714 detected spots, 343 were analyzed, and, from those, 193 were differentially expressed proteins/isoforms. The proteomic data demonstrated significant differences in the protein expression profiles among the representatives of different phylogenetic clades of the genus *Paracoccidioides*. The hierarchical clustering of the proteome profiles of the four isolates of *Paracoccidioides* reflect the phylogenetic relationships between these *Paracoccidioides* species, where S1 is considered an independent species, and PS3 is phylogenetically closer to S1 than to PS2. *Pb01* is highly divergent from all other phylogenetic species (Matute et al., 2006; Teixeira et al., 2009).

Proteomes can give hints as to the physiological state of the cells. In our proteomic analysis, the expression levels of the enzymes that are related to glycolysis suggest the use of a more anaerobic metabolism for energy production from glucose in the *Pb01* isolate. The high level of enzymes involved in glycolysis and alcoholic fermentation led to the assumption that this isolate could have a more anaerobic metabolism than do the others. The levels of ethanol were determined in the yeast cells of the analyzed isolates. As observed, the level of ethanol was higher in *Pb01*, corroborating a more anaerobic metabolism. Transcriptional studies have suggested a more anaerobic metabolism at the yeast phase of isolate *Pb01*, that could produce more ethanol compared to mycelia (Felipe et al., 2005). Consistent with these results, the proteomic analysis detected the accumulation of enzymes of the glycolysis pathway and of alcoholic fermentation at the transition from mycelium to yeast cells in *Pb01* (Rezende et al., 2011). Studies had indicated the relevance of ethanol fermentation for pathogenic fungi. Proteomic studies suggest that ethanol fermentation contributes to the generation of energy in the conidia at dormancy or at the beginning of the germination process in *Aspergillus*

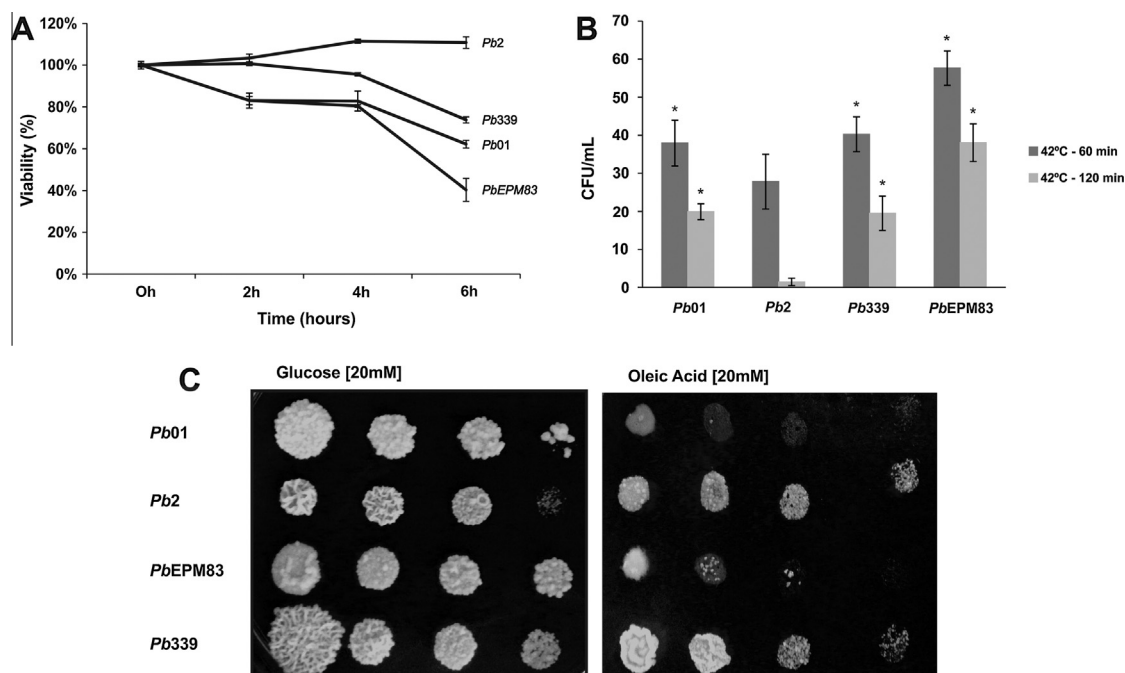


Fig. 4. Cell viability in the presence of oxidative stress, effect of heat shock on the viability of *Paracoccidioides* yeast cells and use of different carbon sources by isolates of *Paracoccidioides*. (A) The oxidative stress response was investigated in yeast cells of *Paracoccidioides* that were exposed to 1 mM menadione (Sigma Aldrich, Co) for 2, 4 and 6 h. The viability was determined by a membrane integrity analysis using propidium iodide (1 mg/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 compared PbEPM83 with the other isolates in this study and were performed by 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. (B) Colony-forming units recovered from the plating of 10^2 viable cells in BHI semisolid medium after incubation under different conditions (36 °C, 42 °C/60 min and 42 °C/120 min), as determined after 7 days. The assay was performed in three independent replicates. Student's *t*-test was used for statistical comparisons of Pb2 with Pb01, PbEPM83 and Pb339, and the observed differences were statistically significant ($p < 0.05^*$). The errors bars represent the standard deviation of three biological replicates. (C) The Pb01 (01-like), Pb2 (PS2), Pb339(S1) and PbEPM83 (PS3) isolates were grown at 37 °C in Fava Netto's liquid medium under gentle shaking for 72 h. The cultures were serially diluted, and each dilution was spotted onto minimal medium containing different carbon sources (glucose and oleic acid) at 20 mM. The plates were incubated for 7 to 15 days at 37 °C.

fumigatus (Teutschbein et al., 2010). In addition, under hypoxic conditions, this fungus prioritizes the activation of ethanol fermentation (Barker et al., 2012).

The most induced metabolic strategies in Pb2 seem to be the pentose phosphate pathway, as revealed by comparative proteomics and confirmed by biochemical analysis. The induction of the non-oxidative branch of the pentose phosphate pathway can provide substrates for glycolysis. Additionally, the high expression of the enzymes of the oxidative branch can provide NADPH for use in biosynthetic processes (Rui et al., 2010). The degradation of amino acids that provide substrates for the TCA cycle is also a differential aspect of the metabolism of Pb2, as revealed by proteomic analysis. A striking feature of Pb339 is beta-oxidation. Growth assays in the presence of oleic acid corroborated the proteomic data. The oxidation of fatty acids is important for the utilization of storage lipids or exogenous fatty acids to generate acetyl coenzyme A (acetyl-CoA) for central carbon metabolism. The transcriptional profiling of *Cryptococcus neoformans* revealed that genes for peroxisomal-oxidation, fatty acid import, and lipid degradation were up-regulated upon pathogen internalization and during pulmonary infection, suggesting that fatty acid metabolism may be important for the virulence of *C. neoformans* in mammals (Kretschmer et al., 2012). If this metabolic aspect can influence the virulence of Pb339, it should be a focus of future investigations. Compared to the other isolates, PbEPM83 seems to preferentially utilize aerobic routes for energy production through carbohydrates, as suggested by the over-accumulation of glycolytic and TCA enzymes.

Protein metabolism appears to be central to growth in the Onygenales. Metabolic assays of the non-pathogenic Onygenales *Uncinocarpus reesii* demonstrated extensive growth on a wide range of proteinaceous substrates compared to carbohydrates

(Desjardins et al., 2011). The metabolism of amino acids seems to be particularly relevant in Pb01 and Pb2 isolates compared to Pb339 and PbEPM83. Whether this metabolic feature provides more attributes to an animal pathogen remains to be elucidated.

Enzymes that are related to the oxidative stress response were more abundant in Pb01, Pb2 and Pb339 compared to PbEPM83, indicating a better response to reactive oxygen species (ROS) in these members of the *Paracoccidioides* complex. The human pathogenic fungi rely on mechanisms for protection against oxidative damage to defend against ROS that are released by host cells in the course of an infection. These species can damage cellular constituents, including DNA, proteins, and lipids, leading to cellular death. The capacity to neutralize ROS represents a stress response in *Paracoccidioides*, as previously described (Chagas et al., 2008). Proteomic studies reported the up-regulation of ROS scavenging enzymes, such as peroxisomal catalase and Cu/Zn and Mn superoxide dismutases, during the treatment of *Paracoccidioides* with hydrogen peroxide, indicating the involvement of those enzymes in protecting the fungus from the general stress generated by the host defense mechanisms (Grossklau et al., 2013). ROS detoxification has an apparent significance for virulence, as phagocytes of the innate immune system produce several oxidants that are presumed to play roles in microbial killing. In this sense, to restrict *Paracoccidioides* infection, macrophages produce several harmful substances, such as hydrogen peroxide, that cause oxidative stress (Pina et al., 2008; Rodrigues et al., 2007). The lower resistance to ROS in PbEPM83, as demonstrated in the proteomic and biochemical assays, warrants further investigation.

A plethora of data have been generated from specific analyses when exposing *Paracoccidioides* to particular stressors (Silva et al., 1994, 1999; Izacc et al., 2001; Nicola et al., 2008). One

obvious stress condition every human pathogen has to endure is the elevated body temperature of the host. The isolate *PbEPM83* showed an over-expression of proteins characterized as HSPs. In fungi, HSPs are modulated in response to various stimuli, including temperature (Burnie et al., 2006). The abundance of HSPs in this isolate can provide this fungus the ability to survive certain environmental stresses, as demonstrated in this work.

In conclusion, we provide evidence for differential metabolic features in members of the phylogenetic complex. The data indicate a significant difference in the global metabolism in members of *Paracoccidioides*. The additional characterization of those differences may lead to the use of biochemical criteria as markers to distinguish between the four cryptic species of the genus *Paracoccidioides*. Additionally, the establishment of 2-DE reference maps for the four isolates will permit correlating the quantitative and qualitative variations in the proteomes with phenotypic characteristics such as pathogenicity, virulence and tolerance to environmental stresses.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.fgb.2013.07.008>.

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Table S1. Proteins/isoforms identified in *Paracoccidioides Pb01, Pb2, Pb339* and *PbEPM83* proteomic maps

Spot ID ^a	Accession number ^b	Protein description	Score PMF ^c	Seq. cov. (%) ^d	Ion score ^e	Matched peptides ^f
1	gi 295664474	Cell division cycle protein	170	29	110	1
2	gi 225679503	Heat shock protein SSB	*	*	339	9
3	gi 225679863	Aminopeptidase	200	26	99	1
4	gi 226295047	Puromycin-sensitive aminopeptidase	236	34	63	1
5	gi 226291641	3-isopropylmalate dehydratase	*	*	61	3
6	gi 60656557	Heat shock protein 90	256	55	619	15
7	gi 295659538	Methionine Cobalamin-independent synthase	204	65	489	14
8	gi 295659538	Methionine Cobalamin-independent synthase	220	39	398	13
9	gi 226293399	Aconitase	152	52	430	15
10	gi 295673716	Hsp70-like protein	308	44	82	1
11	gi 225678945	Clock-controlled gene-9 protein	89	49	*	*
12	gi 295673162	Disulfide isomerase Pdi1	100	57	163	6
13	gi 295659116	Hsp70-like protein	286	58	1042	20
14	gi 225685107	Beta-Ala-His dipeptidase	136	38	88	1
15	gi 295663681	Heat shock protein	185	44	288	10
16	gi 295659837	Heat shock protein SSB1	74	44	110	2
17	gi 295671569	Heat shock protein SSC1	379	60	540	16
18	gi 225681491	Hsp70-like protein	*	*	527	12
19	gi 225682377	Phenylalanyl-tRNA synthetase beta chain	136	21	58	1
20	gi 225685037	Glycyl-tRNA synthetase	109	29	103	1
21	gi 225684009	Aconitase	144	37	320	11
22	gi 295663681	Heat shock protein	195	30	73	1
23	gi 226292595	Bifunctional purine biosynthesis protein ADE17	299	70	245	8
24	gi 295665967	Transketolase	145	26	82	1
25	gi 295659988	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	281	54	370	10
26	gi 295662174	Pyruvate kinase	162	61	231	9
27	gi 295658778	Phosphoenolpyruvate carboxykinase	228	51	336	11
28	gi 295663883	Dihydroxy-acid dehydratase	104	28	73	1
29	gi 225678915	1,4-alpha-glucan-branching enzyme	115	20	102	1
30	gi 295658778	Phosphoenolpyruvate carboxykinase	128	26	92	1
31	gi 295666177	Mitochondrial 2-methylisocitrate lyase	200	59	302	11
32	gi 295672652	Bifunctional purine biosynthesis protein ADE17	74	52	*	*
33	gi 295660969	Isocitrate lyase	337	75	486	16
34	gi 226287911	ATP synthase subunit beta	181	49	98	1
35	gi 295658865	Heat shock protein	334	61	128	3
36	gi 225681422	Kynureninase	97	33	74	1
37	gi 226294989	Peptidyl-prolyl cis-trans isomerase D	114	35	115	1
38	gi 295670601	3-hydroxyisobutyryl-CoA hydrolase	114	26	67	1
39	gi 295671230	Pyruvate decarboxylase	166	56	553	18
40	gi 24528587	Peroxisomal catalase	203	46	83	5
41	gi 225681400	Peroxisomal catalase	192	51	142	6
42	gi 295658821	ATP synthase subunit beta	257	62	328	6
43	gi 225679471	Sulfite oxidase	125	29	72	1
44	gi 295660644	Argininosuccinate synthase	310	65	488	12
45	gi 295666938	Nucleoside diphosphate kinase	*	*	291	5

46	gi 225685085	seryl-tRNA synthetase	130	30	41	1
47	gi 226290885	Glyceraldehyde-3-phosphate dehydrogenase	197	81	*	*
48	gi 146762537	Enolase	171	63	332	6
49	gi 146762537	Enolase	288	73	529	12
50	gi 146762537	Enolase	259	68	368	11
51	gi 226292147	6-phosphogluconate dehydrogenase	120	30	88	1
52	gi 146762537	Enolase	102	55	444	7
53	gi 295665123	Aldehyde dehydrogenase	235	59	495	12
54	gi 225680265	Aldehyde dehydrogenase	154	39	95	1
55	gi 225678657	Formamidase	139	64	*	*
56	gi 226294995	Dihydrolipoyl dehydrogenase	159	38	88	1
57	gi 295664927	ATP-citrate-lyase	*	*	83	4
58	gi 295664927	ATP-citrate-lyase	279	63	330	11
59	gi 295666197	2-methylcitrate dehydratase	221	59	362	11
60	gi 225681947	UDP-galactopyranose mutase	199	26	46	1
61	gi 295668473	Dihydrolipoyl dehydrogenase	196	60	334	7
62	gi 295664022	Glutathione reductase	126	59	177	9
63	gi 295664573	Adenylosuccinate lyase	92	28	113	1
64	gi 295659992	Serine hydroxymethyltransferase	294	60	231	12
65	gi 295671178	Elongation factor 1-alpha	84	36	102	3
66	gi 225560729	Serine hydroxymethyltransferase	163	39	66	1
67	gi 295662102	Rab GDP-dissociation inhibitor	286	74	360	9
68	gi 295672568	ATP-dependent RNA helicase eIF4A	149	36	71	1
69	gi 225683829	Citrate synthase	170	47	72	1
70	gi 226289087	Argininosuccinate synthase	207	48	60	1
71	gi 4164594	Heat shock protein 70	148	30	89	1
72	gi 295668479	Formamidase	175	47	463	13
73	gi 295668877	Actin	179	53	263	8
74	gi 225682808	Adenosylhomocysteinase	201	40	78	1
75	gi 226292231	Prolyl peptidase	156	32	56	1
76	gi 295669670	Adenosylhomocysteinase	214	53	457	11
77	gi 295658821	ATP synthase subunit beta	105	48	291	7
78	gi 295668481	Peptidyl-prolyl cis-trans isomerase D	219	71	428	10
79	gi 14538021	Heat shock protein 70	250	48	523	11
80	gi 295674501	Conserved hypothetical protein	118	81	254	8
81	gi 295669073	12-oxophytodienoate reductase	76	63	66	4
82	gi 226288132	Fumarylacetoacetase	168	39	114	1
83	gi 295669690	Phosphoglycerate kinase	288	70	492	16
84	gi 225678523	Elongation factor 1-gamma 1	195	42	134	1
85	gi 295658897	Citrate synthase	184	52	573	13
86	gi 295668925	Elongation factor 1-gamma 1	186	62	499	15
87	gi 295666179	2-methylcitrate synthase	198	53	236	6
88	gi 295658595	Pyruvate dehydrogenase E1 component subunit alpha	141	68	111	5
89	gi 30351156	14-3-3-like protein	*	*	80	3
90	gi 8576322	43 kDa secreted glycoprotein precursor	188	37	71	1
91	gi 8576322	43 kDa secreted glycoprotein precursor	175	30	74	1
92	gi 295658897	Citrate synthase	126	30	63	1
93	gi 295671352	40S ribosomal protein S0	100	36	179	4
94	gi 295668298	Conserved hypothetical protein	156	57	165	4
95	gi 225677671	3-isopropylmalate dehydrogenase	93	47	138	5
96	gi 295674697	Adenosine kinase	190	56	334	8

97	gi 295659136	Coproporphyrinogen III oxidase	182	64	349	12
98	gi 295669690	Phosphoglycerate kinase	193	85	178	6
99	gi 225685311	Saccharopine dehydrogenase	156	30	86	1
100	gi 295662360	Mannitol-1-phosphate 5-dehydrogenase	324	75	594	12
101	gi 225678712	Ketol-acid reductoisomerase	245	65	232	5
102	gi 295669073	12-oxophytodienoate reductase	320	76	*	*
103	gi 295669073	12-oxophytodienoate reductase	282	74	417	8
104	gi 225678587	12-oxophytodienoate reductase	334	71	75	1
105	gi 295658704	4-hydroxyphenylpyruvate dioxygenase	184	48	291	12
106	gi 295673122	L-threonine 3-dehydrogenase	154	71	328	5
107	gi 295671120	Fructose- 1,6-bisphosphate aldolase	183	47	183	11
108	gi 29826036	Fructose-1,6-biphosphate aldolase 1	237	71	294	6
109	gi 29826036	Fructose-1,6-biphosphate aldolase 1	202	67	*	*
110	gi 295666688	Transaldolase	209	58	218	8
111	gi 295674635	Alcohol dehydrogenase	221	84	228	5
12	gi 225681947	UDP-galactopyranose mutase	94	19	57	1
113	gi 295664270	2-alkenal reductase	127	50	103	3
114	gi 295666161	Alcohol dehydrogenase	129	53	258	8
115	gi 295658119	Glyceraldehyde-3-phosphate dehydrogenase	239	78	580	15
116	gi 225682647	Fructose-1,6-bisphosphatase	162	37	102	1
117	gi 225680961	Inorganic pyrophosphatase	131	38	158	6
118	gi 239609129	Pyruvate dehydrogenase E1 component subunit beta	90	33	93	1
119	gi 225683456	Transaldolase	275	53	90	1
120	gi 295661059	G-protein complex beta subunit CpcB	210	71	143	5
121	gi 295658119	Glyceraldehyde-3-phosphate dehydrogenase	275	84	299	8
122	gi 295658119	Glyceraldehyde-3-phosphate dehydrogenase	204	76	78	2
123	gi 295661300	DNA damage checkpoint protein rad24	151	66	155	5
124	gi 295667868	Elongation factor 1-beta	194	46	228	6
125	gi 295660977	Conserved hypothetical protein	184	58	218	5
126	gi 226293104	Spermidine synthase	88	42	127	5
127	gi 225680783	Proteasome subunit alpha type-3	111	29	55	1
128	gi 225685013	Pyridoxine biosynthesis protein PDX1	196	48	85	1
129	gi 295673937	Malate dehydrogenase	176	42	182	8
130	gi 226289773	40S ribosomal protein S3	164	62	66	1
131	gi 225679636	Translation elongation factor eEF-1 beta chain	115	54	115	10
132	gi 226292478	Proteasome component C1	135	38	95	1
133	gi 225681437	Conserved hypothetical protein	141	41	141	12
134	gi 225683930	Hydrolase	156	31	65	1
135	gi 225679389	Ubiquitin-conjugating enzyme	149	47	149	13
136	gi 225678501	Proteasome subunit alpha type-1	142	46	123	1
137	gi 295663891	2,5-diketo-D-gluconic acid reductase A	135	45	179	5
138	gi 225683092	Pyruvate dehydrogenase E1 component subunit alpha	*	*	65	1
139	gi 225682490	G-protein complex beta subunit CpcB	191	46	63	1
140	gi 295673937	Malate dehydrogenase	175	44	87	1
141	gi 226289845	Gamma-glutamyltranspeptidase	115	15	101	1
142	gi 226295016	Conserved hypothetical protein	88	29	57	1
143	gi 225684267	Ubiquitin carboxyl-terminal hydrolase	151	46	76	1
144	gi 225684846	Fumarate hydratase	*	*	93	1
145	gi 225684609	Proteasome subunit alpha type-2	*	*	61	1
146	gi 225683962	Anthranilate synthase component 1	*	*	66	1
147	gi 225678580	Polysaccharide deacetylase family protein	*	*	98	1

148	gi 226294448	Nicotinate-nucleotide pyrophosphorylase	218	39	96	1
149	gi 295666718	Sorbitol utilization protein SOU2	272	44	172	2
150	gi 295672968	Phosphomannomutase	140	72	251	8
151	gi 295665021	Chorismate mutase	244	69	292	8
152	gi 225677822	6-phosphogluconolactonase	214	63	78	1
153	gi 226289575	Enoyl-CoA hydratase	128	32	67	1
154	gi 226290547	HAD-superfamily hydrolase	156	51	114	1
155	gi 225678961	Predicted protein	153	44	*	*
156	gi 295674837	HAD-superfamily hydrolase	198	61	333	7
157	gi 295656848	TCTP family protein	91	46	47	1
158	gi 225677560	Short-chain specific acyl-CoA dehydrogenase	*	*	71	1
159	gi 225680770	Proteasome subunit beta type-1-A	128	51	*	*
160	gi 295668244	Mitochondrial peroxiredoxin PRX1	211	50	278	6
161	gi 295670663	Triosephosphate isomerase	147	66	147	4
162	gi 295666440	Sporulation-regulated protein	169	61	209	7
163	gi 295674635	Alcohol dehydrogenase	*	*	95	1
164	gi 225678337	Adenosine kinase	140	38	58	1
165	gi 295666522	Conserved hypothetical protein	95	38	69	2
166	gi 226291569	Mitochondrial peroxiredoxin PRX1	109	43	69	1
167	gi 1778408	27 kDa antigen, partial	127	27	66	1
168	gi 1778408	27 kDa antigen, partial	108	28	62	1
169	gi 295666381	GTP-binding nuclear protein GSP1/Ran	169	62	135	5
170	gi 225680288	Wos2	*	*	74	1
171	gi 225684870	V Chain V	77	66	*	*
172	gi 225682872	Mn superoxide dismutase	169	48	91	1
173	gi 225679093	Proteasome subunit alpha type-4	*	*	84	1
174	gi 295658559	Nitroreductase family protein	*	*	121	1
175	gi 17980998	Y20 protein	*	*	224	5
176	gi 295669402	Mn superoxide dismutase	101	42	187	8
177	gi 225682872	Mn superoxide dismutase	76	51	*	*
178	gi 295657799	40S ribosomal protein S12	118	72	117	3
179	gi 226287294	4-carboxymuconolactone decarboxylase family protein	119	60	71	1
180	gi 225678502	Decarboxylase family protein	*	*	65	1
181	gi 226294753	1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase	194	71	83	1
182	gi 225678257	Conserved hypothetical protein	*	*	130	1
183	gi 295672668	Peptidyl-prolyl cis-trans isomerase B	126	45	255	6
184	gi 295672447	Peptidyl-prolyl cis-trans isomerase H	115	42	90	3
185	gi 295658863	Conserved hypothetical protein	106	56	60	2
186	gi 225678115	Peroxisomal matrix protein	161	68	66	1
187	gi 295672073	Hypothetical protein PAAG_01591	97	36	158	4
188	gi 295666684	Cu, Zn superoxide dismutase	117	79	324	6
189	gi 295662699	Peptidyl-prolyl cis-trans isomerase cypE	91	52	109	4
190	gi 295658312	L-PSP endoribonuclease family protein (Hmf1)	108	48	186	3
191	gi 225681129	Predicted protein	105	96	*	*
192	gi 226294299	Conserved hypothetical protein	113	62	114	1
193	gi 226294299	Conserved hypothetical protein	144	62	81	1

*No matches in PMF or MS/MS Mascot databank search;

^aSpots were numbered according to Figure 1;

^bGenBank general information identifier. The accession number refers to higher scores on Mascot search. The identifications were performed using genome databases of three strains (*Pb01*, *Pb03* or *Pb18*).

^cPeptide mass fingerprinting score obtained by Mascot databank search;

^dSequence coverage by peptide mass fingerprinting;

^eMS/MS score obtained by Mascot databank search;

^fNumber of matched peptides performed by MS/MS databank search.

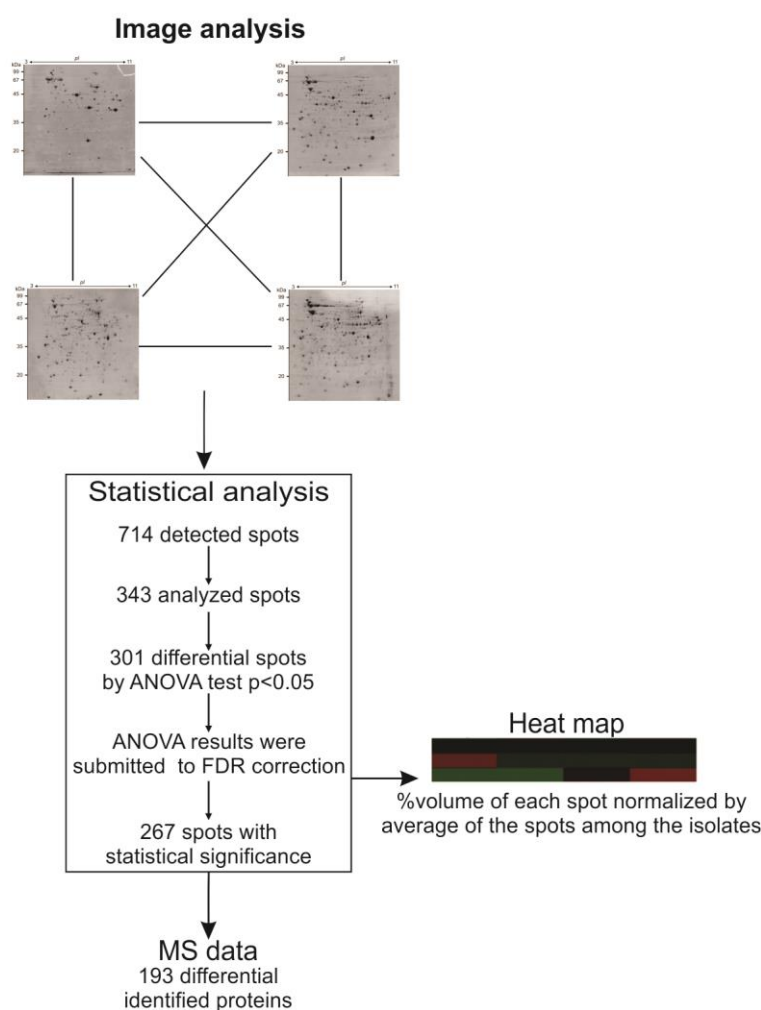


FIGURE S1: A diagram depicting the employed analysis for determining differentially expressed proteins/isoforms. The protein spots were selected based on the staining intensity as determined by the image analysis, as described. Gels in triplicates for each isolate were analyzed separately. The biological triplicates of each isolate were compared among themselves. After that, the isolates were compared so that in each comparison, one of the isolates was considered as a reference. The comparison of protein expression among isolates was validated using a two-way ANOVA test using the FDR (false discovery rate) criterion to correct type 1 errors, considering p values ≤ 0.05 to be statistically significant.

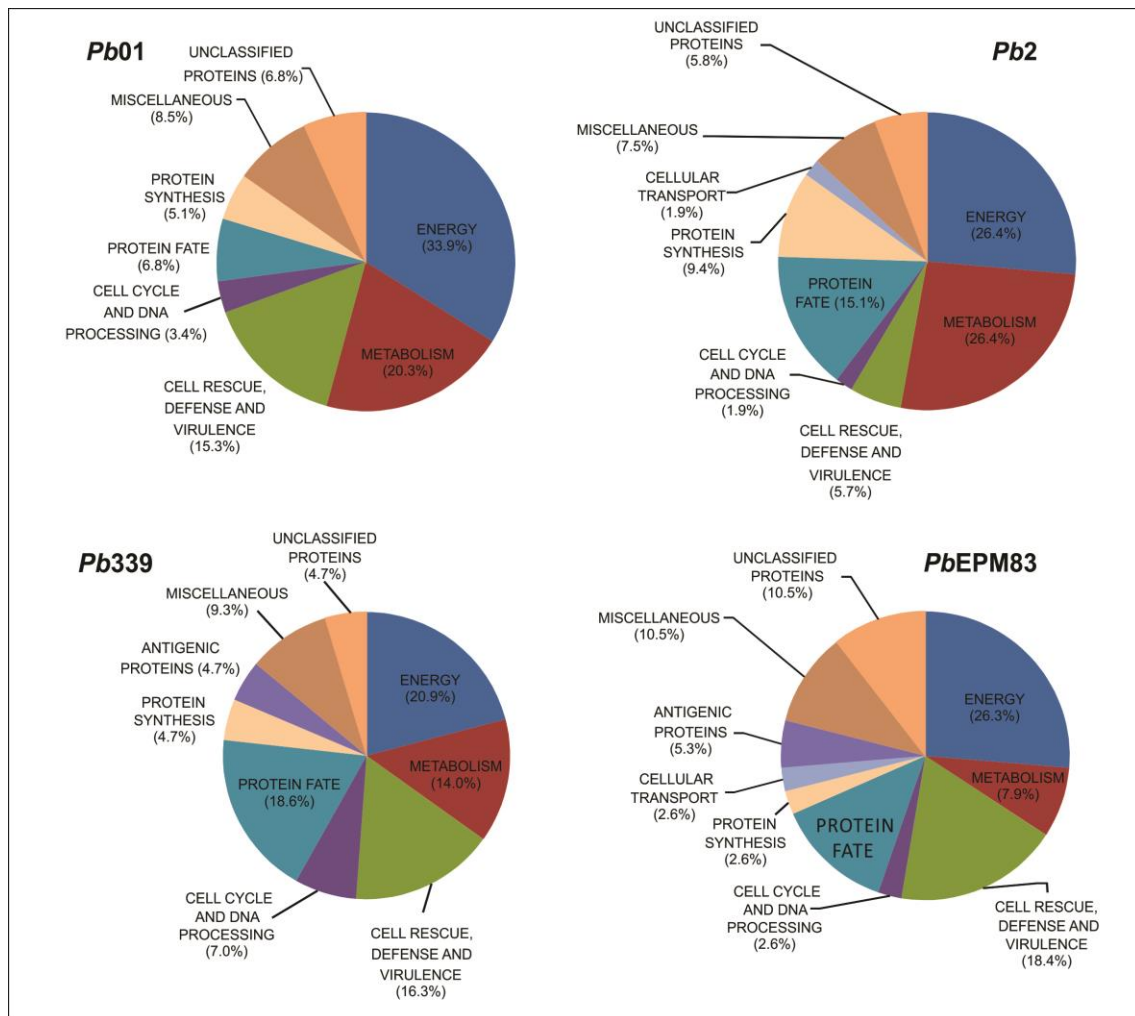
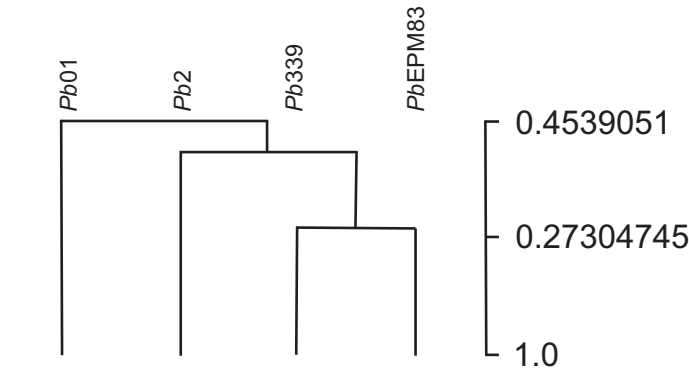
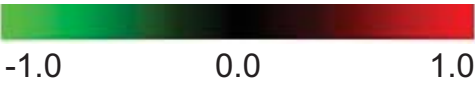


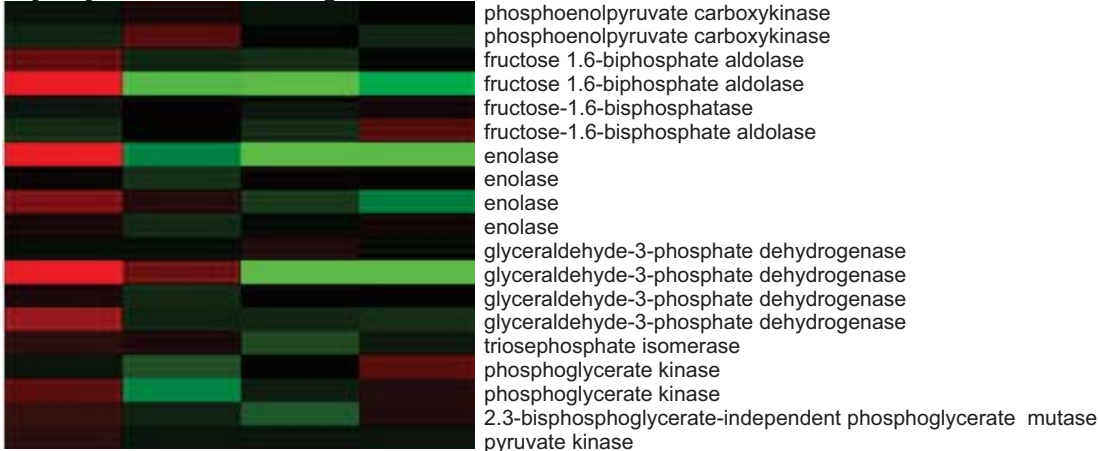
FIGURE S2: Graphic summation of proteomic analysis to representative of each phylogenetic species of *Paracoccidioides*. The identified proteins were classified according to the functional categories of the MIPS functional annotation. The identified proteins were involved in several cellular processes, including energy, metabolism, cell rescue and virulence, cell cycle, protein synthesis, protein fate, cellular communication, cell transport and antigenicity, in different proportions among isolates of *Paracoccidioides*.

FIGURE S3: Differences in the protein profiles in members of the *Paracoccidioides* genus. Hierarchical clustering of the statistically significant proteins/isoforms. A color-coded heat map was derived using the distribution of average of expression for each sample. Shades of green represent down-regulation ratios; shades of red represent up-regulation; and shades of black indicate that the protein was not identified in the studied member of the *Paracoccidioides* genus. The Pearson's correlation, as a similarity measure, was used to group the proteins profile of isolates.

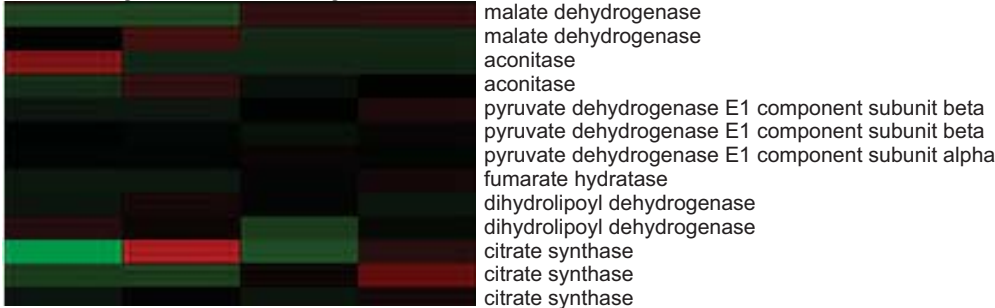


ENERGY

Glycolysis and Gluconeogenesis



Tricarboxylic-acid Pathway



Oxidation of fatty acids



Glyoxylate cycle



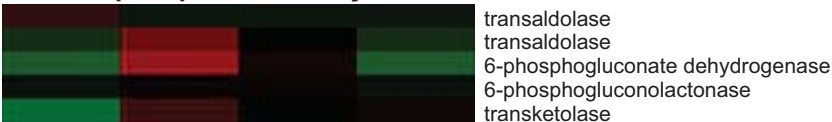
Methylcitrate cycle



Fermentation

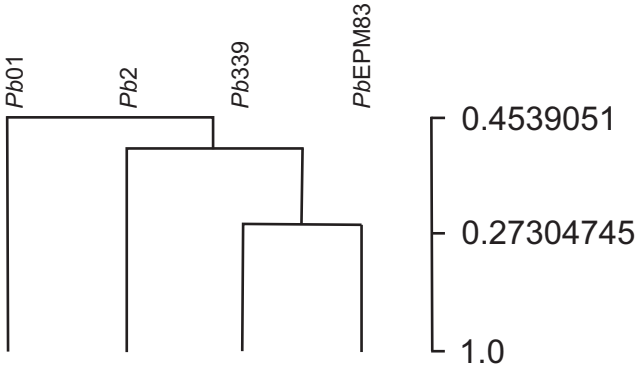


Pentose-phosphate Pathway



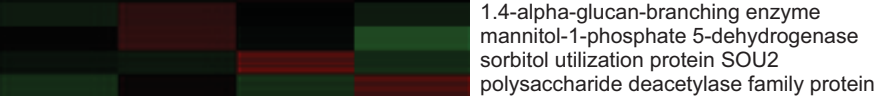
Electron transport and membrane-associated energy conservation



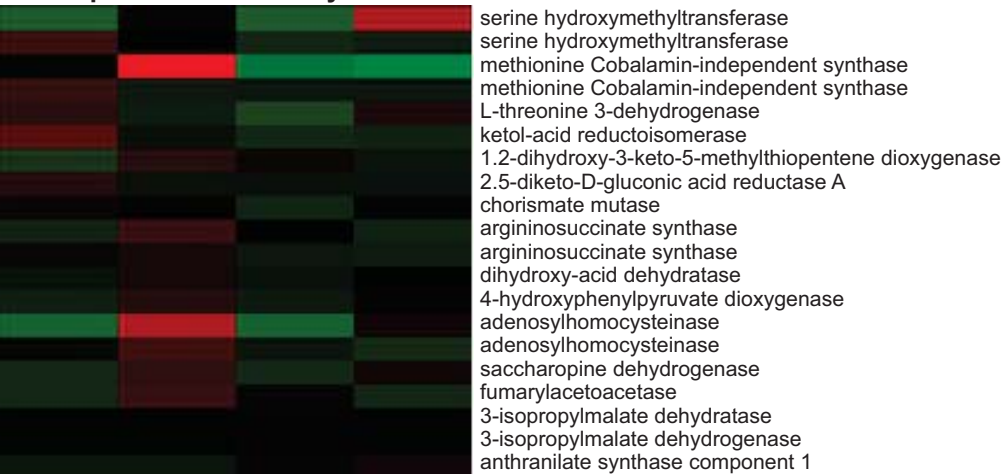


METABOLISM

C-Compound and Carbohydrate Metabolism



C-Compound and Carbohydrate Metabolism



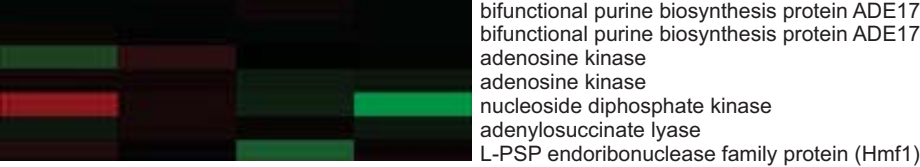
Nitrogen Metabolism



Sulfur Metabolism



Nucleotide Metabolism



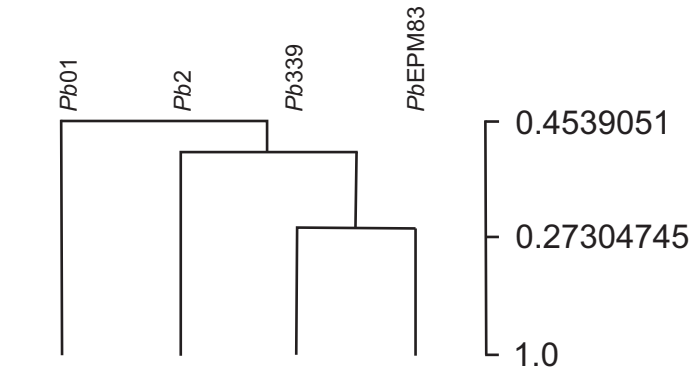
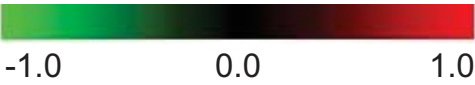
CELL RESCUE, DEFENSE and VIRULENCE

Stress response

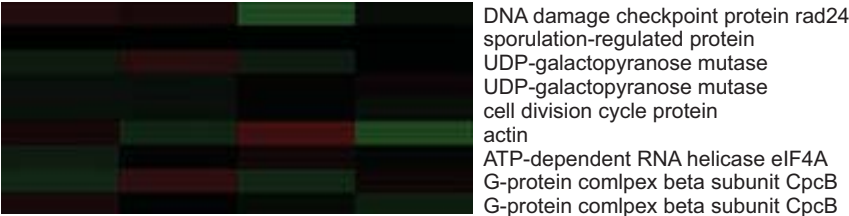


Detoxification

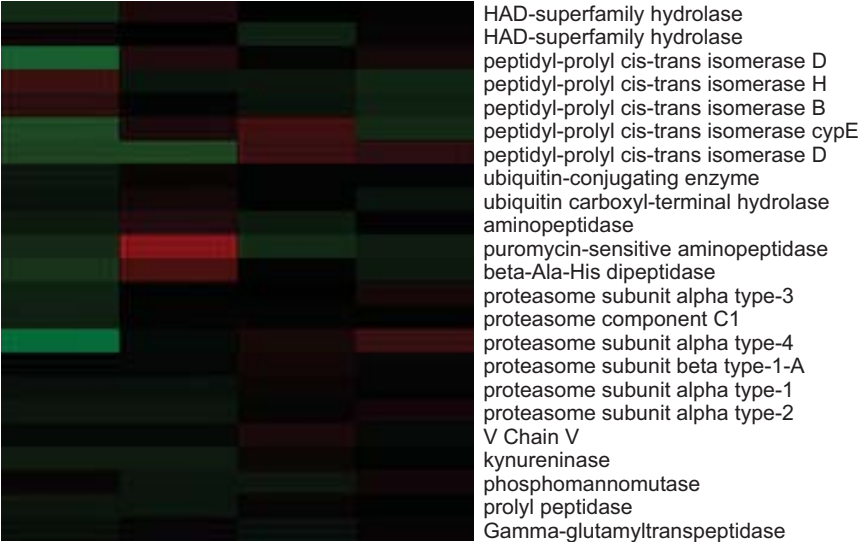




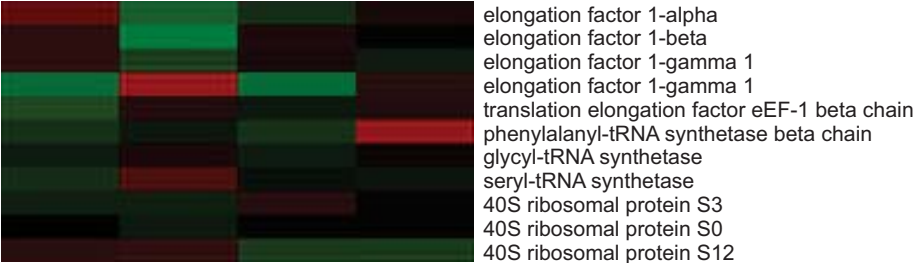
CELL CYCLE AND DNA PROCESSING



PROTEIN FATE



PROTEIN SYNTHESIS

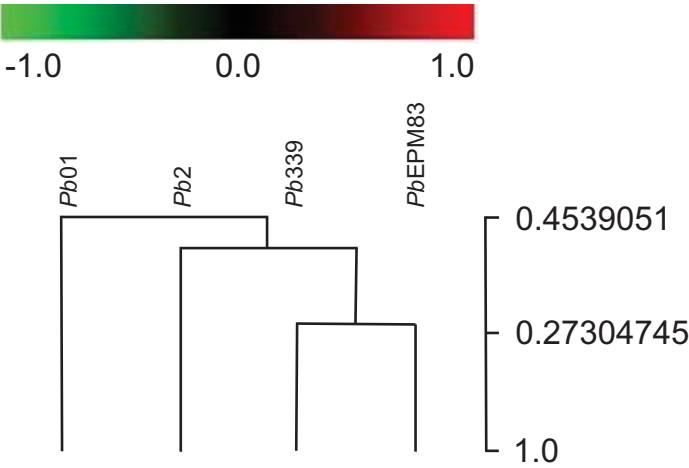


CELLULAR TRANSPORT, TRANSPORT FACILITIES AND TRANSPORT ROUTES

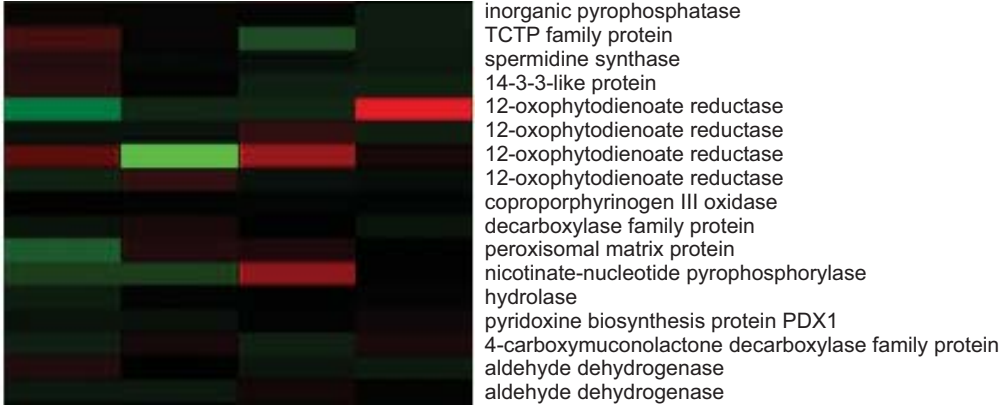


ANTIGENIC PROTEINS

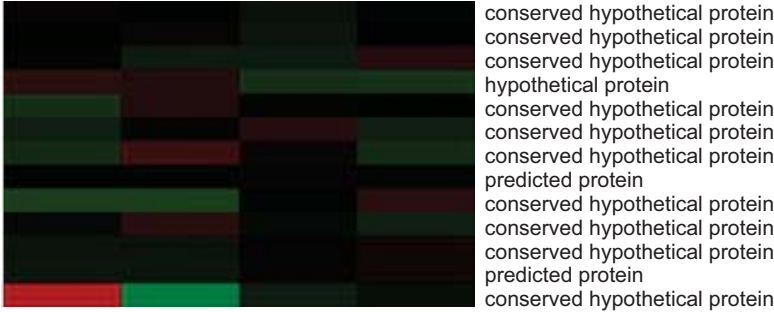




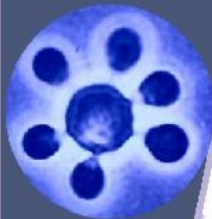
MISCELLANEOUS



UNCLASSIFIED PROTEINS



Discussão



5 – DISCUSSÃO

Estratégias metabólicas e adaptativas comparando membros do gênero *Paracoccidioides* foram discutidas a nível molecular com análises genômicas (Desjardins et al., 2011). No entanto, estas estratégias não têm sido discutidas à luz dos dados experimentais proteômicos até agora. No presente estudo, mostra-se como as proteínas que são acumuladas por um organismo podem indicar o estado fisiológico das células. Os perfis proteômicos comparativos de *Pb01*, *Pb2*, *Pb339* e *PbEPM83* são descritos aqui pela primeira vez. Usando uma análise proteômica, de um total de 714 spots detectados, 343 foram analisados, e, destes, 193 foram proteínas / isoformas diferencialmente expressas. Os dados de proteômica demonstraram diferenças significativas nos perfis de expressão de proteína entre os representantes dos diferentes clados filogenéticas do gênero *Paracoccidioides*. O agrupamento hierárquico dos perfis proteômicos dos quatro isolados de *Paracoccidioides* refletiram as relações filogenéticas entre as espécies *Paracoccidioides*, onde *S1* é considerado uma espécie independente, e *PS3* é filogeneticamente mais perto do que *S1* para *PS2*. *Pb01* é altamente divergente das demais espécies filogenéticas (Matute et al, 2006; Teixeira et al, 2009).

Proteomas podem fornecer subsídios sobre o estado fisiológico das células. Na nossa análise proteômica, os níveis de expressão das enzimas que estão relacionadas com a glicólise sugerem o uso de um metabolismo mais anaeróbico para a produção de energia a partir da glicose no isolado *Pb01*. O elevado nível de enzimas envolvidas na glicólise e fermentação alcoólica levaram à hipótese de que este isolado pode ter um metabolismo anaeróbico mais do que os outros. Os níveis de etanol foram determinados nas células de levedura dos isolados analisados. Como pode ser observado, o nível de etanol foi maior em *Pb01*, corroborando com a proposição de metabolismo mais

anaeróbico. Estudos de transcrição têm sugerido um metabolismo anaeróbico mais na fase de levedura isolado *Pb01*, que poderia produzir mais etanol em comparação com micélio (Felipe et al., 2005). Consistente com estes resultados, a análise proteômica detecta a acumulação de enzimas da via da glicólise e de fermentação alcoólica para a transição de micélio para células de levedura em *Pb01* (Rezende et al. 2011). Estudos indicaram a relevância da fermentação do etanol para fungos patogênicos. Estudos proteômicos sugerem que a fermentação de etanol contribui para a geração de energia em conídios ou no início do processo de germinação em *Aspergillus fumigatus* (Teutschbein et al., 2010). Além disso, sob condições de hipóxia, este fungo privilegia a ativação de fermentação etanol (Barker et al., 2012).

As estratégias metabólicas mais induzidas em *Pb2* parece ser a via das pentoses fosfato, como revelado por proteômica comparativa e confirmado por análise bioquímica. A indução da linha não oxidativa da via das pentoses fosfato pode proporcionar substratos para a glicólise. Além disso, a expressão elevada de enzimas do ramo oxidativo pode fornecer NADPH para uso em processos biossintéticos (Rui et al., 2010). A degradação de aminoácidos que proporcionam os substratos para o ciclo do TCA é também um aspecto diferencial do metabolismo de *Pb2*, conforme revelado por análise proteômica. Uma característica notável das *Pb339* é beta-oxidação. Os ensaios de crescimento na presença de ácido oleico corroboraram os dados de proteoma. A oxidação dos ácidos graxos é importante para a utilização de lipídeos de armazenamento ou ácidos graxos exógenos para gerar acetil coenzima A (acetil-CoA) para o metabolismo do carbono central. O perfil de transcrição de *Cryptococcus neoformans* revelou que os genes para oxidação peroxissomal, importação de ácido graxo, lipídeo e degradação foram mais induzidos após internalização do patógeno e durante a infecção pulmonar, sugerindo que o metabolismo do ácido graxo pode ser importante para a virulência de *C.*

neoformans em mamíferos (Kretschmer et al., 2012). Se este aspecto metabólico pode influenciar a virulência de *Pb339*, ele deve ser um foco de futuras investigações. Em comparação com os outros isolados, *PbEPM83* parece utilizar preferencialmente rotas aeróbicas para a produção de energia através de hidratos de carbono, como sugerido pela acumulação excessiva de enzimas glicolíticas e TCA.

Metabolismo de proteínas parece ser fundamental para o crescimento nas *Onygenales*. Ensaio metabólico do não-patogênico *Onygenales Uncinocarpus* demonstrou crescimento extensivo em uma ampla gama de substratos proteicos em comparação com carboidratos (Desjardins et al., 2011). O metabolismo dos aminoácidos parece ser particularmente relevante no *Pb01* e *Pb2* isolados em relação às *Pb339* e *PbEPM83*. Se este recurso metabólico fornece mais atributos para um agente patogênico animal ainda não foi esclarecido.

Enzimas que estão relacionados com a resposta ao estresse oxidativo foram mais abundantes no *Pb01*, *Pb2* e *Pb339* comparação com *PbEPM83*, indicando uma melhor resposta às espécies reativas de oxigênio (ROS) nestes membros do complexo *Paracoccidioides*. Os fungos patogênicos humanos dependem de mecanismos de proteção contra danos oxidativos para se defender contra ROS que são liberadas pelas células hospedeiras, no decurso de uma infecção. Estas espécies podem danificar os constituintes celulares, incluindo DNA, proteínas e lipídios, levando a morte celular. A capacidade para neutralizar ROS representa uma resposta de stress em *Paracoccidioides*, como descrito anteriormente (de Chagas et al., 2008). Estudos proteômicos relataram a indução de enzimas relacionados à detoxificação de ROS, tais como a catalase peroxissomal e Cu / Zn e Mn superóxido dismutase, durante o tratamento de *Paracoccidioides* com peróxido de hidrogênio, indicando o envolvimento dessas enzimas para proteger o fungo do stress geral gerado pela os mecanismos de

defesa do hospedeiro (Grossklau et al., 2013). Detoxificação de ROS tem um significado aparente para virulência, como fagócitos do sistema imune inato produzir vários oxidantes que se presume a desempenhar papéis na morte microbiana. Neste sentido, para restringir a infecção por *Paracoccidioides*, macrófagos produzem várias substâncias nocivas, tais como peróxido de hidrogênio, que o stress oxidativo causa (Pina et al, 2008;.. Rodrigues et al, 2007). A menor resistência à ROS em *PbEPM83*, como demonstrado nos ensaios de proteômica e bioquímicos, merece uma investigação mais aprofundada.

Uma grande quantidade de dados foram gerados a partir de análises específicas ao expor *Paracoccidioides* a determinados fatores de stress (Silva et al, 1994;.. Silva et al, 1999;.. Isaac et al, 2001;.. Nicola et al, 2008). Uma condição de estresse evidente a cada patógeno humano tem de suportar é a temperatura corporal elevada do hospedeiro. O isolado *PbEPM83* mostrou alta expressão de proteínas caracterizadas como HSP. Em fungos, as HSPs são moduladas em resposta a vários estímulos, incluindo a temperatura (Burnie et al., 2006). A abundância de HSPs em que este isolado pode fornecer este fungo a capacidade de sobreviver certas tensões ambientais, tal como demonstrado no presente trabalho.

Em conclusão, nós fornecemos evidências de características metabólicas diferenciais em membros do complexo filogenética. Os dados indicam uma diferença significativa no metabolismo global de membros de *Paracoccidioides*. A caracterização adicional de essas diferenças pode levar ao uso de critérios bioquímicos como marcadores para distinguir entre as quatro espécies crípticas das *Paracoccidioides* gênero. Além disso, o estabelecimento de mapas 2-DE de referência para os quatro isolados permitirá correlacionar as variações quantitativas e qualitativas nos proteomas com características fenotípicas, como a patogenicidade, virulência e tolerância a estresses ambientais.



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