Molecular analysis of the *Penicillium marneffei* glyceraldehyde-3-phosphate dehydrogenase-encoding gene (*gpdA*) and differential expression of *gpdA* and the isocitrate lyase-encoding gene (*acuD*) upon internalization by murine macrophages

Sophit Thirach,1 Chester R. Cooper, Jr2 and Nongnuch Vanittanakom1

1Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand
2Department of Biological Sciences, Youngstown State University, Youngstown, OH, USA

Penicillium marneffei is an intracellular dimorphic fungus that can cause a fatal disseminated disease in human immunodeficiency virus-infected patients. The factors that affect the pathogenicity of this fungus remain unclear. Here, we report the isolation and characterization of the *gpdA* cDNA and genomic clones encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in *P. marneffei*. Phylogenetic analysis of GAPDH amino acid sequences demonstrated the evolutionary relationship of *P. marneffei* to other fungi, including the intracellular pathogen *Ajellomyces capsulatus*. To assess the central importance of phagocytic cells in defence against *P. marneffei* infection, we used Northern blotting to investigate the response of the isocitrate lyase-encoding gene (*acuD*) and *gpdA* to nutrient deprivation inside macrophages. The results revealed that after macrophage internalization, the gene involved in the glyoxylate cycle, *acuD*, showed higher expression levels as early as 2 h from the start of co-incubation, and the differential expression could be observed again at 8 h after infection. In contrast, the expression of *gpdA* was downregulated in the yeast phase, as well as during macrophage infection after 2, 4 and 8 h of infection. The induction of *P. marneffei* *acuD* was shown to be coordinated with the downregulation of the glycolytic *gpdA* gene, implying that the cytoplasmic environment of macrophages is deficient in glucose and the glyoxylate pathway could be used by this pathogen to allow subsistence on two-carbon compounds within the host cell following its intracellular persistence.

INTRODUCTION

*Penicillium marneffei* is the causative agent of a fatal systemic mycosis known as penicilliosis marneffei, which is endemic in South-East Asia (Vanittanakom et al., 2006). The infection is initiated presumably via inhalation of airborne conidia that are small enough to reach the alveoli. After the conidia are inhaled, they undergo transformation into yeast cells within the infected tissues. The disease begins in the lungs, and then disseminates to other organs and systems. In order to establish a successful infection, the fungal cells that colonize the dynamic substrate of the human host must have the ability to adapt to and modify gene expression in response to changes in their environment. In recent years, several approaches have been developed to identify genes that are putatively related to the host–fungus interaction (Cooper & Vanittanakom, 2008). The transcriptional profile of *P. marneffei* yeast cells and mycelium revealed genes that are potentially related to fungal virulence, in addition to providing a comprehensive view of fungal metabolism (Cooper & Haycocks, 2000; Liu et al., 2007). Recently, Chandler et al. (2008) utilized a proteomics approach to identify proteins associated with the early stages of yeast and mycelial development. Despite these reports, a lack of information exists regarding *P. marneffei* gene expression when the organism interacts with the host.

Analysis of the response of *P. marneffei* to macrophages provides a window into the alterations necessary for the organism to survive its first encounter with the immune system. Once inside the macrophage, the conidia of *P.
**P. marneffei** differentiate into the yeast form, which can pierce the macrophage, thus allowing it to resume proliferation (Vanittanakom et al., 2006; Cooper & McGinnis, 1997). Free yeast cells continue to grow and spread via a haematogenous route. Additionally, infected macrophages can aid in the systemic spread of *P. marneffei* throughout the host. This internalization and escape can be observed in an *in vitro* system in which *P. marneffei* and cultured macrophages are mixed. It is clear, however, that the morphogenetic change is only part of the response to phagocytosis. One method for analysing this encounter completely is to identify the alterations in transcription while *P. marneffei* undergoes phagocytosis.

Housekeeping enzymes are constitutively expressed in all organisms to perform essential metabolic functions for the purpose of survival. In recent years, a number of reports have suggested that some of these enzymes are multifunctional and may play a role in enhancing virulence for many pathogens (Pancholi & Chhatwal, 2003). For example, enzymes that are located on the surface of pathogens could interact with host components, whereas others may interact directly with the host cells to trigger signal transduction processes that enable colonization, invasion and persistence within the host.

Among the enzymes in the glycolytic pathway that may play a role in these processes is glyceraldehyde-3-phosphate dehydrogenase (GAPDH). This enzyme catalyses the oxidative phosphorylation of glyceraldehyde-3-phosphate into 1, 3-biphosphoglycerate in the presence of nicotinamide adenine dinucleotide and inorganic phosphate. GAPDH appears to be a multifunctional protein found in several subcellular locations in eukaryotes, displaying functions unrelated to glycolysis, such as membrane transport and fusion, as well as nuclear RNA transport (McDonald & Moss, 1993; Singh & Green, 1993; Sirover, 1997, 1999). Also, in some pathogens, such as *Candida albicans*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Paracoccidioides brasiliensis*, GAPDH has been found on the cell wall, where it may have various roles in host-pathogen interactions (Gil-Navarro et al., 1997; Gozalbo et al., 1998; Modun & Williams, 1999; Modun et al., 2000; Taylor & Heinrichs, 2002; Barbosa et al., 2004, 2006). GAPDH has long been thought of as the product of a housekeeping gene that exhibits invariant transcript levels. However, several studies have shown that the expression of the *Paracoccidioides brasiliensis* *gpdA* gene and the cognate protein are developmentally regulated in different growth phases of *Paracoccidioides brasiliensis*, with a higher expression in the parasitic yeast phase, thereby reinforcing possible new functions attributed to the enzyme (Barbosa et al., 2004, 2006). Until now, the GAPDH-encoding gene and its potential role in the infection process have not been characterized in *P. marneffei*.

Although little is known about the factors required for the intracellular persistence of *P. marneffei*, a study by Cánovas & Andrianopoulos (2006) indicated that the *acuD* gene, which encodes the glyoxylate bypass enzyme isocitrate lyase, is highly expressed in the yeast phase of this fungus. This observation suggests a link between carbon metabolism, cellular development and pathogenicity in this dimorphic fungal pathogen. The glyoxylate cycle allows two-carbon (C2) compounds to be used as carbon sources in gluconeogenesis. In fact, several lines of evidence indicate that intracellular pathogens may preferentially utilize C2 compounds, such as products of fatty-acid degradation, for energy production (Lorenz & Fink, 2001). Specifically, *Mycobacterium tuberculosis*, *Candida albicans* and *Paracoccidioides brasiliensis* are examples of other micro-organisms that respond to phagocytosis by inducing glyoxylate cycle genes, suggesting that their activity is required for persistence and survival within macrophages (Graham & Clark-Curtiss, 1999; Lorenz & Fink, 2001; Lorenz et al., 2004; Schnappinger et al., 2003; Barelle et al., 2006; Muñoz-Elias & McKinney, 2005; Derengowski et al., 2008). This metabolic shift also includes the downregulation of glycolytic genes.

In this study we have isolated and characterized cDNA and genomic clones, designated *gpdA*, encoding GAPDH in *P. marneffei*. Subsequently, we constructed a phylogenetic tree to assess evolutionary relationships among several fungal GAPDH-encoding genes. Also, we analysed the expression of *gpdA* in different developmental phases of *P. marneffei*. Finally, in order to evaluate the transcriptional status of genes related to other types of carbon metabolism, we investigated the expression of both *gpdA* and the glyoxylate cycle gene, *acuD*, after co-culture of *P. marneffei* conidia with murine macrophages. Our collective results suggest that the persistence of *P. marneffei* within host cells may rely upon the induction of the glyoxylate cycle and the concomitant downregulation of glycolysis.

**METHODS**

**Fungal strain, media and growth conditions.** The strain of *P. marneffei* used in this study, CBS 119456, the preparation of a conidial inoculum from this isolate, and the culture of this fungus in brain heart infusion broth have been described previously (Thirach et al., 2007).

**Isolation and characterization of *P. marneffei* *gpdA*.** Genomic DNA was isolated from *P. marneffei* by a slight modification of a previously described method (Vanittanakom et al., 2002). A *gpdA* fragment was amplified using a previously described method (Thirach et al., 2007) with primers G1 (5‘-ACHHGGYGYTTCACCCACA-3’) and G2 (5‘-ATGCATCTTCCACGTCCT-3’). These primers were designed based upon the alignment of *gpdA* genes from several fungi. The PCR fragment was cloned, sequenced and used as a probe in screening a cDNA library (Pongpom et al., 2005) in order to obtain a full-length *gpdA* cDNA clone. Plaque hybridization screens were carried out using the ECL direct nucleic acid labelling and detection kit (Amersham Pharmacia Biotech). The complete genomic sequence encoding GAPDH was obtained by PCR amplification of *P. marneffei* genomic DNA. A 1400 bp PCR fragment was amplified by a sense primer (5′-CTATCCCGGAATATCGG-3’) and the C-terminal by an antisense primer (5′-GAGCATACCTGGGAAGATG-3’). The amplified PCR product was purified, cloned and sequenced.

http://jmm.sgmjournals.org
Analysis of sequenced data and phylogenetic tree construction. The cloned GAPDH-encoding gene from *P. marneffei* was analysed at both the nucleotide and deduced amino acid sequence levels. The software used in searching for homology/similarity included the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST), as well as DNA translation software (http://www.expasy.org/tools/dna.html). The GAPDH protein sequences of *P. marneffei* and 12 other fungal species were aligned for phylogenetic analysis using the neighbour-joining method from the MEGA3 program (Kumar et al., 2004).

Macrophage culture and recovery of *P. marneffei* cells after infection. The mouse macrophage cell line derived from a reticulum sarcoma, J774.1 (ATCC TIB-67), was used in this study. Cells were infected with *P. marneffei* conidia and fungal RNA extracted from both co-cultures and controls as previously described (Thirach et al., 2007). An aliquot of each RNA sample was checked for the absence of mouse DNA or RNA using murine *gpdh*-specific primers in a PCR or RT-PCR assay, respectively.

Differential expression of the *P. marneffei* acuD and gpdA genes in phase transition and during macrophage infection. Fifteen micrograms of total RNA extracted at cells at different phases (mould, conidia and yeast), as well as from conidia co-incubated with or without macrophages (2, 4 and 8 h), were separated by electrophoresis on a denaturing 1% agarose gel using the NorthernMax-Gly system (Ambion), then transferred onto nylon membranes (Hybond-N). Incubation with or without macrophages (2, 4 and 8 h), were fixed to the membrane by UV cross-linking. To determine the differential expression of *P. marneffei* acuD and gpdA, the immobilized RNA was probed with a labelled PCR-generated DNA fragment of the *acuD* gene and reprobed with that of the GAPDH-encoding *cDNA* clone consecutively. The *acuD* probe was generated by the PCR amplification of *P. marneffei* genomic DNA using primers that were designed from the nucleotide sequence (originally designated *icl1*) deposited in GenBank (accession number AF375018; forward primer, 5′-TATCATGCTACCGACCTCCTTT-3′; reverse primer, 5′-AGATCGGCGAATGCTGATAAGC-3′). Labelling, hybridization and detection of specific probes were carried out by following the directions from the ECL direct nucleic acid labelling and detection kit (Amersham).

RESULTS AND DISCUSSION

Identification and characterization of *P. marneffei* gpdA

The 1011 bp *P. marneffei* cDNA encoding GAPDH, designated gpdA, had both a typical start codon (ATG) and stop codon (TAG), indicating a full-length sequence was present (see Supplementary Fig. S1 available with the online journal). Alignment of its deduced 337 amino acid sequence with five other fungal GAPDH sequences by CLUSTAL W analysis revealed a high level of homology, with the highest identity to the protein found in *Ajellomyces capsulatus* (data not shown). The genomic fragment corresponding to the entire ORF region present in the *cDNA* sequence was amplified by PCR, and then sequenced. Comparison between the nucleotide sequences from the *cDNA* library and the genomic DNA revealed the compatibility of both, except for those of five non-homologous regions from the genomic DNA at nucleotides 49–122, 135–194, 249–289, 608–657 and 1085–1137.

Analysis of these regions strongly suggested that they are introns of 74, 60, 41, 50 and 53 nucleotides, respectively. Their 5′ and 3′ ends conformed to the basic consensus, GT/AG, for the eukaryotic splice donor and acceptor site (Breathnach & Chambon, 1981; Mount, 1982; Kuper et al., 2004). These regions contained an internal putative splice box, which matched the filamentous fungus consensus sequence (CTRAY), upstream from the 3′ end of the intron (Gurr et al., 1987; Kuper et al., 2004). A search at the PROSITE database revealed the presence of a potential site for substrate binding (ASCTTNCL), as described for GAPDH sequences (Goudot-Crozel et al., 1989), at positions 149–156. Amino acids potentially associated with catalysis were at amino acid positions 151 (C) and 178 (H). Potential phosphorylation sites were found located at 10 positions (102–106, 138–140, 183–185, 191–194, 240–242, 245–250, 265–268, 292–295, 311–314 and 321–329). The amino acid residues at positions 34 (D) and 315 (N) corresponded to the putative nicotinamide adenine dinucleotide binding sites. Positions 150, 152, 196, 210 and 225 were found to be probable sites for inorganic phosphate binding (S, T, R, T, G). Positions 181, 233 and 247 were found to be residues that putatively related to the binding of the phosphate from the substrate (T, R, and R) (see Supplementary Fig. S1 available with the online journal).

Phylogenetic analysis

The analysis of GAPDH sequences has been shown to be useful for determining evolutionary relationships between different organisms (Ridder & Osiewacz, 1992) and among fungi (Smith, 1989; Barbosa et al., 2004). These associations may also reflect functional differences among the different taxa. The GAPDH protein sequences of *P. marneffei* and 12 other species were aligned for phylogenetic analysis using the neighbour-joining method. Based on the comparison of deduced GAPDH sequences it was possible to cluster the sequences in three clades comprising Eurotiomycetes, Dothideomycetes and Sordariomycetes. This analysis supported the classification of *P. marneffei* in the Eurotiomycetes clade, close to another dimorphic pathogen, *Ajellomyces capsulatus*. As expected, the pathogen *Coccidioides immitis*, another dimorphic fungus, as well as the monomorphic moulds *Emericella nidulans* and *Aspergillus niger*, occupied a position inside the same clade (Fig. 1a). A comparison of the mosaic structure of *P. marneffei* gpdA and genes from other fungi was performed. Fig. 1 shows the positions of the introns in relation to the amino acid sequences of the GAPDH proteins. A comparison to other species in the Eurotiomycetes revealed that *P. marneffei* shared introns at positions 16/17, 20/21, 42/43, and 270/271, and the analysed Dothideomycetes yielded similar results, except for differences in the intron at position 16/17. The Sordariomycetes had the lowest number of introns (1–2), showing intron similarity to other clades at position 42 (Fig. 1a, b). Interestingly, the presence of well-defined clades comprising
Eurotiomycetes, Dothideomycetes, and Sordariomycetes could be correlated with the patterns of introns in the cognate genes. Our data also confirmed the systemic relatedness of intron positions among the gpdA genes of the Eurotiomycetes examined.

**Differential expression of P. marneffei gpdA and acuD upon internalization by murine macrophages**

Although gpdA has been considered as a constitutively expressed gene in most organisms, an increasing amount of evidence supports the contention that the regulation of this gene depends on several cellular conditions. Barbosa and colleagues demonstrated that the expression of GAPDH and its transcript is higher in the parasitic yeast phase of *Paracoccidioides brasiliensis* (Barbosa et al., 2004, 2006). These observations raise the intriguing question of whether the fungal GAPDH may also perform other important non-glycolytic functions.

In our study, differential expression of gpdA was observed. The transcript of this gene was highly expressed in the mould phase (mycelial phase) of *P. marneffei* as compared to the yeast phase (Fig. 2). Conidia also possess higher levels of the gpdA transcript. These results indicate that gpdA may respond to nutritional limitation or some stage of the life cycle in the yeast form of *P. marneffei*. The function of *P. marneffei* GAPDH and its involvement in the response to nutrition in the yeast phase needs further investigation.

The ability of *P. marneffei* to survive and successfully adapt within phagocytic cells depends on its mechanisms to respond to the metabolic constraints imposed by macrophages. Since it is believed that the microenvironment inside host cells is a hostile habitat, differential regulation of specific genes is probably involved in the establishment and adaptation of this pathogen in the context of the host. Recently, we characterized the oxidative stress response gene, sodA, which encodes the Cu, Zn-superoxide dismutase of *P. marneffei*. The upregulation of sodA during

---

**Fig. 1.** Phylogeny of the GAPDH of *P. marneffei* and analysis of the intron positions. (a) A neighbour-joining tree derived from MEGA 3 analysis of 13 species inferred from the protein sequences of GAPDH is shown. The bootstrap values are shown above the branches. Organism sources and National Center for Biotechnology Information database accession numbers for the sequences are: *Cryphonectria parasitica* (X53996), *Sordaria macrospora* (AJ313527), *Neurospora crassa* (U67457), *Claviceps purpurea* (X73282), *Podospora anserina* (X82824), *Phaeosphaeria nodorum* (AJ271155), *Curvularia lunata* (X88718), *Cochliobolus heterostrophus* (X63516), *Emericella nidulans* (M19694), *Aspergillus niger* (X99652), *P. marneffei* (EU000324), *Ajellomyces capsulatus* (AF273703) and *Coccidioides immitis* (Q1DTF9). The numbers of introns in the cognate gpdA genes are indicated on the right hand side. (b) Structural diagram of gpdA genes. The corresponding amino acid residues indicate the position of introns. The sequences utilized were those described above.

---

**Fig. 2.** Expression analysis of acuD and gpdA during macrophage infection and different phases of the life-cycle of *P. marneffei* by Northern blotting. During macrophage infection, 15 μg total RNA was extracted from conidia in cell-free medium (C) and intracellular conidia (I) at 2, 4 and 8 h of incubation. Total RNA was also extracted from mycelium (25 °C, 96 h), conidia and yeast cells (37 °C, 96 h). *P. marneffei* acuD and gpdA cDNA were used as probes in Northern hybridization (a, b). The 28S rRNA loading control is shown in (c).
macrophage infection suggests that this gene may play an important role in stress responses and the adaptation of *P. marneffei* to the internal macrophage environment (Thirach et al., 2007). Besides detoxification-related genes, it is often assumed that the modulation of metabolism-related genes can be associated with the micro-organism’s response to a changing environment. Cánovas & Andrianopoulos (2006) described that the *P. marneffei* acuD gene, encoding isocitrate lyase, is regulated by both carbon source and temperature. In their studies, Northern blotting revealed that the acuD gene was strongly induced at 37 °C, with relatively small differences in the transcript levels when the fungus was incubated at 37 °C with glucose or acetate as carbon sources. Accordingly, the data we obtained showed a higher expression of the acuD gene in conidia and yeast than in the mycelial cells (Fig. 2).

In order to evaluate *P. marneffei* transcriptional response following co-culture with murine macrophages, expression analysis of the glyoxylate cycle and the glycolytic pathway, using acuD and gpdA as the respective markers, was undertaken by Northern blotting. The results revealed that after macrophage internalization of conidia, acuD had higher expression levels at as early as 2 h from the start of co-incubation when compared to cells grown in cell-free medium as a control, and the differential expression could be observed again at 8 h after infection (Fig. 2). This acuD expression pattern might be affected by the concentration of substrates inside macrophages. The induction of acuD observed following phagocytosis suggests a role of this metabolic pathway in *P. marneffei* adaptation inside macrophages, and provides further evidence of the state of nutritional deprivation inside the phagosome. Since this fungus is a facultative intracellular pathogen and glycolytic substrates are supposed to be absent or scarce in the phagolysosome environment (Schnappinger et al., 2003), the glyoxylate cycle may be required for the utilization of C2 compounds derived from fatty acids in energy production. The role of this pathway in the survival of micro-organisms within macrophages has been shown in pathogens such as *M. tuberculosis* and *Candida albicans* (Graham & Clark-Curtiss, 1999; Lorenz & Fink, 2001; Lorenz et al., 2004; Schnappinger et al., 2003; Barell et al., 2006). In *Paracoccidioides brasiliensis*, induction of this cycle in response to macrophage microenvironments was shown to coordinate with the upregulation of the gluconeogenic phosphoenolpyruvate carboxykinase encoding-gene (pck) (Derengowski et al., 2008). Induction of the glyoxylate cycle upon phagocytosis has been described as an important pathogen adaptation to the glucose-poor environment within macrophages since it facilitates the assimilation of C2 compounds (Lorenz et al., 2004). This may indicate that the organism has identified simple carbon sources in this environment and is activating the pathways necessary to utilize them. An intriguing possibility is that substrates derived from the breakdown of fatty acids via β-oxidation, which results in acetyl-CoA, would require utilization of the glyoxylate cycle. If the fatty acid breakdown of host lipids or lipids from the micro-organism itself occurs within the phagolysosome, it might provide a carbon source accessible to the pathogen while it is inside the macrophage.

The macrophage phagosome is believed to be a poor source of glucose and amino acids (Fan et al., 2005; Lorenz et al., 2004). Such nutrient deprivation inside the macrophage induces a similar adaptive response from intracellular bacterial and fungal pathogens. Shortly following phagocytosis, *Listeria monocytogenes* and *Candida albicans* have a strong reduction in the expression of genes involved in glycolysis (Chatterjee et al., 2006; Lorenz et al., 2004). We show in this study that phagocytized *P. marneffei* cells also sense and respond to the glucose-depleted environment of the macrophage phagosome. Transcription of gpdA, the gene encoding a key enzyme (GAPDH) in the glycolysis pathway, was downregulated in internalized *P. marneffei* (Fig. 2). Similar results were found in *Paracoccidioides brasiliensis* (Tavares et al., 2007).

In summary, these data suggested that upon internalization by macrophages, *P. marneffei* responds to phagocytosis with the induction of an alternate carbon metabolism, and there is a dramatic downregulation of the *P. marneffei* glycolytic gene, gpdA. This alternate pathway involves induction of the glyoxylate cycle in response to nutrient deprivation inside the macrophages. More generally, discovering the role of the glyoxylate cycle in microbial virulence should refocus attention on the critical importance of basic metabolic pathways in the development of disease. If a pathogen is unable to synthesize the precursor nucleic and amino acids required for growth, it is unlikely to proliferate or persist. For this reason, elucidation of a pathogen’s nutritional requirements *in vivo*, as well as the mechanism by which microbes acquire these nutrients once inside a host, is critical to the understanding of virulence and disease. Further investigation will evaluate the transcriptional status of genes related to other central carbon metabolic pathways, including gluconeogenesis in *P. marneffei*. Additional studies are planned to assess the relationship of these metabolic changes with regard to possible alterations of the host intracellular pH as part of the concerted survival and virulence mechanisms of *P. marneffei*.

**ACKNOWLEDGEMENTS**

This work was supported by a Royal Golden Jubilee PhD research assistant fellowship from the Thailand Research Fund (grant numbers BGJ4680013 and PHD/0093/2543) and the Faculty of Medicine, Chiang Mai University. Portions of this work were also supported by the University Research Council of Youngstown State University, as well through a National Science Foundation award (DBI 0330883) to C. R. C.

**REFERENCES**

Barbosa, M. S., Cunha Passos, D. A., Felipe, M. S., Jesuíno, R. S., Pereira, M. & de Almeida Soares, C. M. (2004). The glyceraldehyde-


