VII. SUMMARY

7.1. A lambda-based expression cDNA library was constructed from the 3-day-old yeast phase of *P. marneffei*. The primary library titer was $2.5x10^5$ pfu/ml with up to 98 % of recombinant clones, and the average insert size of 1.1 ± 0.4-kb. After amplification, the library titer was increased to $5x10^9$ pfu/ml. The percentage of recombinant clones and the average insert size were not changed, indicating no preferential amplification of the smaller clones in the amplification process.

7.2. Quality control of the constructed library was assessed as follows:

A) Before the cDNA synthesis, the integrity of mRNA template was revealed by the formaldehyde gel analysis. Two rRNA bands and the large molecules of mRNA species appeared intact.

B) During the cDNA synthesis, the first strand conversion yielded the high molecular mass of cDNA molecules examined by alkaline gel electrophoresis. Size distribution of the products layed between 0.5- to 9-kb consistent with the size distribution of the mRNA examined by formaldehyde gel electrophoresis.

C) After synthesis, size of the primary library was large enough to represent the genes that are expressed in typical eukaryotic cells. The cloning efficiency was high up to 98%, and the average insert size was higher than 1 kb. In addition, 2 house keeping genes, *actin* and *hsp70*, were readily identified from the library by DNA hybridization and antibody screening method, respectively.

Taken together, quality of the constructed cDNA library was proved to be good enough for further genetic studies of *P. marneffei*.

7.3. From screening of a yeast-phase expression cDNA library of *P. marneffei* with a pooled *P. marneffei*-infected sera (n = 5), twenty-eight positive clones were isolated. Clone P22 was discarded as an artifact since it contained only 17-nucleotide long of an insertion part. Thus, only 27 clones were regarded to be the antigenic protein-encoding clones obtained from this study.

7.4. The 27 positive clones were categorized into 17 distinct groups (P22 of group 15 was excluded) according to the data obtained by dot blot hybridization.

7.5. The longest clone of each group was subjected to the DNA sequencing and analyzes. The clones of first 5 groups were whole-clone sequenced, and the clones of the remaining groups were five-prime, single pass sequenced. Using the web-based analysis programs performed analysis of the obtained DNA sequences. The most important analysis that was similarity search to determine the possible gene function. The proteins of known function included the catalase-peroxidase, heat shock protein 30, fructose-1,6-bisphosphatase, 60S ribosomal protein, cytochrome C oxidase, NADH-ubiquinone oxidoreductase, Mp1p-like protein, glutathione peroxidase, thymine synthase, and stearic acid desaturase which were encoded from the clones of group 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 group 2 (P3), 4 (P6), 5 (P7), 8 (P11), 11 (P14), 14 (P21), and 17 (P26).

7.6. The clones in group 1 encoded putative catalase-peroxidase. It was designated *cpeA*. DNA sequence analysis of the *cpeA* revealed an open reading frame encoding a 748 amino acid-polypeptide with a predicted molecular mass of 82.4 kDa. The *cpeA* sequence had 80, 81 and 83 % similarity to those of reported from *Aspergillus nidulans*, *Aspergillus fumigatus*, and *Neurospora crassa*, respectively. The deduced amino acid sequence was 45-69 % identical to that of catalase-peroxidases from some bacteria and fungi, such as

Neurospora crassa, *Streptomyces reticuli*, *Geobacillus stearothermophilus*, *Mycobacterium tuberculosis*, *etc.*

7.7. The *cpeA* gene had a single copy in the genome as determined by Southern blot analysis. It displayed a high level of expression, when the temperature was shifted to 37 °C or in the pathogenic yeast form of *P. marneffei*. The transcript was also detected at only 48 h of incubation at 25 °C as illustrated by Northern blot analysis. RT-PCR result confirmed these findings. Differential expression of this gene *in vitro* may resemble to that occurs *in vivo*.

7.8. The *cpeA* gene fragment, which contained nucleotide-encoding amino acid 461-748 at C-terminal of the encoded protein, was subcloned into the pRSET expression vector. The CpeA-His_6 fusion protein was synthesized by driving an expression in *E. coli* via a strong T7 promoter. The induction condition was 2 h at 37 °C in the presence of 0.05 M IPTG. The fusion protein had a molecular weight of 34 kDa. It was expressed in large amount and aggregated in inclusion bodies. Purification of the fusion protein was performed via metal binding affinity chromatography under denaturing condition. Total amount of the isolated fusion protein was 3.67 mg from a 50-ml *E. coli* culture.

7.9. The fusion protein was used in immunoblot assay to test its immunoreactivity. The protein was recognized by 8 of 15 (53.3%) serum samples from *P. marneffei*-infected AIDS patients. The serum obtained from non-AIDS patient without *P. marneffei* infection and with several fungal infections gave negative result. *Mycobacterium tuberculosis*-infected AIDS patients' sera also failed to react with the fusion protein. Therefore, the CpeA may be a potential diagnostic marker for penicilliosis marneffei.