VI. DISCUSSION

Heat shock proteins have been associated with morphological changes in pathogenic fungi and protozoa with the onset of infection (Maresca and Kobayashi, 1994). They have also been shown to act as immunodominant antigens of pathogenic organisms: these include fungi, such as *Candida albicans* (Eroles *et al.*, 1995), *H. capsulatum* (Gomez *et al.*, 1992; Allendoerfer *et al.*, 1996) and *C. neoformans* (Kakeya *et al.*, 1997); parasites, such as *Trypanosoma*, *Plasmodium*, *Schistosoma* and *Leishmania* (MacFarlane *et al.*, 1990; Polla, 1991) and bacteria (Polla *et al.*, 1995), such as *Mycobacterium* (Britton *et al*, 1986; Young *et al.*, 1988). Hsp70 is a stress protein that plays a critical role in normal cellular function and in recovery and survival after heat shock (Feder and Krebs, 1997; Mayer and Bukau, 1998). As a molecular chaperone, Hsp70 prevents protein aggregation and refolds damaged protein, thus presumably facilitating the survival of organism under stressful condition. For these reasons, the aim of this investigation was the isolation, characterization and differential expression of *hsp70* gene from *P. marneffei.* A previous isolation of this *hsp70* gene was performed using anti-*H. capsulatum* Hsp70 monoclonal antibody (Pongpom, 2004). It is not surprising that the *hsp70* could be isolated from *P. marneffei* because there has been high sequence conservation of Hsp during evolution (Linquist and Craig, 1988). This conservation was confirmed further by high similarity observed between the deduced amino acid sequences of the *hsp70* genes from several organisms.

The structure of *P. marneffei hsp70* was identified in both nucleotide and amino acid level. Sequence conservation was highest at the N-terminus and decreased towards the C-terminus similar to Hsp70 family (Kiang and Tsokos, 1998). The number of nucleotides and amino acids for an open reading frame of *P. marneffei* Hsp70 are correspond to well-known fungal Hsp70. The presence of all conserved sequence motifs of the Hsp70 family in deduced amino acids of *P. marneffei hsp70* suggests that this gene is indeed the *hsp70.* Moreover, we found the presence of some motifs that conserved for all fungal cytosolic Hsp70. We presume that these

conserved sequence motifs are likely to the putative conserved sequence motifs for fungal cytosolic Hsp70s. Some motifs (IV and V) are likely to the putative unique motifs for fungal cytosolic Hsp70s. However, the conclusion of this hypothesis desires more information of *hsp70* from undetected-*hsp70* fungi.

In the phylogenetic tree of fungal Hsp70s, *P. marneffei* Hsp70 was clustered with the other known cytosolic Hsp70s and it was closely related with *A. nidulans* Hsp70. This finding suggests that protein synthesized from the *P. marneffei hsp70* gene belongs to the cytosolic subgroup of the Hsp70 family. In addition, the high identity was found between the sequences from *P. marneffei* and filamentous fungi, in particular the Hsp70 from *A. nidulans*. These findings support a previous study of mitochondrial genome of *P. marneffei* (Woo *et al.*, 2003). Mitochondrial genome of *P. marneffei* is much more closely related to molds, especially to that of *A. nidulans*, than to yeasts. The set of protein coding genes in the *P. marneffei* mitochondrial genome is exactly the same as that in the *A. nidulans* mitochondrial genome. The gene order of the protein genes, except for a gene encoding ATP synthase subunit 9 (*atp9*), is also the same as that in the *A. nidulans* mitochondrial genome. Furthermore, the phylogenetic tree of concatenated amino acid sequences of 14 protein coding genes in the mitochondrial genome of *P. marneffei* and 24 other fungi showed the closest relatives of *P. marneffei* to *A. nidulans* and other molds, whereas the yeasts were more distantly related. For these findings, it can be predicted that the genes in *P. marneffei* genome may closely related to *A. nidulans* genes.

One of the interesting and unsuspected features observed during this work is the presence of microexon that contained only 3 nucleotides. This is a new finding of fungal microexon of 3-nt, in the *P. marneffei hsp70* gene. This finding was confirmed by the nucleotide sequence analysis of the cloned gene from genomic DNA and of the RT-PCR fragments using RNA and genomic DNA as templates. There are a few reports of microexons, especially the 3- nt exon. The complete genomic organization of the *Drosophila* troponin T (TnT) gene showed the presence of a 3-nt microexon which was conserved among Drosophilidae (Benoist *et al.*, 1998). In vertebrate, the microexons have been reported, such as a 18-nt of human *n-src* N1 exon (Modafferi and Black, 1997: 1999), a 12-nt of human agrin exon 28 (Wei *et al.*, 1997), a 24-nt of γ_2 $GABA_A$ (Zhang *et al.*, 1996), a 7-nt of fast skeletal troponin I exon 3 and a 6-nt of

chicken cardiac troponin T exon 17 (Sterner and Berget, 1993; Carlo *et al.*, 1996). In addition, the presence of microexons have also been reported in plant genes such as 19-nt of *Apx1b* exon 9, two exons in a putative DNA helicase of 8 nt and 4 nt, 3-nt exon from *RNS2* (Simpson *et al.*, 2000), a 5-nt exon in a wheat cathepsin B homolog (Cejudo *et al.*, 1992) and the 9-nt of invertase microexon in a number of plants (Simpson *et al.*, 2000).

The coding region of a *P. marneffei hsp70* gene is interrupted with different base length of two introns. Not surprisingly, filamentous fungal *hsp70* genes are usually interrupted with the different number and base length of introns. On the other hand, coding regions of several yeast *hsp70* genes are not interrupted with any introns, such as *Saccharomyces cerevisiae* (Slater and Craig, 1989a; 1989b; Boorstein and Craig, 1990a; 1990b; Jonniaux *et al.*, 1994; Normington *et al.*, 1989; Craig *et al.*, 1989), *Pichia angusta* (Titorenko *et al.*, 1996; van der Heide *et al.*, 2002), *C. albicans* (Maneu *et al.*, 1997) and *Schizosaccharomyces pombe* (Powell and Watts, 1990; Oishi *et al*, 1996; Usui *et al.*, 1997). In addition, the size distribution of intron-containing fungal *hsp70* genes was similar in *A. nidulans*, *Neurospora crassa* and *C. neoformans* introns, which had narrow ranges of intron length with a dominant peak distribution between 50 and 70 nucleotides (Kupfer *et al.*, 2004). Conversely, the size distribution of *S. cerevisiae* introns has distinct bimodal pattern, with approximately 25 % of the *S. cerevisiae* introns falling in the size range of 401 to 2,000 nucleotides (Rodriguez-Medina and Rymon, 1994; Lim and sharp, 1998; Spingola *et al.*, 1999; Kupfer *et al.*, 2004).

The Southern blot analysis of genomic DNA suggested that the genome of *P. marneffei* contains more than one copy of *hsp70* gene that encode cytosolic Hsp70s. In different fungal species, the copy numbers of *hsp70* genes are different. The copy numbers of *B. emersonii* (Stefani and Gomes, 1995) and *T. rubrum* (Rezaie *et al.*, 2000) were determined as single-copy cytosolic *hsp70* gene. *S. cerevisiae* (Boorstein *et al.*, 1994) genome contains four copies of differentially regulated cytosolic *hsp70* genes, whereas the genome of *R. nigricans* (Cernila *et al.*, 2003) contains 3 copies of cytosolic *hsp70* genes. In a study of dimorphic *P. brasiliensis* (Florez *et al.*, 2003), two *hsp70* gene homologues were found.

Northern blot analysis indicated that *hsp70* mRNA accumulated in the conidia of *P. marneffei*. This finding is similar to that found in other fungal stress genes; in *cpeA* of *P. marneffei* (Pongpom *et al.*, 2005) and *catA* of *A. nidulans* (Navarro *et al.*, 1996). The *A. nidulans catA* transcript accumulated in the conidial form as well as in response to multiple types of stress, while the *P. marneffei cpeA* transcript accumulated in conidia and during development to the yeast form. However, the observation of the *hsp70* mRNA in conidial form has not been reported in any other fungi. In addition, heat-shock proteins (Bonato *et al.*, 1987) and *hsp70* (Stefani and Gomes, 1995) mRNA were not presented in the zoospore of *B. emersonii*. From these findings, we presume that the accumulation of transcripts from stress genes, especially *hsp70* and *cpeA* could be found in conidial dormant form of *P. marneffei*, but their possible roles in fungal survival remain unknown.

The downregulation of *hsp70* transcript during the development from conidia to mycelial phase at 25°C indicates that *hsp70* is constitutive expressed during growth in natural conditions. This data correlate with other dimorphic fungi, *H. capsulatum* (Caruso *et al.*, 1987) and *P. brasiliensis* (da Silva *et al.*, 1999), that low level of *hsp70* mRNA transcripts could be observed in mycelium. However, some *hsp70* transcripts could be seen approximately at 48 h. This observation is the same as those seen in the transcript of *cpeA* (Pongpom *et al.*, 2005). The *hsp70* upregulation may occur from the adaptation of this fungus to starvation. In the fungus *B. emersonii*, the *hsp70* transcripts were upregulated at 60 and 90 h during sporulation at normal temperature (Stefani and Gomes, 1995). These results indicate that Hsp70 may response to nutritional limitation or some stages of fungi's life cycle at normal temperatures.

This study showed the differential expression of *hsp70* during the transition phase from conidia or mycelia to yeast phase. The *hsp70* transcripts were slightly increased during phase transition from conidia or mycelia to yeast cells. The slightly upregulation of *hsp70* transcripts occurred 1 h and remained high until 24 h after temperature upshifted from 25°C to 37°C. This pattern is similar to the expression of *hsp70* genes from other fungi, such as *H. capsulatum* (Caruso *et al.*, 1987), *P. brasiliensis* strain pb349 (Florez *et al.*, 2003), and *T. rubrum* (Rezaie *et al.*, 2000). However, in contrast to higher levels in the yeast phase, the absence of *hsp70* mRNA in the mycelial phase and low levels at 24 and 48 h after temperature shift to 37°C

were observed by Northern blot analysis in the *P. brasiliensis* strain pb01 (da Silva *et al.*, 1999). In addition, the expression of *P. marneffei hsp70* gene was dowregulated at the yeast phase from 48 to 96 h after yeast induced-temperature from 25°C to 37°C. This result corresponds to the expression of this gene in dimorphic *H. capsulatum* (Caruso *et al.*, 1987), whereas the *hsp70* mRNA accumulated in dimorphic *P. brasiliensis* (da Silva *et al.*, 1999; Florez *et al.*, 2003). The slightly upregulation of *P. marneffei hsp70* transcripts during an early stage in the upshifted temperature to 37°C may involve in cells survival during infection in host body at 37°C.

In an experiment imitated the normal homeostatic condition (37°C) and during simulated human fever (39°C), the *hsp70* transcripts were rapidly abundant upregulated within 30 min after the temperature upshifted from 37°C to 39°C. The similar results were found in *B. emersonii* (Stefani and Gomes, 1995) after heat shock (38°C) for 30 min, and in *H. capsulatum* temperature resistant strain G222B after heat shock (40°C) for 3 h, whereas the absence of *hsp70* mRNA in temperature sensitive Downs strain (Caruso *et al.*, 1987). High expression of *hsp70* may be important *in vivo* as it would facilitate the fungal survival in host-body during host response by fever. This result strongly suggests that Hsp70 is a putative virulence factor of *P. marneffei* for cell survival during host response by heat shock at 38-39°C.

The investigation of *hsp70* gene expression during severe heat shock condition (42°C) showed the shut off of *hsp70* mRNA synthesis. This phenomenon was also found in a dimorphic *P. brasiliensis* (da Silva *et al.*, 1999). The result showed downregulation of the expression of most of the cellular proteins after increasing the incubation temperature to 42°C. It revealed the over-limitation condition for biosynthesis of these fungi. Conversely, there was an evidence of an upregulation of *N. crassa hsp70* transcript when heat shock treatment was administered to 14-h-old mycelial cultures at 48°C (Kapoor *et al.*, 1995). This occurrence can be explained by the limitation of thermoresistant for each fungus.

It is well known that heat shock affects RNA processing in organisms such as *Drosophila* (Yost and Lindquist, 1986) and *S. cerevisiae* (Yost and Lindquist, 1991), mRNA splicing is blocked at high temperature. On the other hand, it has been shown in *H. capsulatum* that the mRNA of *hsp82* and a gene encoding cyclin-dependent protein kinase (*cdc2*) were properly spliced during a severe heat shock at 42°C

(Minchiotti *et al.*, 1991; Di Lallo *et al.*, 1994). In *P. brasiliensis*, *hsp70* mRNA was correctly spliced during severe heat shock (42°C) of the yeast form (da Silva *et al.*, 1999). However, a transient accumulation of unspliced *hsp70* mRNA of *P. brasiliensis* was observed during yeast phase transition (26°C to 36°C). This unspliced *hsp70* mRNA decreased progressively with differentiation to the yeast form, with no unspliced transcripts observed in yeast. In the present investigation, when conidia of *P. marneffei* were submitted to a temperature induced mycelial (25°C) or yeast (37°C) phase transition, RT-PCR showed some unspliced *hsp70* mRNA in both mycelial and yeast phases. Surprisingly, the DNA sequence of intron-containing fragment showed that it contained only a longer intron II (198 bp) which lies downstream from a 3-nt microexon. In addition, no fragment was corresponding to the full-length precursor *hsp70* mRNA that contained two introns of *P. marenffei hsp70* gene. These results were concomitant to one study of a gene encoding cAMP phosphodiester class II (*cgs2*) from *S. pombe* (Romfo *et al.*, 2000). A fission yeast intron that lies downstream from a 9-nt microexon of *cgs2* gene was inefficiently spliced. It is likely that inefficient splicing of the intron which lies downstream from microexon may be a characteristic of transcripts that contained microexon in several organisms including *P. marneffei*. Further, the absence of a smaller intron I (57 bp) in this study suggested that splicing may favorable occur at the smaller fragment of introns that flanks to microexon.