IV. MATERIALS AND METHODS

4.1. Fungal strains and culture condition

Penicillium marneffei CBS 119456 is a clinical isolate derived from penicilliosis marneffei patient of Maharaj Nakorn Chiang Mai Hospital, Thailand. The fungus was purified and identified as *P. marneffei* by its characteristic morphology and dimorphic property. The culture was maintained in a mold phase at 28^{o}C on Sabauraud's dextrose agar (SDA; Becton Dickinson and Company, MD, USA). *Penicillium citrinum* was isolated from environment and identified by sequencing of 18S ribosomal DNA and its morphology. The conidia of both fungi were collected by washing and scraping the surface of colonies, which were grown on malt extract agar (MEA; Oxoid, Hampshire, UK) slants, with a small volume of phosphate buffer saline (PBS), followed by filtering through sterile glass wool. The filtered conidial suspension was counted with the heamocytometer; the conidia were used immediately for macrophage infection or for RNA extraction. To investigate saprophytic and parasitic phase transition, conidial suspensions of *P. marneffei* were inoculated into Brain Heart Infusion (BHI, Oxoid) broth and incubated for 96 h at 25^{o}C or 37^{o}C. All cultures were maintained in a shaking incubator (Jeio Tech, Kyunggi-Do, South Korea) or shaker bath (Precision Scientific 360) with continuous operation at 135 rpm. At the indicated time, the cells were harvested by centrifugation at 12,000 rpm for 5 min.

4.2. Macrophage culture

J774.1 (ATCC TIB-67) is a mouse macrophage cell line derived from a reticulum sarcoma. Cells were maintained at 37^{o}C in 10% CO_2 in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Invitrogen Corporation, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO) and 100 μg/ml penicillin-streptomycin (GIBCO). The macrophage cells were cultured in a 75 mm^2

canted neck culture flask (Corning Inc., MA, USA) to near confluence. The medium was then removed and replaced with 10 ml of new culture medium. Two or three times weekly cells were dislodged from the flask substrate with a cell scrapper (Corning Inc.), aspirated and dispensed into new flasks with a subcultivation ratio of 1:3 to 1:6. The cell line was used between passages 4 and 15. The macrophage cells were tested for viability with 0.4% trypan blue (GIBCO) staining (Tolnai, 1975). The phagocytosis activity of prepared macrophages was tested with *Candida albicans* yeast cells (ATCC 90028) as an activity control before used (Sasada and Johnston, 1980).

4.3. Phagocytosis and killing assay

For the phagocytosis and killing assay, macrophages were cultured in 24-well culture plates (Corning Inc.) under conditions described above for 3 days. Each phagocytosis assay comprised 2 samples, each spiked with an identical number of conidia, and either cell-free medium for the control or adherent macrophage cells for the test (Martin and Bhakdi, 1991). The phagocytosis assay was initiated by the addition of 5-6 x 10^5 conidia suspension in DMEM medium to 5-6 x 10^4 macrophage cells in each well (MOI=10). The samples were then incubated at 37^{o}C with 5% CO_2.

To study phagocytosis, after incubation for different time points (0, 30, 60, 120 and 240 min), the macrophage cells were washed with warm DMEM medium to remove an excess of conidia and then fixed by adding 2% (wt/wt) paraformaldehyde (PFD; Sigma-Aldrich Gmbh, Steinheim, Germany) solution in PBS to the final concentration of 0.6%. The adherent macrophage cells were scraped and ten microliters of each sample was withdrawn for determining the percentage of phagocytosis (PP) which is the total number of macrophage cells from 100 cell count that can internalize fungal conidia. Moreover, phagocytic index (PI) was determined by counting the intracellular conidia and calculating for the average number of conidia per macrophage cell as follow:

$$\text{Phagocytic index (PI)} = \frac{\text{Total no. of intracellular conidia}}{\text{no. of phagocyte cells}}$$

To quantify killing activity, each well of the test and control was combined with 0.5% sodium lauryl sulfate (SDS; Sigma) for 3 min at room temperature. The SDS solubilized macrophage cells and the remaining particles were counted as by viable and nonviable conidia. Ten microliters of detergent-treated samples were diluted 200-fold in PBS, and 20 μl was then plated in duplicate for colony forming unit (CFU) determination on SDA plates after 3-5 days incubation at 25-27^{o}C. The percentage of killing in CFU was calculated as follow:

$$\text{Percentage of killing (PK)} = 100 \times \left(1 - \frac{\text{CFU test}}{\text{CFU control}} \right)$$

4.4. Genomic DNA extraction

Fungal genomic DNA was isolated from *P. marneffei*, *Histoplasma capsulatum*, *Aspergillus fumigatus* mycelia and *Saccharomyces cerevisiae* yeast cells by slight modification of a previously described method (Vanittanakom *et al*., 1996). Briefly, fungal cells from the surface of the agar medium were suspended in 0.5 ml of lysis buffer (1.5% sodium dodecyl sulfate, 0.25 M Tris [pH 8.0]), and boiled for 30 min. The suspension was vortexed for 2 min. An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1 ratio) was added, and the suspension was vortexed for 10 min to extract the DNA. After centrifugation, the upper phase was removed and DNA precipitation was carried out with cold acetone. The DNA pellet was air dried and resuspended in 50 μl of water.

4.5. Total RNA extraction

Total RNA was isolated from *P. marneffei* mycelia and *S. cerevisiae* yeast cells by mechanical disruption method using the RNeasy mini kit (Qiagen, GmbH, Germany). Approximately 600 μl acid-washed glass beads were pipetted to 1.5 ml screw-cap microtube. The cell pellet was resuspended in 600 μl of Buffer RLT and mixed by vortexing. This sample was added to the glass beads prepared in the screw-cap microtubes. Then, the samples were agitated using a Bead Beater (Biospec, Bartlesville, OK, USA) for 5 times at top speed for 30 s with cooling until cells were completely disrupted. The lysate was centrifuged at maximum speed for 2 min, and

the supernatant was transferred to a new microcentrifuge tube. Seventy percent ethanol (350 µl) was added to the lysate and mixed by pipetting to adjust the binding conditions. The samples were applied to RNeasy mini column placed in a 2 ml collection tube, and centrifuged at 10,000 rpm for 15 s for adsorption of RNA to membrane. Buffer RW1 (350 µl) was added to RNeasy mini column, and the column was centrifuged at 10,000 rpm for 15 s. The mixture of DNase I (10 µl) and Buffer RDD (70 µl) was added to the column to remove any traces of genomic DNA. After 15 minutes, 350 µl of buffer RW1 was added to RNeasy mini column, and the column was centrifuged at 10,000 rpm for 15 s. The column was washed twice by using 500 µl of RPE buffer, and centrifuged at 10,000 rpm for 15 s and 2 min, respectively. The column was dried by centrifugation at full speed for 1 min. RNase-free water (30 µl for each sample) was added to the column and total RNA was eluted by centrifugation at full speed for 1 min. The RNA concentration and purity were determined by measuring the absorption at 260 and 280 nm (Eppendorf, Hamburg, Germany). The quality of RNA was determined by electrophoresis on denaturing 1% agarose gel using the NorthernMax-Gly system (Ambion, Austin, TX, USA). The suspension was then stored at -80^{o}C until used.

4.6. Isolation of homologous probes for fungal genes of interest

4.6.1) Primer design

Degenerate primers for Cu, Zn superoxide dismutase gene. The first step in identifying primers for a set of homologous genes is to compute a sequence alignment of the protein sequences. For the superoxide dismutase gene (*SOD*), the primers were designed on the basis of identity/similarity to the superoxide dismutase protein sequences from selected other fungi, considering the highly conserved region. Six protein sequences of superoxide dismutase were used for primer design (*Aspergillus nidulans*; EAA66114, *Aspergillus flavus*; AAM94904, *Aspergillus fumigatus*; AAL38991, *Saccharomyces cerevisiae*; NP012638, *Paecilomyces sinensis*; AAR15471 and *Cryptococcus neoformans* var. *neoformans*; AF248046). The two conserved regions were identified in the alignment such that the target region to be amplified is flanked by them. Primers need to satisfy several requirements: a feasible

annealing temperature, an appropriate range for its GC-content, and reasonably sticky ends to avoid degeneracy at the end of the primers (Table 1 and Appendix C).

Degenerate primers for glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*). The primers were designed from two conserved regions of the alignment of 5 fungal *GAPDH* nucleotide sequences (*Ajellomyces capsulatus*; AF273704, *Aspergillus oryzae*; AB032274, *Aspergillus fumigatus*; XM743052, *Coccidioides immitis*; AY536450 and *Paracoccidioides brasiliensis*; AF396657) (Table 1 and Appendix C).

Degenerate primers for *P. marneffei* calmodulin gene *(cam)*. To amplify a DNA fragment of the calmodulin gene, the degenerate primers were designed from the calmodulin gene of *P. brasiliensis* (Cavalho *et al*., 2003) (Table 1).

Primers for *P. marneffei* catalase gene *(cat)*. The primers were designed from the nucleotide sequences of *P. marneffei* sequence tagged site (STS) AL683912 with the expected product size of 550 bp (Table 1).

4.6.2) PCR amplification

To amplify DNA fragments of potential virulence genes, *P. marneffei* genomic DNA (100 ng) was subjected to the following reaction: 10 μM of primers CAT-F and CAT-R or 100 μM for the degenerate primers (SOD-F/SOD-R, CAM-F/CAM-R or GAP-F/GAP-R); 250 μM dNTP; 1X PCR buffer; 3 mM $MgCl_2$ and 2U *Taq* DNA polymerase (Invitrogen) in the final volume of 25 μl. The PCR condition was: (1) 95^{o}C for 5 min; (2) 95^{o}C for 30 s; (3) appropriate annealing temperature for each pair of primers for 30 s (Table 1); (4) 72^{o}C for 1 min; (5) 30 times from second to fourth step; (6) 72^{o}C for 10 min. Total RNA from *S. cerevisiae* or genomic DNA (100 ng) from *H. capsulatum* and *A. fumigatus* were used as templates for control reaction of SOD, CAM and GAPDH degenerate primer amplification, respectively (Table 1). The annealing temperature gradients were necessary for finding out appropriate PCR conditions using GeneAmp® PCR system 2700 (Applied Biosystems, Foster city, CA, USA) and Mastercycler® gradient (Eppendorf). The products were analyzed by agarose gel electrophoresis and purified by using NucleoSpin® PCR purification kit (Macherey-Nagel, Easton, PA, USA) for further analysis.

4.6.3) Cloning and sequencing

The PCR products were cloned into pCR®2.1-TOPO® (Invitrogen) and used to transform TOP10 *Escherichia coli* competent cells. The recombinant plasmids were checked by *Eco*RI restriction enzyme digestion. A released DNA fragment of expected size was selected for DNA sequencing. DNA sequencing was performed by dideoxynucleotide chain termination method (Sanger *et al*., 1977). The procedure for extension products was performed according to manufacturer's protocol of BigDye™ Terminator Version 3.1 (Perkin-Elmer, Applied Biosystems). The conditions for amplifying extension products were as follows: 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 4 min. The extension products were purified by adding 2 µl of 150 mM EDTA, and then transferred to microcentrifuge tube containing 50 µl of ice-cold absolute ethanol with 2 µl of 3M sodium acetate. The suspensions were mixed by vortexing and placed on ice for 10 min. The extension products were pelleted by centrifugation at 13,000 rpm for 30 min, and washed twice with 70 % ethanol, then air-dried for 20 min. The pellet was resuspended in Template Suppression Reagent (TSR) in 25 µl and then vortexed and spun. The suspension of purified-extension products were heated at 95°C for 2 min to denature. The sequencing products were analyzed on the automated sequencer (Genetic analyzer Model 310, Beckman). The PCR fragments of uncharacterized *P. marneffei* genes were used as probe in cDNA and gDNA library screening.

4.7. Isolation of potential virulence genes from *P. marneffei*

4.7.1) Screening for full-length transcript genes from cDNA library

A cDNA library was constructed by Pongpom *et al.* (2004). The system for construction of cDNA library was SuperScript™ Lambda by using ZipLox vector (GIBCO). The procedure of plaques hybridization was carried out following to the ECL Direct Nucleic Acid Labeling and Detection Kit (GE Healthcare UK Limited, Amersham Place, Buckinghamshire, England)

Titering of the cDNA library. Each 100 µl of tenfold serial dilutions (10^{-1} to 10^{-8}) of the cDNA library were used to infect the equal volume of *E. coli* Y1090 host (OD600 = 4) for 15 min at 37°C. The infected bacteria were mixed with 3 ml of 0.6%

LB top agar and plated on the LB agar in a 100-mm plate. The plates were incubated overnight at 37°C. The titer of cDNA library was calculated by the number of plaques at the appropriate dilution (30 to 300 plaques/plate) as follows;

$$\text{Titer of the cDNA library (pfu/ml)} = \frac{\text{Number of plaques x dilution factor x } 10^3}{\text{100 µl (infection volume)}}$$

Preparation of labeled probe. A probe was prepared from PCR product purified from agarose gel with DNA gel extraction kit (Qiagen). Before use, the fragment was labelled with the enzyme horseradish peroxidase, then utilized in accord with the manufacturer's protocol (ECL Direct Nucleic Acid Labelling and Detection Kit, GE Healthcare).

Plaque hybridization. The plaque lifted membrane containing DNA from the cDNA library was prepared as follows (adapted from cDNA library protocols, Ian and Carolin, 1997). Approximately 10,000 pfu of phage library were mixed with 0.6 ml of *E. coli* Y1090 (OD_{600} of 4) and incubated for 15 min at 37°C. Infected bacteria were added with 8 ml of molten top agarose (0.6% agarose). The mixture was poured onto a 150-mm LB agar plate and allowed to solidify at room temperature. The plate was incubated 8 h at 37°C to generate plaques. A 132-mm Hybond-N^+ membrane (GE Healthcare) was laid down on the surface of the agar for 2 min. The orientation of the nylon membrane was marked by piercing 3 locations asymmetrically with a 21-guage needle, and the membrane was carefully lifted from the plate. The membrane bound with library DNA was denatured by laying it on filter papers saturated with 0.5 M NaOH for 5 min and neutralized with 2X SSC. DNA was fixed to the membrane by UV crosslinker (GS Gene Linker™ UV Chamber, BIO-RAD, Hercules, CA, USA) at C-L mode. Hybridization, washing and detection steps were done according to the manufacturer's protocol (Direct Nucleic Acid Labelling® and Detection kit, GE Healthcare). Briefly, the membrane was prehybridized and hybridized at 42°C for 1 h and overnight, respectively. The membrane was washed twice under high stringency conditions by using a primary washing buffer with urea at 42°C for 20 min, and

washed twice with 2X SSC at room temperature for 5 min. The membrane was exposed for 1 h to Hyperfilm (ECL, GE Healthcare). Positive signals on the Hyperfilm were aligned with the agar plate and plaques corresponding to the positive signals were picked and suspended in 250 µl SM buffer containing 0.3% chloroform. Phage clones from primary screening were screened for the largest insert size by PCR using T7 and specific primers for each gene.

Screening for the largest clone by PCR using T7 and gene specific primers. Phage clones were subjected to the PCR by using sense primer T7, a binding site located in the λZiplox vector, and the antisense specific primer. PCR was performed in a 25 µl reaction volume by using a PCR master mix reagent (Qiagen). The PCR parameter was 94^{o}C for 5 min, followed with 35 cycles of 94^{o}C for 30 s, 55^{o}C for 15 s, 72^{o}C for 2 min and 30 s, and final extension at 72^{o}C for 10 min. PCR products were determined by agarose gel electrophoresis, and stained with ethidium bromide. The size of amplicon was determined by comparing it to the size of 100 bp or DNA ladder and λ*Hind*III DNA marker (New England Biolabs). The longest clone was subjected to repeated screening to obtain the purified positive clones. The insert size of the purified clone was determined by PCR using a pair of primers, T7 and SP6, which their binding sites were located in the λZiplox vector.

***In vivo* excision of the phage clone.** A phage clone containing full-length transcript of cDNA clone was excised from the λZiplox vector. The *in vivo* excision process was performed by using *E. coli* DH10B according to the manufacturer's protocol (Invitrogen). The resulting plasmid, pZL1, contained the inserted cDNA.

Isolation and purification of plasmid DNA. The method was adapted from the manual of Sambrook and Russell, 2001 (Appendix E). A single colony of *E. coli* DH10B containing plasmid pZL1 was cultured overnight in LB medium containing ampicillin 100 µg/ml at 37^{o}C, and then harvested by centrifugation at 3,000 rpm for 2 min. The cell pellet was resuspended by vigorous vortexing in 100 µl of ice-cold Solution I containing 50 µg/ml of RNaseA (Sigma). A freshly prepared lysis buffer (Solution II, 200 µl) was added, and then mixed by inverting the tube five times rapidly. The suspension was placed on ice, and an ice-cold Solution III (150 µl) was added. The suspension was gently inverted 10 times, and stored on ice for 5 min.

Plasmid DNA solution was separated by centrifugation at 12,000 rpm for 10 min. The supernatant was transferred to a new tube. An equal volume of phenol: chloroform was added, and then mixed by vortexing. After centrifuging at 12,000 rpm for 10 min at 4°C, the supernatant was transferred to a new tube. Plasmid DNA was precipitated by two volumes of absolute ethanol at -20°C for at least 20 min, and then centrifuged at 12,000 rpm for 30 min at 4°C. The plasmid DNA pellet was washed twice with 70 % ethanol, and air dried. Plasmid DNA was resuspended in distilled water. The concentration and quality of plasmid DNA were determined by measuring the absorbance at 260 with a spectrophotometer (Eppendorf) and by gel electrophoresis. The size of cDNA clone was checked by using *Not*I and *Sal*I enzymes to excise the insert from plasmid pZL1.

Cloning of genes. The complete nucleotide sequence encoding a given gene was obtained by PCR amplification of genomic DNA of *P. marneffei*. Primers were designed based on the cDNA sequence. The PCR was performed with 100 ng of genomic DNA of *P. marneffei*, and the amplification conditions were started at 95°C for 5 min; followed with 35 cycles at 95°C for 30 s, annealing at appropriate temperature for each pair of primers for 30 s, and extension at 72°C for 5 min; and a final extension at 72°C for 10 min. The amplification was performed with automatic Thermocycler (GeneAmp PCR System 2700, Applied Biosystems, Singapore). An amplified PCR product was gel-purified by using DNA gel extraction kit (Qiagen), subcloned into pCR®2.1-TOPO® vector (Invitrogen) and sequenced by the dideoxynucleotide chain termination method (Sanger *et al*., 1977).

4.7.2) Screening of genes from gDNA library

A gDNA library was constructed by Yuen *et al.* (2003). The system for construction of gDNA library was pBK-CMV vector by using ZAP Express® vector (Stratagene, CA, USA). The procedure of plaques hybridization was carried out following to the ECL Direct Nucleic Acid Labeling and Detection Kit (GE Healthcare).

Titering of the gDNA library. Each 100 µl of tenfold serial dilution (10^{-1} to 10^{-8}) of the gDNA library were used to infect an equal volume of *E. coli* XL1-Blue MRF′ host (OD_{600} of 0.5) for 15 min at 37°C. The infected bacteria were mixed with

3 ml of 0.6% NZCYM (GIBCO) top agar and plated on the NZCYM agar in a 100-mm plate. The plates were incubated overnight at 37°C. The plaques were counted and the concentration of the library (pfu/ml) was determined based on the dilutions.

Preparation of labeled probe. A probe was prepared by using PCR amplified products. The gene fragments were purified by using DNA gel extraction kit (Qiagen). Before use, the gene fragment was labelled with the enzyme horseradish peroxidase, and utilized an accord with manufacturer's protocol (ECL Direct Nucleic Acid Labelling and Detection Kit, GE Healthcare).

Plaque hybridization. The plaque lifted membrane containing DNA from the gDNA library was prepared as follows. Approximately 10,000 pfu of phage library were mixed with 600 µl of freshly prepared XL1-Blue MRF´ at an OD_{600} of 0.5 and incubated for 15 min at 37°C to allow the phage to attach to the cells. 8 ml of molten NZCYM top agarose (~48°C) were added to the bacteria and phage mixture. Then, the mixture was quickly poured onto a dry, prewarmed 150-mm NZCYM agar plate, which was at least 2 days old. The plates were left at room temperature for 10 minutes. Then, they were inverted and incubated at 37°C for ~8 h. After incubation, the plates were chilled for 2 hours at 4°C and a nitrocellulose membrane was placed onto each plate for 2 minutes to allow the transfer of the phage particles to the membrane. A needle was used to prick through the membrane and agar for orientation. The nitrocellulose-bound DNA was denatured after lifting by submerging the membrane in a 1.5 M NaCl and 0.5 M NaOH denaturation solution for 2 min. The nitrocellulose membrane was neutralized for 5 min by submerging the membrane in a 1.5 M NaCl and 0.5 M Tris-HCl (pH 8.0) neutralization solution. The nitrocellulose membrane was rinsed for 30 s by submerging the membrane in a 0.2 M Tris-HCl (pH 7.5) and 2X SSC buffer solution and blotted briefly on a Whatman® 3MM paper. The DNA was cross-linked to membrane using the UV cross linker for 30 s. Hybridization, washing and detection steps were done according to the manufacturer's protocol (Direct Nucleic Acid Labelling® and Detection kit, GE Healthcare). Positive signals on the Hyperfilm were aligned with the agar plate and plaques corresponding to the positive signals were picked and suspended in 250 µl SM buffer containing 0.3% chloroform.

4.8. Analysis of sequenced data and phylogenetic tree construction

DNA sequences were analyzed on nucleotide sequence levels by the BLAST web-based computer program. The search parameters are discussed below.

Homology/Similarity searching. The sequences of genes were analyzed for homology or similarity to known genes or proteins in the databases. BLAST software was used for searching homology/similarity. BLAST is maintained by the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST). The BLAST program comprises 5 separate programs. For this work, BLASTN was used to compare the queried DNA to all DNA sequences in the databases, and BLASTX was employed to compare all six reading frames as they are translated from the queried DNA to all polypeptide sequence in the protein databases.

Deduced amino acid sequence. Software for DNA translation, http://www.expasy.org/tools/dna.html, was used. The program uses universal codons to translate 6 frames of the queried DNA to generate a deduce the amino acid sequence. The acquired information predicts possible physical/biochemical and virulence characteristics.

Multiple sequences alignment. The DNA sequences or deduced amino acid sequences were aligned by the ClustalW program (http://www.ebi.ac.uk/clustalw/). This program produces multiple sequence alignments of divergent sequences. The best match of the selected sequences was calculated and aligned with the other sequences. The identical and similar residues in the multiple-alignment chart were represented by different colors or shadings with BOXSHADE 3.21 (www.ch.embnet.org/software/BOX_form.html).

Phylogenetic tree construction. The construction of phylogenetic trees was carried out with MEGA3 program (Kumar *et al.*, 2004). Robustness of branches was estimated using 1000 boot-strapped replicates.

4.9. Primer design for gene expression study

RT-PCR amplification of a particular mRNA sequence requires two PCR primers that are specific for that mRNA sequence. The primer design should also allow differentiation between the amplified product of cDNA and an amplified

product derived from contaminating genomic DNA. The primers of *gapdh* and *sodA* were designed from full-length cDNA sequences by programs Primer3, located at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi, and GeneFisher, located at http://bibiserv.techfak.uni-bielefeld.de/genefisher. After examining the detailed alignment of cDNA against genomic sequences, *gapdh* and *sodA* primers were designed to flank a region that contains one and two introns, respectively. With these primers, any product amplified from genomic DNA would be much larger than a product amplified from intronless mRNA (Figure 1). Primers for *P. marneffei acuD* and *hsp70*, the genomic and cDNA sequences from GenBank were aligned by ClustalW program. Primers were designed to span exon-exon boundaries on the mRNA; such designed primers should not amplify genomic DNA because the intron intervenes between the pairing of primer and DNA (Figure 1). Primers for non full-length, *cat* and *cam* or intronless *cpeA* genes were directly designed from cDNA sequences from gene characterized data (Appendix C).

4.10. Macrophage infection and recovering *P. marneffei* cells after infection

Macrophage cells were cultured in 125 mm^2 canted neck culture flask for 3 days, the medium was then removed and replaced with 20 ml DMEM culture medium containing approximately 10^8 *P. marneffei* conidia (MOI=10). Cells were then incubated at 37^{o}C in the presence of 5% CO_2. Flasks of cell-free media inoculated with conidia served as controls. Samples were taken from 2, 4 and 8 h after the conidia of *P. marneffei* were added to adherent macrophage. The culture medium containing unadherent cells was gently removed and the macrophages were washed repeatedly with sterile PBS pre-warmed at 37^{o}C. Following extensive washing with PBS, infected macrophages were lysed with 0.5% SDS solution. The released conidia of *P. marneffei* were recovered by centrifugation at 900 x g for 15 min, and then used for total RNA preparation. *P. marneffei* conidia which were incubated at 37^{o}C in the cell free culture medium (without the macrophage cells), were similarly processed as the pre-infection control. RNA was isolated from both conidial samples by using RNeasy mini kit as described above. An aliquot of each RNA sample was checked for the absence of mouse DNA or RNA using mouse glutaraldehyde-6-phosphate

dehydrogenase (GAPDH) specific primers in a PCR or RT-PCR assay, respectively (Table 1).

4.11. Differential genes expression of the *P. marneffei* in different phases and during macrophage infection

4.11.1) RT-PCR analysis

The RNA samples from *P. marneffei* in different phases (conidia, mycelia and yeast cells), as well as intracellular and control conidia (2, 4 and 8 h of incubation), were amplified with duplex specific primers using the OneStep RT-PCR kit (Qiagen) according to the manufacturer's protocol. 50, 100 or 200 ng of DNaseI treated total RNA was used as the template for RT-PCR. The gene fragment was amplified with specific primers. The internal control consisted of a 630 bp PCR product amplified with specific primers for 18S rRNA (RRF1 and RRH1, Table 1). The RT was performed at 50^{o}C for 30 min followed by an initial PCR step at 95^{o}C for 15 min. The subsequent 25 cycles of PCR amplification were performed at 94^{o}C for 30 s, annealing at 60^{o}C for 30 s, 72^{o}C for 1 min and a final extension at 72^{o}C for 10 min. A PCR without RT was performed to detect DNA contamination in RNA samples. The RT-PCR products were subjected to electrophoresis on a 2% agarose gel and visualized with ethidium bromide staining. Fluorescent intensity of the products was analyzed by using a program in GelDoc1000 (BIO-RAD). To observe the differential expression, a relative expression level of expressed genes was calculated from the ratio of band intensity between the gene of interest and 18S rRNA control gene.

4.11.2) Northern blot analysis

The conditions of fungal growth and total RNA extraction were described above. Fifteen micrograms of total RNA from extracted cells at different phases (mycelium, conidia and yeast) and co-incubated conidia with or without macrophage (2, 4 and 8 h) were separated by electrophoresis on denaturing 1% agarose gel using the NorthernMax-Gly system (Ambion) and transferred onto nylon membranes (Hybond-N^{+}) by capillary blotting. RNA was fixed to the membrane by UV crosslinker at C-L mode. The immobilized RNA was probed with a PCR-generated DNA fragment of genes of interest. Probe labelling, hybridization, and detection of

the chemiluminescent signal were performed according to the manufacturer's protocol (GE Healthcare).

4.12. Statistical analysis

4.12.1) Phagocytosis results

All data were expressed as mean ± SD of the number of determinations carried out in triplicate for percentage of phagocytosis, phagocytic index and duplicated for percentage of killing. Variables were tested for normality and then the different groups were compared using Paired Sample t-test (SPSS version 11.0), where $P<0.05$ was considered as statistically significance between groups (Appendix D).

4.12.2) Gene expression results

The mean and standard deviations of relative gene expression levels were carried out in duplicate experiments. The differences between groups were compared using Paired Sample t-test (SPSS version 11.0), where $P<0.05$ was considered as statistically significance between groups (Appendix D).

4.13. Accession number

The nucleotide sequence of the *sodA* and *gapdh* gene isolated from the cDNA library was submitted to the GenBank database under the accession number DQ413185 and EF987476, respectively. The *gapdh* gene from genomic DNA, the nucleotide sequence containing open reading frame disrupted with 5 introns and 5', 3' flanking, was submitted to the GenBank database under the accession number EU000324.

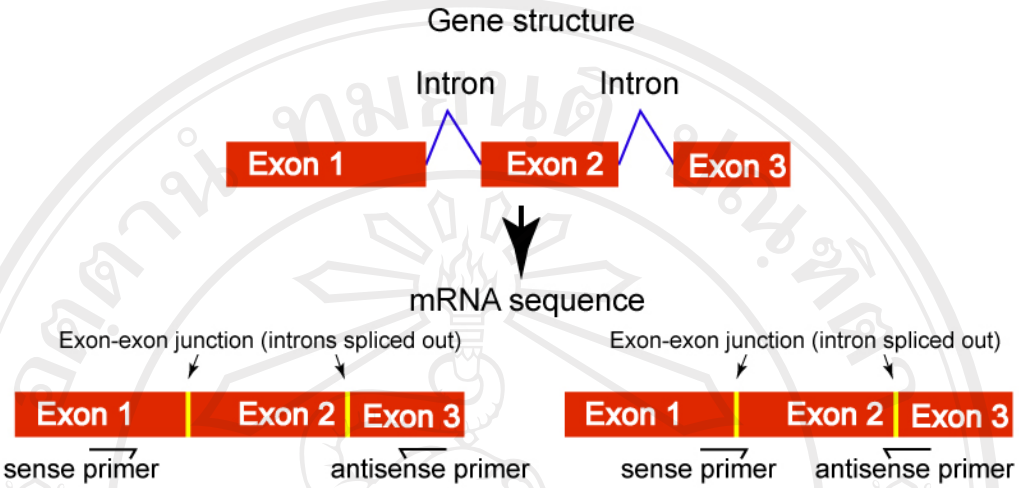

Figure 1. Reverse transcription primers design. There are 2 approaches to design primers for RT-PCR analysis. Primers should be designed to flank a region that contains at least one intron or design primers so that one half hybridizes to the 3' end of one exon and the other half to the 5' end of the adjacent exon.

Table 1. Primers for genes amplification of potential virulent genes

Genes	Sequences (5'-3')	Size of amplicon (bp)	Annealing temperature (°C)	Reference/Accession No.
SOD	SOD-F: WSIGCIGGICCICAITWYAAYCC SOD-R: CKIGSICCIGCRTTICCIGTYTT	250	60	This study
	SOD-gF: AATGGTCAAGGCTGTCGCTG SOD-gR: ACGTTGCAGTTGAGATGACGAC	~500	60	This study
GAPDH	GAP-F: ACHGGYGTYTTCACCACCA GAP-R: ATGACCTTKCCDACRGCCT	400	60	This study
	GAP-gF: CTATCACCGCAATCATGG GAP-gR: GAGCATACTGCGAAGAGTGG	~1400	60	This study
CAM	CAM-F: AAGGAGGCCTTCTCYCTATT CAM-R: GCCTCRCGRATCATCTCRTC	600	65	Carvalho *et al*., 2003
CAT	CAT-F: GGTACCACCAATCAAGCCTGAG CAT-R: CGTCGAACATTGTAGAACGCACAC	550	55	AL683912
	CAT-Fw1: AAGCCTCGTGTACCAACT CAT-Fw2: TATGGCGTGGTTACTGCAGC	-	55	This study

Standard mix base definitions: R=A/G; Y=C/T; K=G/T; S=C/G; W=A/T and I=Inosine

Table 2. Primers sequences applied in RT-PCR analysis

Gene	Sequence (5'-3')	Length	*Tm* (^{o}C)	Product size (bp)	Reference sequences
sodA	SOD-RT1; GTCAAGGCTGTCGCTGTCCTC SOD-RT2; ACTGGCAACGCTGGTCCTCGT	21 21	75 79	448	This study
gapdh	GAP-RT1; GAGACTGGTGCCGCTTACATTG GAP-RT2; GAACACGCATGGACATTCCAGT	22 22	75 75	420	This study
acuD	ICL-RT1; TATCATGGGTACCGACCTCCTTG ICL-RT2; AGATCGGCGAATGGTGCATAAGC	23 23	72 80	450	AF373018
hsp70	HSP70-RT1; GAAGGATTTGACCACCAATGCTCGTGC HSP70-RT2; AGGAGCAACGTCGAGAAGCAAGAT	23 23	80 79	440	AY960136
cpeA	CPE-RT1; CAAGAGCAGACTGACGTCCA CPE-RT2; CCATCAGAGCTACCGTACACCT	20 22	60 62	400	AF537129
cat	CAT-RT1; TGTACCAACTGTGGCCAGCAGA CAT-RT2; ACGACTCAACGACACCAGTGCT	22 22	64 65	420	This study
cam	CAM-RT1; CTGGTTGTCGCAATGTTGTG CAM-RT2; CATAACGTGGCGCAATTCAGCA	20 22	60 63	450	This study
18S rRNA	RRF1; ATCTAAATCCCTTAACGAGGAACA RRH1; CCGTCAATTTCTTTAAGTTTCAGCCTT	22 27	72 78	630	Vanittanakom *et al*., 2002
Mouse *GAPDH*	Mgapdh-RT1; ACCACAGTCCATGCCATCACTGC Mgapdh-RT2; GCTACAGCAACAGGGTGGTGGA	23 22	79 77	420	BC083065