VI. DISCUSSION

6.1. Construction of the complementary DNA library of *Penicillium marneffei*

The main goal in the construction of the cDNA library was to find the genes that encode for antigenic proteins of *P. marneffei*. Based on this reason, the yeast phase of *P. marneffei* was used to make the cDNA library since only the yeast form is found in human and animals. From previous studies, the conidia of *P. marneffei* turned completely to the yeast form after cultivation for 24 hours at 37 °C (Cooper & Haycock, 2000). Here, the 3-day-old *P. marneffei* culture was chosen for construction of the library. In this culture, microscopically yeast phase of *P. marneffei* was observed. It was composed of 70% of fragmented hyphae and 25% of arthroconidia and 5 % of oval yeast cells.

In the steps of cDNA library construction, reverse transcriptase converted the mRNA to first strand cDNA. The quality of the mRNA used as the template influenced profoundly the yield and size distribution of the first strand product. Total RNA and mRNA preparation were analyzed by formaldehyde gel electrophoresis (Figure 11). In the lane of total RNA, two predominant bands of intact RNA (3.8 and 2.0 kb) were found. In addition, the diffused mRNA population of high molecular weight (>1 kb) that spread throughout the gel indicated an integrity of the RNA preparation. The gel electrophoresis of the poly(A)$^+$ RNA revealed an enrichment of mRNA molecules. During the cDNA library construction, the integrity of mRNA directed the synthesis of long molecules of first-strand cDNA (Figure 12).

The quality of the constructed cDNA library was assessed by different parameters (Fulle, 2003). We found that there was a high frequency of recombinant clones (98%). Most recombinant clones had DNA inserts with average size of 1.1 kb. Moreover, two house keeping protein-encoding genes, *actin* and *hsp70*, were readily found after screening the cDNA library by using the DNA hybridization and antibody screening methods. Screening of the library by DNA hybridization yield two clones encoding the actin gene. This result was similar to the study of the actin gene in

Histoplasma capsulatum (El-Rady & Shearer, 1997), which also had the similar size of gene transcript, i.e., 1,700 nucleotides in length. Putting together with the sequence alignment result (Figure 14C), the conclusion was made that the obtained actin-encoding clone from *P. marneffei* contains the full-length gene. Obtaining the full-length clone implied good quality of the cDNA library. Three clones encoding Hsp70 were identified by antibody screening of 10,000 pfu of the library. However, 25 positive clones were detected when performing DNA hybridization screening (data not shown). This result was expected since in theory, one-third of directional clones could be expressed and detected by the antibody screening method. Thus, DNA hybridization screening should yield more positive clones than the antibody screening. Even though the full-length clones encoding Hsp70 could not be achieved from this antibody screening, they could finally obtained by using the DNA hybridization approach. The success in antibody screening of the Hsp70 from the constructed library indicated that the expression worked well.

6.2. Immunological screening of lambda phage cDNA expression library and sequence analysis

The objective of this study was to identify the proteins of *Penicillium marneffei* that elicit patients' humoral immune response. In previous studies, Vanittanakom *et al.* (1997) identified a number of secreted proteins that could be useful in the development of serodiagnostic tests by using immunoblotting assay. Strenuous effort was required in the purification of some of these proteins. They could only be partially purified by using at least two methods of protein purification, liquid isoelectric focusing, preparative gel electrophoresis and Mini-Protean II preparation (Jeavons *et al.*, 1998; Poolsri, 1999). For this reason, an alternative method such as molecular cloning and antibody screening were used. These methods were successfully applied in cloning of gene encoding an antigenic protein Mp1p from *P. marneffei* (Cao *et al.*, 1998).

To select the appropriate *P. marneffei*-infected patient's immune sera for the library screening, the Western immunoblotting assay with the crude cytoplasmic *P. marneffei* antigens was performed. Five sera that possessed different patterns of reactivities were used in screening of the cDNA library. Immunoglobulin G was purified from the pooled sera and an endogenous anti-*E.coli* antibody was then removed. Omitting these procedures can cause unacceptable background in the screening process that was occurred in the previous report (Cao *et al.*, 1998). In this study, twenty-eight clones of interest were isolated from the screening of up to 100,000 plaque forming units derived from the cDNA library. These positive clones were categorized into 18 distinct groups.

DNA sequencing was carried out on the entire insert of clones of the first five groups. In the remaining groups, only 5'-end sequencing was performed in each clone. Five-prime, single pass sequencing (400-500 bases) is usually enough for the BLAST analysis. Biological information could be extracted from the DNA sequence analyses via online bioinformatic tools. Once the sequence information was collected, the task at hand was searching through the databases to locate the similar sequences that are predicted to have a similar biological function through a close evolutionary relationship. If a queried sequence could be readily aligned to a database sequence of known function, it was predicted to have the same function. In case the function of the analyzed protein could not be defined from the similarity searching, then motifs scan through PRODOM and Pfam structural databases was performed.

Sequencing result showed that one of the positive clones named P22 contained only 17 nucleotides in the insert part. The possibility that a 17-nt will serve as a gene is low. BLASTN reported this DNA segment as a lambda gap. Thus, the P22 clone was regarded as an artifact and it was discarded. The reason why this clone gave positive result by antibody screening is unknown. This positive reactivity was seen in the repeated screening. The additional residues resulting from this short insert may influence the antigenic property of the expressed protein or optimized interaction with the immunoglobulins that was used in the screening process.

With biocomputational approaches it is possible to find the nucleotides or amino acids in databases which show a high degree of similarity towards a newly discovered DNAs by comparing their sequences. Sequence analyses of seventeen antigenic protein-encoding groups showed that 10 of them encoded proteins of conceived possible functions, while 7 of them had no significant similarity to any genes or proteins of known function in the databases. The genes of known functions included the genes that encoded for catalase-peroxidase, heat shock protein 30, fructose-1,6-bisphosphatase, 60S ribosomal protein, cytochrome C oxidase, NADH-ubiquinone oxidoreductase, Mp1p-like cell-wall associated protein, glutathione peroxidase, thymine synthase, and stearic acid desaturase which were encoded from the clones of groups 1 (P1, P2, P4, P8, P16, P19, P20), 3 (P5, P23), 6 (P9), 7 (P10), 9 (P12, P25, P27), 10 (P13), 12 (P15, P18), 13 (P17), 16 (P24), and 18 (P28), respectively. The clones of unknown function were in groups 2 (P3), 4 (P6), 5 (P7), 8 (P11), 11 (P14), 14 (P21), and 17 (P26). These clones displayed novel genes with no similarity to any previously identified genes.

Most functions of the isolated clones are involved in cell metabolism. Fructose-1,6-bisphosphatase (FBPTASE) is a critical enzyme in the gluconeogenic pathway that removes one phosphate from fructose 1,6-bisphosphate to form fructose-6-phosphate (Marcus & Harrsch, 1990). The ribosomal L18A is one of the components in eukaryotic 60S ribosome. Cytochrome C oxidase and NADH-ubiquinone oxidoreductase are enzymes in the electron transport, the process of ATP generation in the cellular respiration process (Babcock & Wikström, 1992). Thymine synthase is involved in nucleic acid metabolism (Maundrell, 1990). Stearic acid desaturase is the enzyme that synthesizes monounsaturated fatty acids, the main component of cell membrane, from saturated fatty acids (stearic acid or palmitoic acid). Knockout of this gene causes mortality in *Saccharomyces cerevisiae* (Stukey, McDonough & Martin, 1990).

Two groups of the isolated antigenic protein-encoding genes encoded the enzymes that possessed the antioxidant function, the catalase-peroxidase and glutathione peroxidase. Reactive oxygen species (ROS), including superoxide, hydrogen peroxide, and hydroxyl radicals, are produced as by-products in both aerobic respiration and respiratory burst process that occur during a killing process in the phagosome of phagocytes. When cells encounter the oxidative stress, they must produce the antioxidant enzymes to detoxify the ROS. Finding these expressed proteins suggested that the various oxidative substances might attack the *P. marneffei* cells. The role of the antioxidant enzymes for example, the catalase-peroxidase, plays a role in detoxifying the ROS intracellulary needs to be proven further. Isolation of the heat shock 30 protein also supported the encountering of stress conditions during the growth of yeast phase. Based on this observation, it is postulated that the survival of *P. marneffei* at elevated temperature may depend largely on stress responses.

The clone containing *MP1*-like gene possibly encodes another cell wall protein of *P. marneffei*. Nucleotide alignment of the P15 DNA and *MP1* gene (Figure 30) showed global similarity. Alignment of the encoded polypeptide from P15 to the Mp1p-translated polypeptide also showed matching over the entire length (data not shown). The alignment results confirmed the isolation of a gene that is closely related to the *MP1* gene. The antigenic property of Mp1p was reported and used successfully in a serodiagnostic ELISA assay (Cao *et al*, 1998a, 1998b, and 1999). However, the polyclonal antisera produced from this recombinant protein could not strongly react to the crude antigen of *P. marneffei* in Thai isolate (tentative result, data not shown). One possible explanation is that the antigenic wall-associated protein might be different in different geographical strains of *P. marneffei*.

Even though the current approaches in biocomputing are very helpful in identifying patterns and functions of several proteins and genes, they are still far from being perfect. The resulting data may lead to false interpretations and assumptions. It is therefore still mandatory to use biological reasoning and common sense in evaluating the results delivered by a biocomputing program.

6.3. Characteization of genes encoding catalase-peroxidase, expression pattern, and its overexpression

As a facultative intracellular pathogen, *P. marneffei* usually survives and replicates in the yeast form inside phaogosome of the macrophages (Chan & Chow, 1990; Cooper & McGinnis, 1997). Several studies demonstrated the defense mechanism against *P. marneffei* infection in the macrophages, including the role of nitric oxide and reactive oxygen species (Cogliati *et al.*, 1997; Kudeken, Kawakami & Saito, 1998; Kudeken *et al.*, 1999b; Roilides *et al.*, 2003). In contrast, only two mechanisms that contribute the intracellular survival of *P. marneffei* have been investigated (Rongrungruang & Levitz, 1999; Taramelli *et al.*, 2000). In phagosome of the macrophages, *P. marneffei* cells must struggle to survive in the harsh environment. With respect to the function of the catalase-peroxidase enzyme, it is possible that *P. marneffei* may use this enzyme as a virulence factor to detoxify the hydrogen peroxide during infection. This study described the characterization of the *cpeA* of *P. marneffei* and its differential expression. The obtained data provided preliminary insight into the gene that may play a role in pathogenesis of *P. marneffei*. In addition, its expression in form of fusion protein in *E. coli* was immunoblot assay with patients' sera. The result suggested the possibility of using this protein as a diagnostic marker.

The deduced amino acid sequence of *cpeA* indicated that its product, CpeA, contains conserved residues on both distal and proximal sides of heme at the active site pocket (figure 37). Expression of *cpeA* appears to be regulated at the transcriptional level (Figure 39). Northern blot analysis indicates that the 2.5-kb *cpeA* mRNA transcript is differentially expressed. The *cpeA* expression was nearly absent in the mold phase (25 °C incubation), although a low level of transcript could be detected at 48 h, but not prior to or after this time point. Presumably, some toxic reactive oxygen by-products may have accumulated at this time point from an aerobic cellular metabolism. Such a stress condition might induce the expression of *cpeA*. In contrast, after 12 h and 48 h of incubation time at 37°C, low transcription levels of this gene were observed, whereas no transcript was detected at the 24 h incubation time. At this time, the transcription may be too low to be detected by the

Northern blot assay used in this experiment since *cpeA* mRNA could be demonstrated using the RT-PCR assay (Figure 39B). This very low level of expression might be indicative of an adaptation stage that is required for phase transition from the mold to yeast form, thereby resulting in a transient decrease in any mRNA transcripts (Herruer *et al.*, 1988). In laboratory condition, initial development of either the mold or yeast phase of *P. marneffei* occurs within 18-24 h of incubation of conidia at 25°C or 37°C. Thereafter, cultures committed to growth in the yeast phase could again produce the transcripts for survival and replication, since by this point, if this were an actual infection, *P. marneffei* conidia should have been phagocytosed and exposed to the ROS response of macrophages. Consistent with this explanation is the high level of *cpeA* expression after 48 h and 72 h of incubation at 37°C. By the latter time point, yeast cell formation should be nearly completed.

Microbial pathogens have evolved sophisticated and efficient enzyme systems to avoid oxidative damage from ROS. The catalase-peroxidase is one of the proteins that function in detoxification of hydrogen peroxide, so it could serve as a virulence factor. Among the fungi, catalase-peroxidase encoding genes have been reported in *Penicillium simplicissimum*, *Neurospora crassa, Aspergillus nidulans,* and *Aspergillus fumigatus*. However, only the *A. fumigatus* Cat2p has been shown to be a putative virulence factor by involving in the H_2O_2 degradation *in vitro* and it could transiently protect the fungus against the oxidative burst in the rat model (Paris *et al.*, 2002). This study showed the increased synthesis of *cpeA* transcript in the yeast phase, which is the pathogenic form of *P. marneffei*. Since the yeast phase of *P. marneffei* is often found within macrophages in infected individuals, therefore this enzyme may facilitate the intracellular survival of the fungus by providing a non-toxic environment in the macrophage phagosome. However, the presence of mRNA does not mean that protein expression does occur. Preliminary evidence, though, from enzymatic assays suggests that yeast phase cultures of *P. marneffei* exhibit readily detectable levels of catalase activity, but virtually no activity was observed in the mold phase cultures (Husk T. & Kim T., unpublished data). Collectively, these results support the speculation that the catalase-peroxidase may represent a potential virulence factor of *P. marneffei.*

The result of Western immunoblot assay showed the antigenic property of catalase-peroxidase in this fungus. Its strong antigenicity may be useful serving as a diagnostic marker for penicilliosis caused by *P. marneffei*. Previous attempts to purify sufficient amounts of protein from *P. marneffei* for detailed studies as well as development of diagnostic testing were intensive labor. Isolation of clones containing the *cpeA* facilitates its overexpression *in vitro*, thereby making it readily available for further characterization or evaluation as a diagnostic marker. The recombinant protein made from the *cpeA* gene was tested with patients' sera. This study, 53% of *P. marneffei* infected sera gave positive result. However, a related study with the other set of AIDS-sera (n = 10) found up to 70 % of sera that were positive by immunoblotting assay to the CpeA fusion protein (Praparattanapan, unpublished data). It is not possible to detect antibody in infected sera to 100% sensitivity because of the inferior immune status of AIDS patients. This result is supported by the previous immunoblot assay using 61-, 54- and 50-kDa purified antigen (Jeavon *et al.*, 1997). They found that the proteins were recognized by 86, 71, and 48 % of 21 serum samples, respectively. Therefore using the combination of several antigens could probably help to increase the detection of specific antibodies among AIDS patients. Inversely, using specific antibody to detect *P. marneffei* antigen in AIDS patient's sera or other clinical specimens seems to have more advantage. The proteins produced from the recombinant DNA technology can be used to immunize animals for polyclonal antibody production. Alternatively, monoclonal antibodies can be made. With the combination of antigen and antibody detection assays, the specificity and sensitivity of a diagnostic test may reach 100 %.

No cross-reactivities were detected when tested the CpeA-fusion protein with sera obtained from patients with other mycoses and *M. tuberculosis*-infected AIDS sera. These results suggested that this protein is a potential diagnosis marker by showing no cross-reactivities to other infected-sera and specific to *P. marneffei*-infected sera. Nevertheless, testing with more sera in the future will ensure the specificity of this protein to *P. marneffei* infection.

Further studies will involve the production of specific antibodies to the CpeA protein. These antibodies can be used to determine or isolate of the native protein produced by *P. marneffei*. The development of an enzyme-linked immunosorbent assay will be focused for the diagnostic purpose. Furthermore, the role of *cpeA* in the resistance of *P. marneffei* to macrophage antimicrobial activity and the pathogenesis of this fungus require further investigations, particularly the up-regulation of its expression during the formation of pathogenic yeast phase.

6.4. Future perspectives

Collectively, the results obtained from present study were novel. Availability of the antigenic protein-encoding clones will facilitate and lead to extended studies in the future. Information obtained from the study of antigenic proteins and their encoding genes may improve the basic knowledge involving the virulence factors, immunology, diagnosis, and vaccine development of this fungus. First and the most important, single or combined specific antigens could be used as diagnostic markers in the serological test. Such a test would be of particular value to those infected individuals who are asymptomatic or in the early stages of disease. In addition, a serologic test would be useful in an epidemiological study to determine if people living in the endemic area have been previously exposed to this fungus. The functions of some antigens identified in this present study are likely to be unknown. Further characterization of these new proteins may help in understanding of the pathogenesis of this fungus. Moreover, some antigens might serve as virulence factors. Others may play a role in the host's immunological response to *P. marneffei*. Studies focused on such proteins should help understanding basic host-fungus relationship. Finally, and perhaps most significant, the antigenic proteins could serve as a vaccine candidate or drug target in the prevention and treatment of penicilliosis due to *P. marneffei*.