I. INTRODUCTION

Penicillium marneffei, the only thermal dimorphic pathogen among several hundred species of *Penicillium*, is known to be pathogenic to animals and humans. It is being recognized as a cause of the disseminated and life-threatening infection in both immunocompetent and immunocompromised hosts, particularly in the patients with human immunodeficiency virus (HIV) infection. Almost all cases have been diagnosed in patients who lived or traveled in Vietnam, Thailand, Hong Kong, Indonesia, or Southern China, where *P. marneffei* is endemic. The disease is after cryptococcosis, pneumocystis carinii and candidiasis, the fourth most common opportunistic fungal infection in patients with AIDS in Thailand (HIV/AIDS epidemiology report, Division of Epidemiology, Ministry of Public Health, Thailand [http://epid.moph.go.th]). *P. marneffei* has emerged from relative obscurity to become a common cause of opportunistic infection in HIV-infected patients, especially within endemic area. The typical feature of this pathogenic fungus is thermal dimorphism. At 37°C on artificial medium or in human tissue, the fungus grows in a yeast-like form with the formation of fission arthroconidium cells. The fission yeast cells represent the parasitic form of *P. marneffei*. This form is seen in the intracellular infection of the macrophages. At 25^{o}C, they are present as mycelial form with a general morphology of the genus *Penicillium* with diffusing red pigment production.

Prior to AIDS, infection with *P. marneffei* was rare event. The organism was first isolated from bamboo rats, *Rhizomys sinensis*, in Vietnam (Segretain, 1959a) and subsequently from another species of bamboo rat, *Rhizomys pruinosus* in southern China (Deng *et al*., 1986). In northern Thailand, *P. marneffei* could be isolated from the lung, liver or spleen of two additional species of bamboo rat (*Rhizomys sumatrensis* and *Cannomys badius*). The bamboo rats are believed to be

the important reservoir of this fungus. At the present it is not clear whether the organism resides in soil and bamboo rats are only another natural host of infection with this fungus (Chariyalertsak *et al.*, 1996b).

P. marneffei appears to be a primary pulmonary pathogen that disseminates to other internal organs by hematogenous spread. The exact route and mechanism of infection by *P. marneffei* as well as host immune response are still poorly understood. The patients might probably inhale the conidia from environment. This respiratory portal of entry would be consistent with infection caused by other dimorphic fungi that produce conidia in the saprophytic phase of growth. Phagocytic cells are likely to be the primary line of the host defense against this fungus. *P. marneffei* conidia are able to recognize fibronectin and bind to laminin via a sialic acid specific lectin (Hamilton *et al.*, 1998; 1999). This reaction may play an important role in the attachment of conidia to bronchoalveolar epithelia before ingestion by host mononuclear phagocytes. The pulmonary alveolar macrophages and peripheral blood mononuclear cells may be replete with multiple yeast forms with the characteristic binary septate morphology and yeast cells are disseminated throughout the body, especially to the reticuloendothelial system. Consequently, liver, lymph nodes, bone marrow, and spleen are commonly involved (Powderly, 1997). The profound impairment of cell-mediated immunity that is encountered in HIV infection results in impaired immunoregulation of pulmonary alveolar macrophages. *P. marneffe*i yeast-like cells are observed to be associated with both the intracellular and extracellular environments of both macrophages and histiocytes. The intracellular yeasts are oval or spherical cells of 2 to 3 μm in diameter, which multiply by binary fission. Elongated cells of up to 13 μm long can be observed extracellularly (Deng *et al.*, 1988).

The disease caused by most of dimorphic fungal pathogens is primary respiratory in origin, and thus virulence mechanisms may include common strategies to combat pulmonary defenses. Infection results from the inhalation of reproductive propagules (conidia) which are produced by the mycelial forms and aerosolized upon environmental disturbance. The small size of conidia allows them to penetrate deeply into the alveoli of the lung. In the alveoli, conidia germinate into the parasitic yeast forms, but all forms must avoid elimination by the alveolar macrophages that

constitute up to 95% of normal airspace leukocytes (Twigg *et al*., 2004). Phagocytosis of microbes by macrophages normally results in microbe elimination through the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and the action of lysosomal enzymes following phagolysosomal fusion. Thus, survival within the lung environment depends on virulence mechanisms that counter or inactivate macrophage defenses, and diverse mechanisms likely exist for the dimorphic fungi. The ability to resist to ROS, RNS, or both is predicted to be the virulence mechanisms of pathogenic fungi, particularly in their encounter with host phagocytic cells. As for other intracellular pathogens, surviving within the phagocytes is the primary key to a successful invasion by *P. marneffei*. However, the mechanism of survival of *P. marneffei* under oxidative stress within the macrophage remains unknown. Many fungi have been shown to survive within the phagocytic environment (Nittler *et al*., 2005). The mechanism of resistance for these organisms may function by inhibiting the production of reactive oxygen metabolites or by neutralizing inhibitory host metabolites. For example, gene controlling stress factors, such as heat shock proteins, may be virulence genes in fungi that are just as important as those associated with enzymatic pathways that confer protection from reactive oxygen species (ROS). Oxidant inactivation relies upon a variety of enzymes including catalase, catalase-peroxidase and superoxide dismutases (SODs).

SODs, the first lines of antioxidant defense are metalloenzymes that catalyze the disproportionate of O_2^- to O_2 and H_2O_2, whereas catalases subsequently convert H_2O_2 to water. The primary role of SODs is to protect cells from endogenously generated superoxide anion, which is a by-product of normal aerobic respiration. SODs can be complexed with iron, manganese, and copper plus zinc. The iron and manganese SODs are genetically similar to each other, whereas the Cu, Zn SOD exhibits no significant homology with the other two enzymes (Gralla *et al*., 1992; Lynch and Kuramitsu, 2000). Eukaryotic cells generally possess a Mn SOD in the mitochondrial matrix as well as a Cu, Zn SOD located predominantly in the cytoplasm and, to a lesser extent, in peroxisomes (Chang *et al*., 1991; Keller *et al*., 1991). The peripheral location of the Cu, Zn SOD suggests that it may protect the surface of fungi against extracellular superoxide generated by host cells. Host phagocytic cells exploit the deleterious biological effects of ROS in their nonspecific

host defense against pathogens. It is not surprising, given its role, that SOD has been implicated in intracellular pathogen survival and as a virulence factor in some bacteria and fungi, such as *Mycobacterium tuberculosis* (Harth and Horwitz, 1999; Piddington *et al*., 2001), *Candida albicans* (Hwang *et al*., 2002) and *Cryptococcus neoformans* (Cox *et al*., 2003).

Catalases are antioxidant metalloenzymes that are found virtually ubiquitously within aerobic organisms; their role is to protect cells from oxidative damage arising from exposure to hydrogen peroxide (Schonbaum and Chance, 1976). At high concentration of hydrogen peroxide, the enzyme is catalytic, whereas it is peroxidatic when peroxide concentrations are low. As in other aerobes, the primary function of fungal catalase is to remove hydrogen peroxide generated as a normal by-product of cellular metabolism. As such catalase interacts closely with the functions of peroxidases and superoxide dismutases, and together these enzymes play an essential role in the intracellular oxidative detoxification process. Catalases are also known to contribute to growth regulation and development in a variety of eukaryotes, including *Saccharomyces cerevisiae* (Veenhuis *et al*., 1987). In the study of stress response, this enzyme has been shown to inhibit the killing of *Histoplasma capsulatum* by bronchoalveolar macrophages (Brummer and Stevens, 1995). *H. capsulatum* possesses three catalase genes: *CATA*, *CATB* (encoding the M antigen), and *CATP*. Of these, *CATB* and *CATP* are constitutively expressed, whereas *CATA* expression is restricted to the mycelial phase or when yeast cells are exposed to H_2O_2 (Johnson *et al*., 2003). *Paracoccidioides brasiliensis* also appears to have three catalases, one which is upregulated in the parasitic phase (Moreira *et al*., 2004). Superoxide dismutase- and catalase-encoding genes have not been characterized in *P. marneffei*. In 2005, an antigenic catalase-peroxidase protein-encoding gene (*cpeA*) was isolated by antibody screening of a cDNA library (Pongpom *et al*., 2005). Interestingly, high-level expression of this gene was induced when the temperature was shifted to 37°C, whereby the pathogenic yeast phase of *P. marneffei* is formed. Likewise, the expression of *hsp70* was generally upregulated during the yeast phase transition, with abundant upregulated during temperature increase from 37°C to 39°C. These results indicated that Hsp70 may play an important role in environmental stress response and adaptation of *P. marneffei* during yeast phase transition. It may also be a putative

virulence factor of *P. marneffei* for cell survival in the host cells (Kummasook *et al*., 2007). Nevertheless, the role of *cpeA* and *hsp70* in the pathogenesis of *P. marneffei* require further investigations.

Besides the oxidative stress response, the conversion to the yeast phase is most probably required for progressive infection. The putative virulence factors in dimorphic fungi are genes involved with phase transition. Thus, genes essential for early adaptation to elevated temperatures or conversion to, and maintenance of, the parasitic phase are likely to represent significant virulence determinants. Adaptation to macrophage or tissue environment of the host and transformation to the yeast state may entail the organism to grow and survive in hostile environment. Microorganism's sensing specific molecules (such as phenolic compounds or saccharide, or responding to environmental conditions, such as temperature, osmolarity, pH, oxygen, CO_2, or calcium) may activate genetic mechanisms, such as isocitrate lyase gene which is an important gene encoding enzyme in glyoxylate cycle (Lorenz and Fink, 2001) and calmodulin gene (*CaM*) which is an intracellular calcium signaling modulator and acts on several metabolic pathways and gene expression regulation in many eukaryotic organisms including human fungal pathogens, *C. albicans* and *H. capsulatum* (Kraus and Heitman, 2003). The putative virulence factor, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) enzyme catalyzes the oxidative phosphorylation in the glycolysis pathway. The GAPDH has long been considered to be the product of a housekeeping gene whose transcript level does not vary. However, several studies have shown that the expression of *P. brasiliensis gapdh* gene and the cognate protein were developmentally regulated in the different growth phases of *P. brasiliensis*, with a higher expression in the yeast parasitic phase, thereby reinforcing possible new functions attributed to the enzyme (Barbosa *et al*., 2006).

In order to establish a successful infection, *P. marneffei* that colonize within the dynamic substrate of a human host must have the ability to adapt to and modify gene expression in response to changes in the host environment. In recent years, several approaches have been developed to identify differential expressed genes putatively related to phase transition. The transcriptional profile of *P. marneffei* yeast cells and mycelium reveal genes that are potentially related to fungal virulence, in addition to a

comprehensive view of fungal metabolism. In 2000, Cooper and Haycocks used differential-display reverse-transcriptase-coupled PCR to find genes that were specifically up- or down-regulate during the phase transition. Many of genes whose expression differs during mold-to-arthroconidium (or mycelial-to-yeast) transition are related to energy metabolism. Two importance genes that involve in glyoxylate pathway, malate syntase and isocitrate lyase are suggested to be upregulated during yeast phase formation of *P. marneffei* (Haycocks, 2001). Recently, a study of Cánovas and Anidrianopoulos (2006) indicated that *P. marneffei acuD* gene encoding isocitrate lyase, originally identified by Cooper and Haycocks (2000) as *icl1*, the genes belonging to the glyoxylate bypass are highly expressed during the pathogenic yeast form in *P. marneffei*. These results provide evidence for a link between carbon metabolism, development and pathogenicity in this dimorphic fungal pathogen. Furthermore, 43 differential expression genes were identified by suppression subtractive hybridization combined with real-time semi-quantitative RT-PCR (Liu *et al.*, 2007). These genes were found to be homolog of genes involved in various cellular processes including cell wall synthesis, signal transduction, cell cycle, substance transport, general metabolism and stress response, etc. This suggests that a very complex series of molecular mechanisms is involved in the switching process of *P. marneffei* (Liu *et al.*, 2007). Recently, two-dimensional difference gel electrophoresis was used to investigate the proteins expressed differentially in the yeast and mycelial phases of *P. marneffei* (Xi *et al.*, 2007). The analysis resulted in the identification of two known *P. marneffei* proteins, catalase-peroxidase and isocitrate lyase, which showed a 14.38-fold and 5.32-fold increase, respectively, in the yeast phase compared with the mycelial phase. *P. marneffei* homologues of Hsp90, binding protein, Hsc70, cytochrome P-450 and others demonstrated a significant increase in the yeast phase, whereas several other proteins, including poly(A) polymerase and ATP-dependent chromatin-remodeling factor snf22 were found to be decreased (Xi *et al*., 2007).

Despite those descriptions, a lack of information exists regarding *P. marneffei* gene expression when the organism interacts with the host. Identifying differential expressed genes in a given model system is a powerful tool for the investigating how organisms changes in the response to environmental conditions. Discovering which

genes are involved in such response can lead research down a number of paths, from identifying signaling mechanisms to defining novel drug targets. 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. Within the macrophage cells, the conidial form of *P. marneffei* differentiates into the yeast from, which can proliferate and spread to other organs. It is clear, however, that the morphogenetic change is only part of the response to phagocytosis. One method for analyzing this encounter more completely is to identify the alterations in transcription as *P. marneffei* is undergoing phagocytosis. Eukaryotic gene expression may be controlled at any of several steps leading to the synthesis of a protein. However, in many cases, its regulation occurs at the transcriptional level. Therefore, the amount of mRNA detected for a specific gene under a set of growth conditions is usually an indicator of the level of production of the corresponding protein. Several techniques have been developed to enable researchers to compare patterns of gene expression between different cell types such as, differential display (Liang *et al*., 1992), array hybridization (Schena *et al*., 1996), suppression subtractive hybridization (SSH) (Diatchenko *et al*., 1999). Although these strategies is potentially very powerful, all of differentially expressed genes identified by any of the methods described above do not ensure importance because genes will need to be reconfirmed for their actual differential expression by RT-PCR or Northern blotting.

Our aim is to uncover candidate genes that might contribute to *P. marneffei* adaptation to and survival in the host during infection. The putative virulence genes involved in host infection will be isolated and characterized. The genes of interest include the stress response genes, such as Cu, Zn superoxide dismutase, catalase, catalase-peroxidase and heat shock protein 70 encoding genes. In addition, the genes responsible in cell adhesion and adaptation, such as GAPDH, calmodulin and isocitrate lyase encoding genes will be focused. The degenerate primers are designed to amplify the genes and used as the probes to screen the constructed cDNA and gDNA library. The approach is the use of semi-quantitative RT-PCR and Northern blot analysis to identify certain *P. marneffei* genes that are expressed in different phases (mycelial, conidia and yeast cells) and during the infection process. The transcriptional analysis of phagocytosis involves co-incubation of J774 mouse

macrophage cells, together with *P. marneffei* conidia in tissue culture medium at 37°C. The transcript profiles of phagocytosed cells at various time intervals (at 2, 4 and 8 h post-inoculation) are determined and compared to cells control grown in the same media at the same times in the absence of macrophages. The results will provide novel knowledge that may lead to a better understanding of the possible mechanism of *P. marneffei* infection. The identification of the expressed genes during macrophage infection thus reveals the potent virulent genes, which can be used for drug targets or vaccine epitopes.