V. RESULTS

5.1. Fungal identification

5.1.1) Macroscopic and microscopic morphology

Penicillium citrinum and *Penicillium marneffei* were cultured on SDA agar at 25°C for 3 and 5 days, respectively and the yeast phase transformation of *P. marneffei* was performed by culture on BHI at 37°C for 7 days. After incubation, the fungal colony was picked, stained with lacto phenol cotton blue and observed by light microscopy (Figure 2).

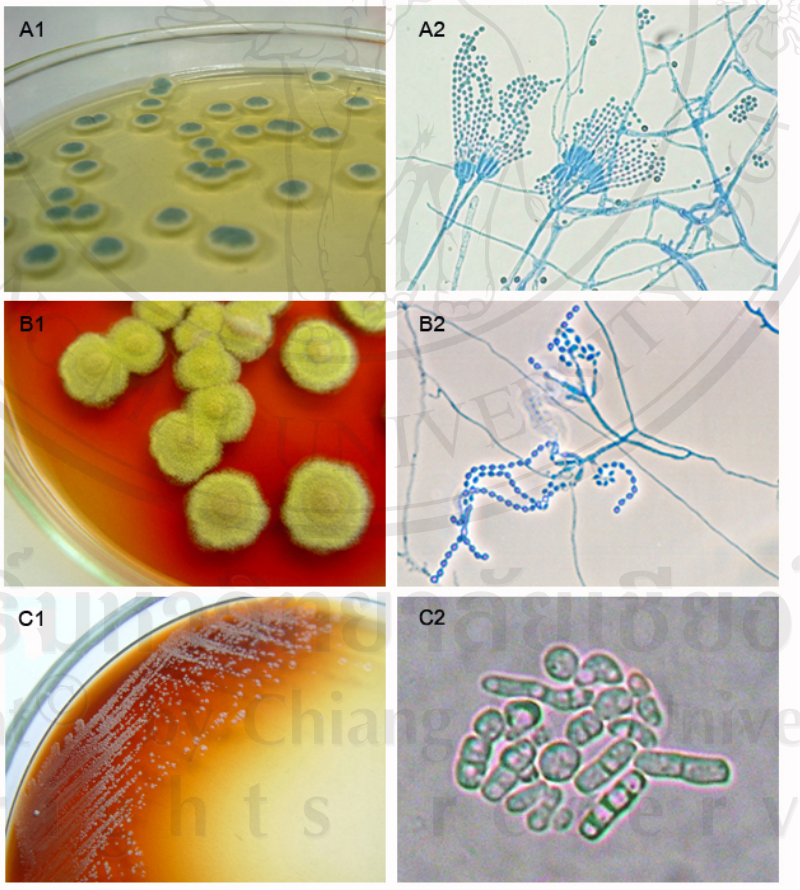

Figure 2. The fungal macroscopic and microscopic morphology examination. *Penicillium citrinum* was cultured on SDA for 3 days at 25°C (A1-2), *Penicillium marneffei* mycelial form was cultured on SDA for 5 days at 25°C (B1-2) and yeast form cultured on BHI for 7 days at 37°C (C1-2) (magnification, x 400).

5.1.2) Sequence analysis of 18S ribosomal DNA

The sequence of partial 18S rDNA and internal transcribed spacer (ITS) sequences of *P. citrinum* showed 100 percent homology with *Penicillium citrinum* strain NRRL 35449 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence accession number DQ123646.1 (Appendix A).

5.2. Macrophage viability and phagocytosis activity

The viability of J774.1 macrophage cell line was 95-98% and the phagocytosis activity in the control experiment with *C. albicans* (ATCC 90028) at the 120 min was 87% (data not shown).

5.3. Phagocytosis assay

Results indicated a high efficiency in phagocytic activity of J774.1 macrophage cells against the conidia of *P. marneffei* and *P. citrinum*. The phagocytosis occurred when the conidia were incubated with the macrophage cells for 30 min at 37^{o}C, and reached to the maximum after 120 min of incubation. In this study, the number of conidia which were phagocytosed by one macrophage was about 0-10 (Figure 3). The percentage of phagocytosis (PP) of *P. citrinum* was 47.00 ± 2.65% at 30 min, 73.00 ± 6.08% at 60 min, 80.00 ± 3.00% at 120 min, and reached to the maximum of 88.00 ± 2.65% at 240 min of incubation while the phagocytic index was 1.64 ± 0.07, 2.09 ± 0.07, 2.31 ± 0.30 and 2.66 ± 0.30, respectively. The PP of *P. marneffei* was 49.67 ± 4.73% at 30 min, 72.33 ± 3.51% at 60 min, 90.67 ± 9.29% at 120 min, and reached to the maximum of 93.67 ± 7.51% at 240 min of incubation, while the PI was 1.60 ± 0.21, 2.50 ± 0.05, 3.98 ± 0.68 and 4.57 ± 0.44, respectively. There was no difference between the percentage of phagocytosis by *P. citrinum* and *P. marneffei* (P>0.05) (Figure 4). However, the phagocytic indices of *P. marneffei* at 60, 120 and 240 min of infection were higher than those of *P. citrinum* (P<0.05) (Figure 5).

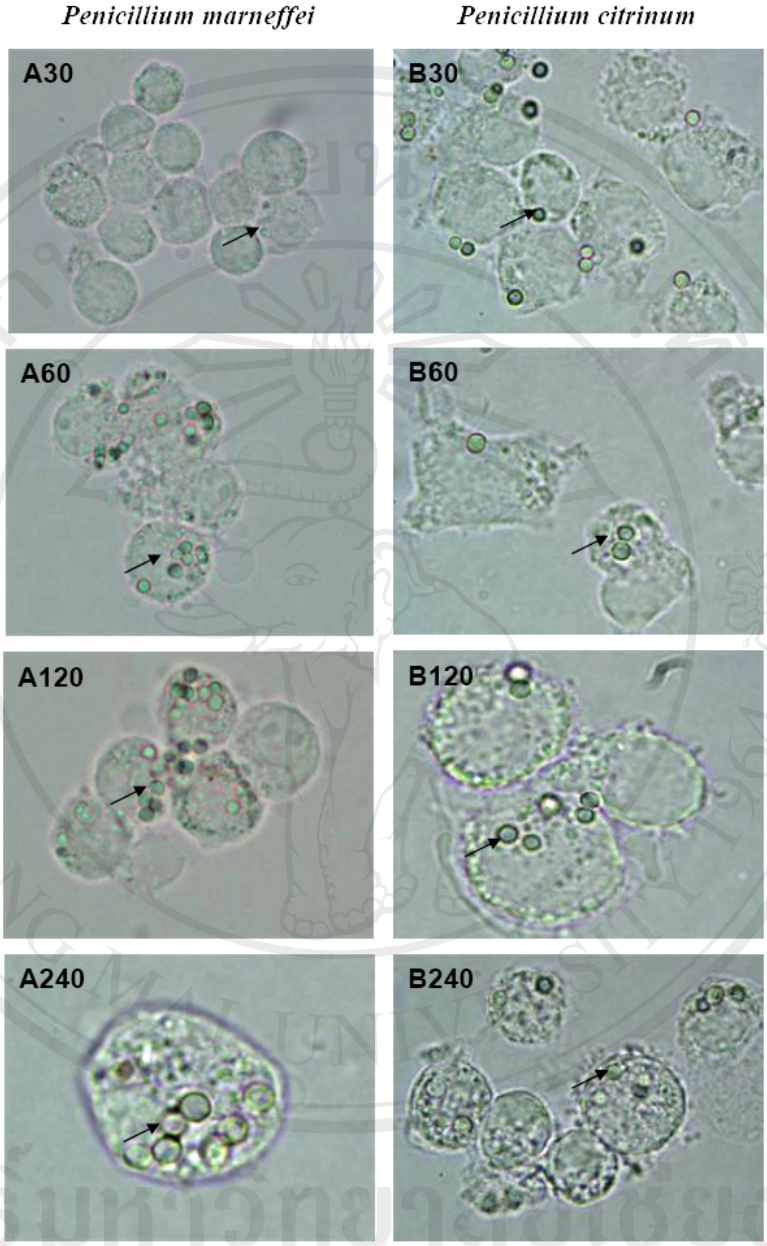

Figure 3. Phagocytosis of *Penicillium marneffei* and *Penicillium citrinum* conidia by mouse macrophage J774.1 cells. Microscopic observation was performed at time 30, 60, 120 and 240 min (column A and B, respectively). There is no phagocytosis at the time zero (data not shown). At 30 min of incubation, all conidia attached the macrophage cells (A30) and some intracellular conidia are seen (B30). More internalized conidia in the macrophages are seen after longer incubation time (A60-240 and B60-240). At 240 min after incubation, some of *P. citrinum* conidia are lysed inside the macrophages (B240). The intracellular conidia were counted and calculated for PP and PI .The arrows indicate the intracellular conidia (magnification, x 1,000).

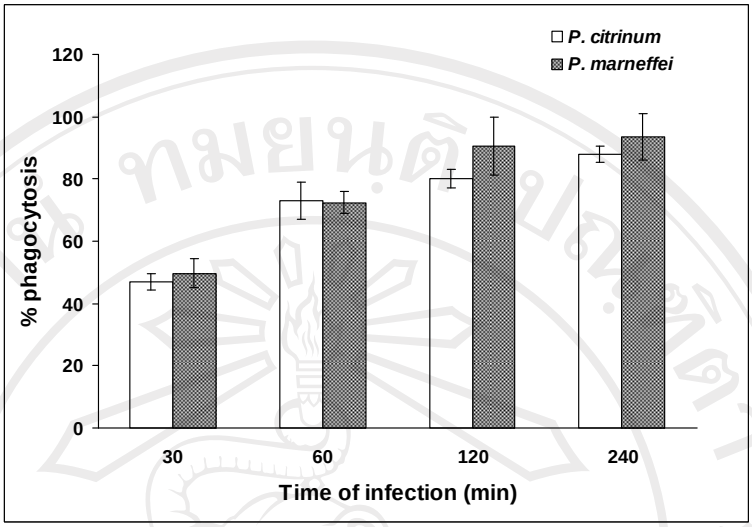

Figure 4. The percentage of phagocytosis (PP) of *Penicillium citrinum* and *Penicillium marneffei.* Phagocytosis occurs at 30 min of incubation and reaches to the maximum at 240 min after incubation. There is no difference in the PP values of both fungi (P>0.05).

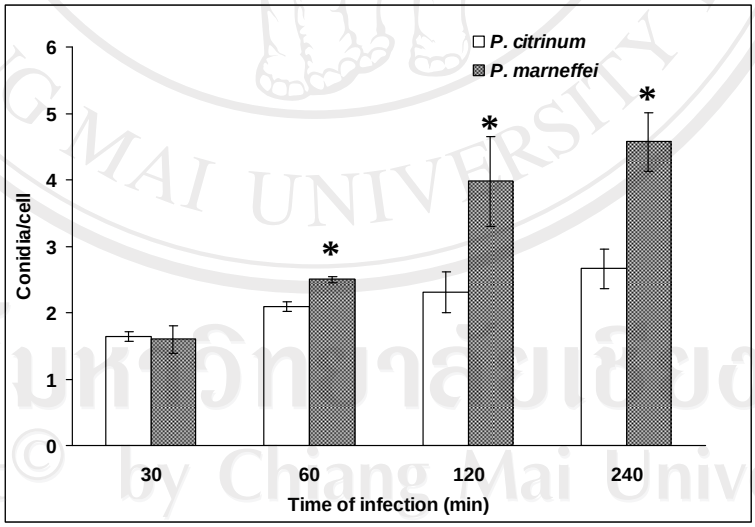

Figure 5. The phagocytic index (PI) of *Penicillium citrinum* and *Penicillium marneffei.* Phagocytosis occurs at 30 min of incubation and the PI results are the time dependent. The graph indicates that the PI of *P. marneffei* is significantly higher than the PI of *P. citrinum* at 60, 120 and 240 min of infection (*****P<0.05).

5.4. Killing assay

The killing activity of macrophages against *P. marneffei* increased to approximately 7.23 ± 1.22%, 16.55 ± 6.05%, 38.85 ± 6.94%, and 61.71 ± 1.54% after 30, 60, 120 and 240 min of incubation, respectively. In the non-pathogen, *P. citrinum*, 42.40 ± 4.23%, 68.56 ± 3.03%, 66.78 ± 2.48% and 64.12 ± 3.42% of conidia were killed after 30, 60, 120 and 240 min of incubation, respectively. In the early stage of phagocytosis, the percentage of killing of *P. citrinum* was significantly higher than those observed in pathogenic *P. marneffei* ($P<0.05$) (Figure 6).

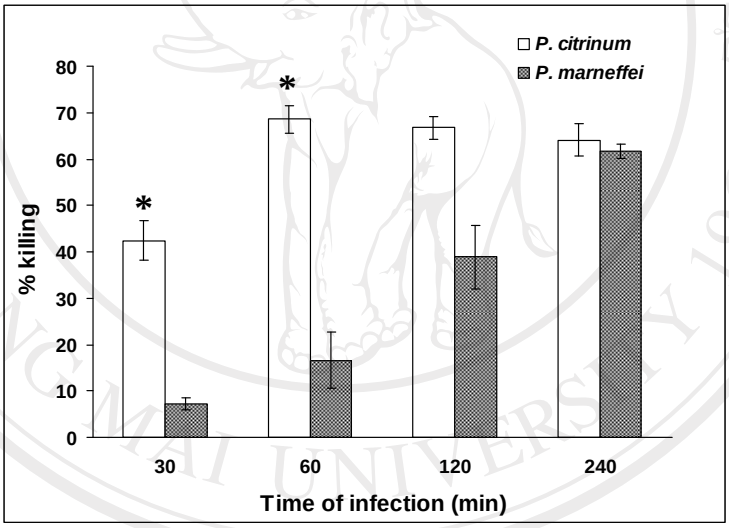

Figure 6. Percentage of killing of *Penicillium citrinum* and *Penicillium marneffei*. The killing activity of the macrophage cells against the conidia of *P. citrinum* is significantly higher than *P. marneffei* at the early incubation time of 30 and 60 min (*****$P<0.05$).

5.5. Isolation and characterization of genes of interest from *P. marneffei*

5.5.1) Cu, Zn superoxide dismutase (Cu, Zn SOD)

Isolation of a specific probe for Cu, Zn *SOD* genes. Cu, Zn *SOD* genes were identified in the RT-PCR experiments by using the RNA templates from *Penicillium marneffei* and *Saccharomyces cerevisiae.* The amplified products of about 250 bp were detected in both fungal RNA samples (Figure 7). These products were cloned and sequenced. The nucleotide sequencing of the fragment produced from *S. cerevisiae* showed 97% homology to the previously derived Cu, Zn *SOD* gene of *S. cerevisiae* (AY690619). By comparison, the RT-PCR product from *P. marneffei* showed 84% homology to the Cu, Zn *SOD* of *Aspergillus terreus* (XM121572), 83% homology to *Aspergillus flavus* (AF401280) and 82% homology to *Aspergillus fumigatus* (XM748622) (Appendix C). Subsequently, this RT-PCR product of *P. marneffei* was used as a probe to screen the cDNA and gDNA library.

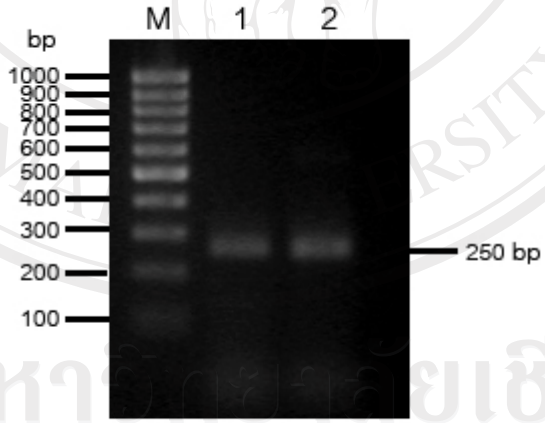

Figure 7. The amplification products of *SOD* gene fragments. The degenerate primers (SOD-F and SOD-R) were designed from fungal SODs alignment and used to amplify *SOD* from *S. cerevisiae* (lane 1) and *P. marneffei* (lane 2) by RT-PCR with expected amplicon size 250 bp.

Screening for full-length genes from cDNA and gDNA libraries The 250 bp Cu, Zn SOD probe was labeled and used in a plaque hybridization procedure to screen 3 x 10^4 recombinant plaques from the *P. marneffei* cDNA library. This screen yielded 13 positive clones. One of these clones, a 667 bp *Not*I/*Sal*I fragment, was purified and subcloned. Sequencing of the plasmid insert identified a portion that spanned 462 bp, which encoded a sequence with homology to fungal Cu, Zn SODs. The cDNA encoding the putative *P. marneffei* Cu, Zn SOD, designated as *sodA*, had both a typical start codon (ATG) and stop codon (TAG), indicating that the full-length sequence was present. The 154-deduced amino acid sequence of a clone containing the full-length *sodA* transcript also displayed strong homology to the Cu, Zn SOD of other fungi. The motif scan search showed conserved domain of Cu, Zn superoxide dismutase (Figure 8). In particular, alignment of the predicted *P. marneffei* protein sequence with five other fungal Cu, Zn SODs by ClustalW analysis revealed a high level of homology (Figure 9). The SodA sequence showed the highest identity to the Cu, Zn SOD of *Aspergillus fumigatus* (87% identity). SodA apparently lacks a signal peptide, but it does have six His at positions 47, 49, 64, 72, 81 and 121 and one Asp residues at position 53 (Figure 9). Presumably, these residues act as the metal binding ligands that are typically found in other fungal Cu, Zn SODs. In addition, two conserved cysteine residues, i.e., Cys58 and Cys147, were noted to surround the active site of this enzyme and likely form an internal disulfide bridge. From gDNA library screening there was no positive clone.

Cu, Zn superoxide dismutase

DNA sequence of Cu, Zn *SOD*

```
  1 GAATTCCCGG GTCGACCCAC GCGTCCGATC ACTATTAACC ATCCCTTTTT AATCATCTAA
 61 AACACTCAAA TCTAGCCAAA ATGGTCAAGG CTGTCGCTGT CCTCCGTGGA GACTCCAACA
121 TCAAGGGTAC CGTCACCTTT GAACAGGCTG ACGAGAACTC CCCTACCACC ATCTCATGGA
181 ACATCACCGG CCACGACGCC AACGCTGAGC GTGGCATCCA CGTTCACCAG TTCGGTGACA
241 ACACCAACGG CTGCACATCT GCCGGTCCTC ACTTCAACCC CTTCGGAAAG ACCCACGGTG
301 CTCCCACCGA TGACGAACGC CATGTCGGTG ACTTGGGTAA CTTCAAGACC GATGCTCAGG
361 GCAATGCTGT CGGCTTCGTC GAGGACAAGC TCATCAAGTT GATCGGTGCT GAGAGCGTTC
421 TCGGACGTAC TATCGTCGTC CACGCCGGTA CTGACGACCT CGGCCGTGGT GGCAACGAGG
481 AGTCCAAGAA GACTGGCAAC GCTGGTCCTC GTCCTGCTTG CGGTGTCATT GGTATCTCTG
541 CTTAGATGAT TAGCCATATG GCTTGAAAAT GATAAAAGTA GTCGTCATCT CAACTGCAAC
601 GTTATGATGA ACATTTAGAT AGAGATAATG CAACAATTGT TTCTATCAAT TGAAAAAAAA
661 AAAAAAA
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Sequence analysis

A. Similarity search

Types	Organism	Description	GenBank acc. no.	E-value	Identity (%)
BLASTN	*Aspergillus flavus*	Cu,Zn-superoxide dismutase mRNA	AF401280	4e-134	402/468 (85%)
	Aspergillus fumigatus	Cu,Zn superoxide dismutase mRNA	AF128886	2e-122	398/472 (84%)
	Aspergillus terreus	Superoxide dismutase mRNA	XM121572	4e-119	381/451 (84%)
BLASTX	*Humicola lutea*	Superoxide dismutase (HlSOD)	P83684	7e-76	133/153 (86%)
	Aspergillus fumigatus	Cu,Zn superoxide dismutase	AAD42060	1e-75	135/154 (87%)
	Aspergillus oryzae	Cu,Zn superoxide dismutase	BAC56176	3e-73	131/154 (85%)

B. Recognition of coding frame

```
  3  ATTCCCGGGTCGACCCACGCGTCCGATCACTATTAACCATCCCTTTTTAATCATCTAAAA

  1                  M  V  K  A  V  A  V  L  R  G  D  S  N  I
 63  CACTCAAATCTAGCCAAAATGGTCAAGGCTGTCGCTGTCCTCCGTGGAGACTCCAACATC

 15   K  G  T  V  T  F  E  Q  A  D  E  N  S  P  T  T  I  S  W  N
123  AAGGGTACCGTCACCTTTGAACAGGCTGACGAGAACTCCCCTACCACCATCTCATGGAAC

 35   I  T  G  H  D  A  N  A  E  R  G  I  H  V  H  Q  F  G  D  N
183  ATCACCGGCCACGACGCCAACGCTGAGCGTGGCATCCACGTTCACCAGTTCGGTGACAAC

 55   T  N  G  C  T  S  A  G  P  H  F  N  P  F  G  K  T  H  G  A
243  ACCAACGGCTGCACATCTGCCGGTCCTCACTTCAACCCCTTCGGAAAGACCCACGGTGCT

 75   P  T  D  D  E  R  H  V  G  D  L  G  N  F  K  T  D  A  Q  G
303  CCCACCGATGACGAACGCCATGTCGGTGACTTGGGTAACTTCAAGACCGATGCTCAGGGC

 95   N  A  V  G  F  V  E  D  K  L  I  K  L  I  G  A  E  S  V  L
363  AATGCTGTCGGCTTCGTCGAGGACAAGCTCATCAAGTTGATCGGTGCTGAGAGCGTTCTC

115   G  R  T  I  V  V  H  A  G  T  D  D  L  G  R  G  G  N  E  E
423  GGACGTACTATCGTCGTCCACGCCGGTACTGACGACCTCGGCCGTGGTGGCAACGAGGAG

135   S  K  K  T  G  N  A  G  P  R  P  A  C  G  V  I  G  I  S  A
483  TCCAAGAAGACTGGCAACGCTGGTCCTCGTCCTGCTTGCGGTGTCATTGGTATCTCTGCT


543  TAGATGATTAGCCATATGGCTTGAAAATGATAAAAGTAGTCGTCATCTCAACTGCAACGT


603  TATGATGAACATTTAGATAGAGATAATGCAACAATTGTTTCTATCAATTGAAAAAAAAAA


663  AAAAA
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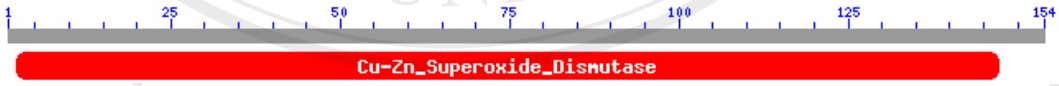
C. Function analysis



Descriptions

Cu, Zn superoxide dismutase (cd00305); superoxide dismutases catalyse the conversion of superoxide radicals to molecular oxygen. Three evolutionarily distinct families of SODs are known, of which the copper, zinc-binding family is one.

Figure 8. Characterization of *P. marneffei* Cu, Zn superoxide dismutase gene.

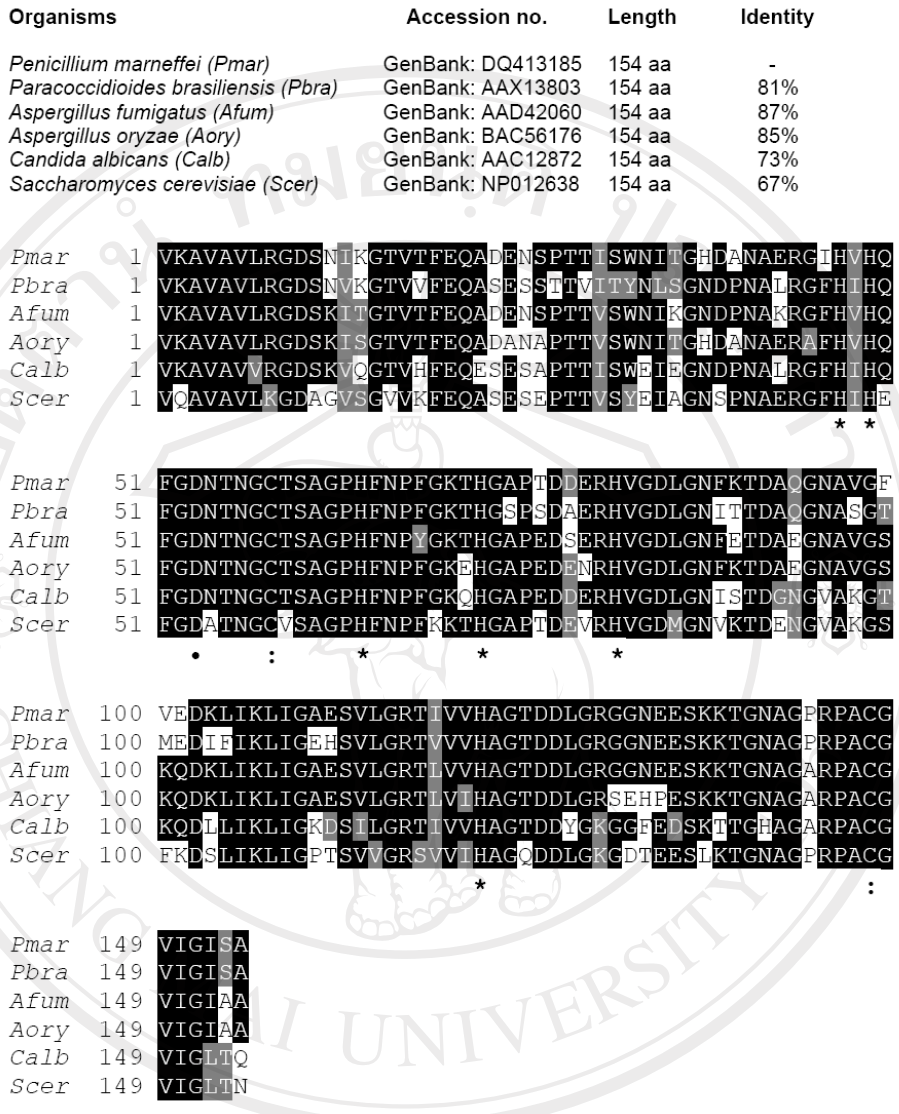


Figure 9. The alignment of fungal Cu, Zn SOD amino acid sequences. Shaded residues indicate ≥ 75% homology (black) or ≥ 50% homology (gray). Sequences were aligned using ClastalW and were shaded by BOXSHADE. The percent identity between *P. marneffei* (Pmar) and other sequences was determined by pairwise comparison using DIALIGN. The putative conserved amino acid sequences are marked. The Cu, Zn SOD sequences have six His (*****) and one Asp residues (•), which act as the metal binding ligands, and two Cys residues (**:**) form the disulfide bond.

Phylogenetic analysis of SodA. In the consensus tree, the Cu, Zn SOD peptide sequences of vertebrates and fungi form two separate clades (Figure 10). Within the fungal clades, the Cu, Zn SODs form two sister groups that represent genes from ascomycetous and basidiomycetous fungi. The *P. marneffei* SodA identified in this study is clustered in the ascomycetous group and appears most closely related to sequences from *Aspergillus fumigatus* and *Aspergillus oryzae*.

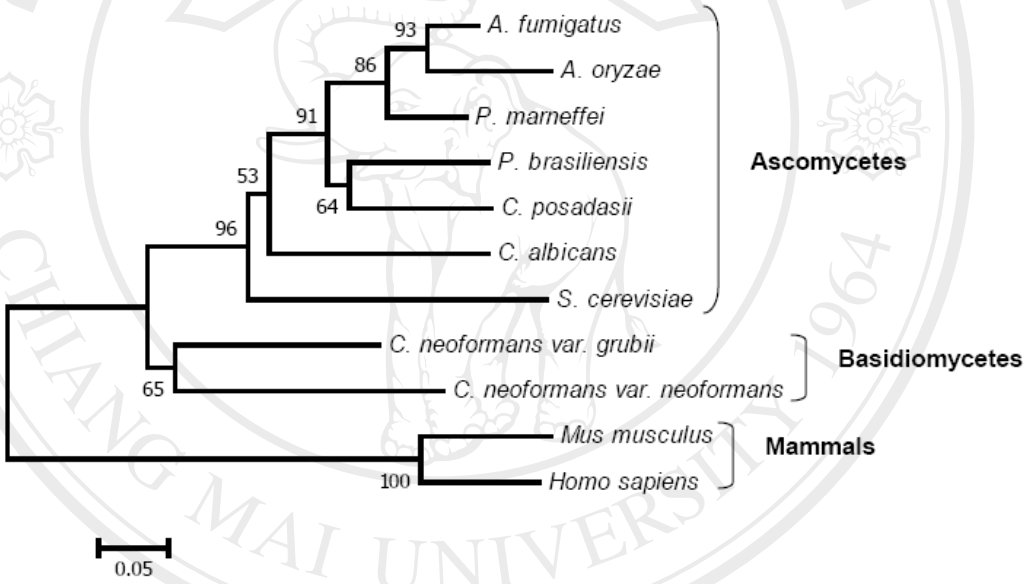


Figure 10. Phylogenetic relationship among Cu, Zn SOD peptide sequences. The Neighbor-joining tree derived from MEGA3 analysis of 154 amino acid positions is shown. The tree was arbitrarily rooted using mammals as an outgroup. The bootstrapping values are shown above the branch. Scale bar indicates the number of substitutions per site. Organism sources and NCBI database accession numbers for sequences are: *Aspergillus fumigatus* (Q9Y8D9), *Aspergillus oryzae* (BAC56176), *Penicillium marneffei* (ABD67502) *Paracoccidioides brasiliensis* (AAX13803), *Coccidioides posadasii* (ABF7315), *Candida albicans* (ACC12872), *Saccharomyces cerevisiae* (NP012638), *Cryptococcus neoformans* var. *grubii* (Q9C0N4), *Cryptococcus neoformans* var. *neoformans* (AF248045), *Mus musculus* (NP035564) and *Homo sapiens* (P83684).

Cloning and characterization the *sodA* from genomic DNA. The genomic fragment corresponding to the full-length cDNA sequence was amplified by PCR using the primers SOD-gF and SOD-gR (Table 1) and sequenced. Comparison between nucleotide sequences from the cDNA library and genomic DNA revealed the compatibility of both, except for those of three non-homologous regions in the genomic DNA between nucleotide 194-246, 419-477 and 575-622 (Figure 11). Analysis of these regions revealed that they are introns of 52, 58 and 47 nucleotides, respectively. Their 5' and 3' ends conformed to the basic consensus, GT/AG, for the eukaryotic splice donor and acceptor sites (Breathnach and Chambon, 1981; Mount, 1982). These regions contained an internal putative splice box, which matched the filamentous fungus consensus sequence (CTRAY) upstream from the 3' end of the intron (Gurr *et al*., 1987).

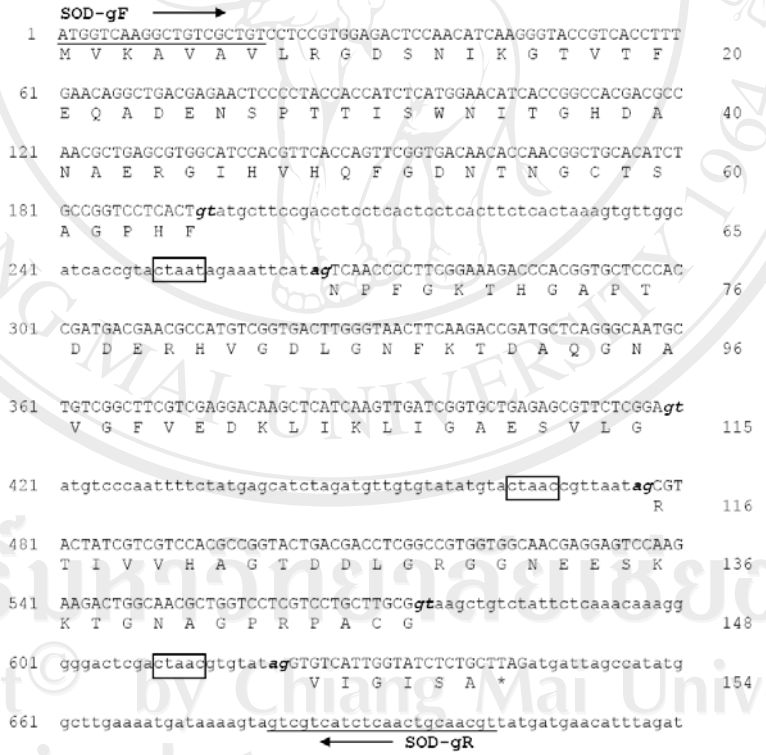


Figure 11. The nucleotide sequence and deduced amino acid of the *sodA* gene from *P. marneffei*. Upper case letters indicate the exon, while lower case letters indicate the intron. The amino acid sequence is indicated beneath the DNA sequence. Nucleotides in bold italics represent the conserved 5' and 3' consensus of the intron. The internal splice (lariat) sequences are boxed.

5.5.2) Glyceraldehyde-3-phosphate dehydrogenase gene (*gapdh*)

Isolation of a specific probe for *gapdh*. Part of *P. marneffei gapdh* could be identified in the PCR experiment using the designed degenerate primers (Figure 12). The nucleotide sequencing analysis of the 420-bp PCR product showed 83% homology to the *gapdh* gene of *Aspergillus fumigatus* (XM748622) and *Aspergillus oryzae* (AB226266) (Figure 13). This PCR fragment was used as a probe to screen the cDNA library.

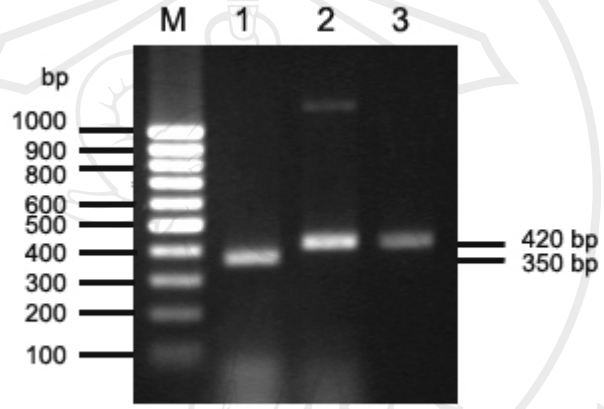


Figure 12. The amplification products of *gapdh* gene. The degenerate primers (GAP-F and GAP-R) were designed from fungal *gapdh* genes alignment and used to amplify *gapdh* from *A. fumigatus* (lane 1) and *P. marneffei* (lane 2) by RT-PCR with an expected amplicon size of 350 and 420 bp, respectively. The 420 bp PCR product form *P. marneffei* was purified and cloned for sequencing (lane 3).

Glyceraldehyde-3-phosphate dehydrogenase

DNA sequence of *gapdh*

```
   1 GCTAAGCAAT GTGGGTGACC CAGTCGTTGC GTCAACAACA GAACATTCAC CGAATGTGCC
  61 TCTTTCGTCA AAGCTACCCC GCCAGGCCAC CCTTTCTTAT TAAGACAAAA TCTATCTCCT
 121 GATTTTTTTC CCTTTCCTTC TCTTCTCTTT CTTCTCTACA CCCATCAATT CAACTTCCAT
 181 CCACCTCATT CTTCGAATTT CTTCCTCAGC AAACAAACTA CTATCACCGC AATCATGGTT
 241 ACCAAGGTTG GCATCAACGG TTTCGGCCGT ATCGGTCGTA TTGTCTTCCG CAACGTCATC
 301 GAGCACGATG ATGCTGAGGT TGTCGCTGTC AACGACCCCT TCATTGAGAC TCACTACGCT
 361 GCCTACATGC TCAAGTATGA CACCCAGCAC GGTCAGTTCA AGGGCACCAT CGAGGTCGAG
 421 GCTTCCGACC TCATCGTCAA TGGCAAGCGC GTCAAGTTCT ACCAGGAGCG TGACCCCGCC
 481 AACATCAAGT GGTCCGAGAC TGGTGCCGCT TACATTGTCG AGTCCACCGG TGTCTTCACC
 541 ACCACCGAGA AGGCCTCTGC TCACTTGAAG GGTGGCGCCA AGAAGGTCGT CATCTCTGCT
 601 CCTTCCGCAG ATGCTCCTAT GTTCGTCATG GGTGTCAACC ACACAACCTA CAAGAGCTCA
 661 GACACCATCA TCTCCAACGC TTCTTGCACC ACCAACTGCT TGGCTCCCCT CGCCAAGATT
 721 GTCAACGACA ACTGGGGTCT TGTTGAGGGT CTCATGACCA CCATTCACTC CTACACTGCT
 781 ACCCAGAAGA CCGTTGATGG TCCCTCCGCC AAGGACTGGC GTGGTGGTCG TACCGCTGCT
 841 CAGAACATCA TTCCCAGCAG CACTGGTGCC GCCAAGGCTG TCGGAAAGGT CATTCCTGCC
 901 CTTAACGGAA AGCTCACTGG AATGTCCATG CGTGTTCCTA CCTCCAACGT CTCCGTTGTT
 961 GACTTGACCT GCCGCACTGA GAAGCCCGTC AGCTACGACG AGATCAAGGC CACAGTCAAG
1021 AAGTACGCCG AGGGCGAGCT CAAGGGAATC ATGGGCTACA CTGAGGACGA CGTTGTCTCC
1081 ACTGACATGA ACGGCAACAG CAACAGCTCC GTCTTCGATG CCAAGGCTGG TATCGCTCTT
1141 AACTCCAACT TCATCAAGCT CGTCAGCTGG TACGACAATG AGTGGGGTTA CTCTCGCCGT
1201 GTTGTTGACC TCATCGTCTA CATCTCCAAG GTCGACGGCA ACGCTTAGGA ATCAGCGGCC
1261 ACTCTTCGCA GTATGCTCTA AATGCTGAAT GCTATCCTTG CCCGAGATAA AGGGAAGGAA
1321 ACTCGTTTCA ATTTTTGATC TGATCTGGAA TGAGAGTTAC GAATTGGCAA CTTATGATGT
1381 GCTCCGAAAA TTAGAACGAG AATGTTATTA TCTCTTCCAA TGACAATAAA TAGTTTAATG
1441 AAAAAAAAAA AAAAA
```

Sequence analysis

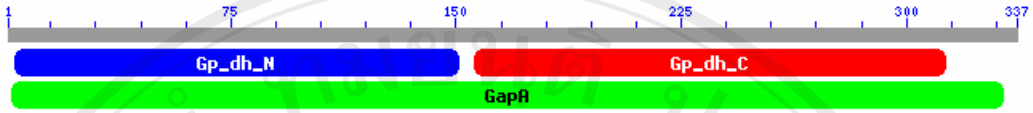
A. Similarity search

Types	Organism	Description	GenBank acc. no.	E-value	Identity (%)
BLASTN	*Aspergillus oryzae*	*gpdA* mRNAfor glyceraldehyde-3-phosphate dehydrogenase	AB032274	0.0	852/1024 (83%)
	Neurospora crassa	glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	XM951884	0.0	839/1014 (82%)
	Aspergillus terreus	glyceraldehyde-3-phosphate dehydrogenase mRNA	XM1218438	0.0	787/939 (83%)
BLASTX	*Ajellomyces capsulatus*	glyceraldehyde-3-phosphate dehydrogenase	AF273703	2e-160	274/334 (82%)
	Emericella nidulans	glyceraldehyde-3-phosphate dehydrogenase	AAA33307	3e-158	274/334 (82%)
	Cryphonectria parasitica	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GPD-1)	CAA37943	3e-158	271/337 (80%)

B. Recognition of coding frame

```
   1  GCTAAGCAATGTGGGTGACCCAGTCGTTGCGTCAACAACAGAACATTCACCGAATGTGCC

  61   TCTTTCGTCAAAGCTACCCCGCCAGGCCACCCTTTCTTATTAAGACAAAATCTATCTCCT

 121  GATTTTTTTCCCTTTCCTTCTCTTCTCTTTCTTCTCTACACCCATCAATTCAACTTCCAT

   1                                                          M  V
 181  CCACCTCATTCTTCGAATTTCTTCCTCAGCAAACAAACTACTATCACCGCAATC**ATG**GTT

   3   T  K  V  G  I  N  G  F  G  R  I  G  R  I  V  F  R  N  V  I
 241  ACCAAGGTTGGCATCAACGGTTTCGGCCGTATCGGTCGTATTGTCTTCCGCAACGTCATC

  23   E  H  D  D  A  E  V  V  A  V  N  D  P  F  I  E  T  H  Y  A
 301  GAGCACGATGATGCTGAGGTTGTCGCTGTCAACGACCCCTTCATTGAGACTCACTACGCT

  43   A  Y  M  L  K  Y  D  T  Q  H  G  Q  F  K  G  T  I  E  V  E
 361  GCCTACATGCTCAAGTATGACACCCAGCACGGTCAGTTCAAGGGCACCATCGAGGTCGAG

  63   A  S  D  L  I  V  N  G  K  R  V  K  F  Y  Q  E  R  D  P  A
 421  GCTTCCGACCTCATCGTCAATGGCAAGCGCGTCAAGTTCTACCAGGAGCGTGACCCCGCC

  83   N  I  K  W  S  E  T  G  A  A  Y  I  V  E  S  T  G  V  F  T
 481  AACATCAAGTGGTCCGAGACTGGTGCCGCTTACATTGTCGAGTCCACCGGTGTCTTCACC

 103   T  T  E  K  A  S  A  H  L  K  G  G  A  K  K  V  V  I  S  A
 541  ACCACCGAGAAGGCCTCTGCTCACTTGAAGGGTGGCGCCAAGAAGGTCGTCATCTCTGCT

 123   P  S  A  D  A  P  M  F  V  M  G  V  N  H  T  T  Y  K  S  S
 601  CCTTCCGCAGATGCTCCTATGTTCGTCATGGGTGTCAACCACACAACCTACAAGAGCTCA

 143   D  T  I  I  S  N  A  S  C  T  T  N  C  L  A  P  L  A  K  I
 661  GACACCATCATCTCCAACGCTTCTTGCACCACCAACTGCTTGGCTCCCCTCGCCAAGATT

 163   V  N  D  N  W  G  L  V  E  G  L  M  T  T  I  H  S  Y  T  A
 721  GTCAACGACAACTGGGGTCTTGTTGAGGGTCTCATGACCACCATTCACTCCTACACTGCT

 183   T  Q  K  T  V  D  G  P  S  A  K  D  W  R  G  G  R  T  A  A
 781  ACCCAGAAGACCGTTGATGGTCCCTCCGCCAAGGACTGGCGTGGTGGTCGTACCGCTGCT

 203   Q  N  I  I  P  S  S  T  G  A  A  K  A  V  G  K  V  I  P  A
 841  CAGAACATCATTCCCAGCAGCACTGGTGCCGCCAAGGCTGTCGGAAAGGTCATTCCTGCC

 223   L  N  G  K  L  T  G  M  S  M  R  V  P  T  S  N  V  S  V  V
 901  CTTAACGGAAAGCTCACTGGAATGTCCATGCGTGTTCCTACCTCCAACGTCTCCGTTGTT

 243   D  L  T  C  R  T  E  K  P  V  S  Y  D  E  I  K  A  T  V  K
 961  GACTTGACCTGCCGCACTGAGAAGCCCGTCAGCTACGACGAGATCAAGGCCACAGTCAAG

 263   K  Y  A  E  G  E  L  K  G  I  M  G  Y  T  E  D  D  V  V  S
1021  AAGTACGCCGAGGGCGAGCTCAAGGGAATCATGGGCTACACTGAGGACGACGTTGTCTCC

 283   T  D  M  N  G  N  S  N  S  S  V  F  D  A  K  A  G  I  A  L
1081  ACTGACATGAACGGCAACAGCAACAGCTCCGTCTTCGATGCCAAGGCTGGTATCGCTCTT

 303   N  S  N  F  I  K  L  V  S  W  Y  D  N  E  W  G  Y  S  R  R
1141  AACTCCAACTTCATCAAGCTCGTCAGCTGGTACGACAATGAGTGGGGTTACTCTCGCCGT

 323   V  V  D  L  I  V  Y  I  S  K  V  D  G  N  A
1201  GTTGTTGACCTCATCGTCTACATCTCCAAGGTCGACGGCAACGCT**TAG**GAATCAGCGGCC

1261   ACTCTTCGCAGTATGCTCTAAATGCTGAATGCTATCCTTGCCCGAGATAAAGGGAAGGAA

1321  ACTCGTTTCAATTTTTGATCTGATCTGGAATGAGAGTTACGAATTGGCAACTTATGATGT

1381   GCTCCGAAAATTAGAACGAGAATGTTATTATCTCTTCCAATGACAATAAATAGTTTAATG

1441   AAAAAAAAAAAAAAA
```

C. Function analysis



Descriptions

- GpdhC (pfam02800); glyceraldehyde 3-phosphate dehydrogenase, C-terminal domain. GAPDH is a tetrameric NAD-binding enzyme involved in glycolysis and glyconeogenesis.
- GpdhN (pfam00044); glyceraldehyde 3-phosphate dehydrogenase, NAD binding domain.
- GapA (COG0057); glyceraldehyde-3-phosphate dehydrogenase/erythrose-4-phosphate dehydrogenase (Carbohydrate transport and metabolism).

Figure 13. Characterization of *P. marneffei* glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene.

Screening for full-length gene from cDNA and gDNA libraries. A screening of 10^4 recombinant plaques from the *P. marneffei* cDNA library yielded 117 positive clones. One of these clones, a *Not*I/*Sal*I fragment of 1500 bp was purified and subcloned. Sequencing of the plasmid insert identified a portion that spanned 1011 bp, which encoded a sequence with homology to fungal GAPDHs. The cDNA encoding the GAPDH, designated as *gapdh*, was subjected to six-frame translation to determine the coding frame and encoded polypeptide sequence. The forward frame 1 revealed an open reading frame of 337 amino acids with typical start codon (ATG) and stop codon (TAG), indicating that the full-length sequence was present. In addition, the result from BLASTX search showed that the polypeptide from the forward frame 1 was functionally identical to the glyceraldehyde-3-phosphate dehydrogenase. The motif scan search found the conserved domain for glyceraldehydes-3-phosphate dehydrogenase in the deduced amino acid sequence. This match thus confirmed the possible glyceraldehyde 3-phosphate dehydrogenase function of this gene (Figure 13). Alignment of its deduced amino acid sequence with five other fungal GAPDHs by ClustalW analysis revealed a high level of homology. The sequence showed the highest identical score to *Ajellomyces capsulatus* GAPDH (87% identity) (Figure 14).

Phylogenetic analysis. The GAPDH protein sequences of *P. marneffei* and 12 other species were aligned for phylogenetic analysis using the Neighbor-Joining method. Based on the comparison of the deduced GAPDHs it was possible to cluster the sequences in three clades comprising the Eurotiomycetes, the Dothideomycetes, and the Sordariomycetes. *P. marneffei* was classified in the Eurotiomycetes clade, close to *Ajellomyces capsulatus*. *Coccidioides immitis*, *Emericella nidulans*, and *Aspergillus niger* occupied a derived position inside the Eurotiomycetes clade (Figure 15A). A comparison of the mosaic structure of *P. marneffei gapdh* and genes from other fungi was performed. Figure 15B presents the positions of the introns in relation to the amino acid sequences of the GAPDH proteins. A comparison to other species in the Eurotiomycetes revealed that *P. marneffei* shares introns at positions 16/17, 20/21, 42/43, and 270/271. Comparison with the analyzed Dothideomycetes yielded similar results, except for differences in the intron at position 16/17. The Sordariomycetes, having the lowest number of introns (1-2), showed intron similarity at position 42 (Figure 15A and B) to other clades.

Cloning and characterization the *gapdh* from genomic DNA. The genomic DNA fragment corresponding to full-length cDNA sequence, was amplified by PCR using the primers Gap-gF and Gap-gR (Figure 16, Table1) and then it was sequenced. Comparison between nucleotide sequences from the cDNA library and genomic DNA revealed the compatibility of both, except for those of five non-homologous regions from the genomic DNA between nucleotide 49-122, 135-194, 249-289, 608-657, and 1085-1137 (Figure 16). Analysis of these regions reveled that they are introns of 74, 60, 41, 50 and 53 nucleotides, respectively. A search at the PROSITE database revealed the presence of a potential site at positions 149–156 for substrate binding (ASCTTNCL), as described for the GAPDHs (Goudout-Crozel *et al.*, 1989). Amino acids potentially associated with catalysis were at amino acid positions 151 (C) and 178 (H). Several potential phosphorylation sites were found at 10 positions (102–106, 138–140, 183–185, 191–194, 240– 242, 245–250, 265–268, 292–295, 311–314, and 321- 329). The amino acid residues at positions 34 (D) and 315 (N) corresponded to the putative NAD^+ binding sites. Positions 150, 152, 196, 210, and 225 were found to be probable sites for inorganic phosphate binding (S, T, R, T, G). Positions 181, 233, and 247 were found to be residues putatively related the binding of the phosphate from the substrate (T, R, and R).

Organisms	Accession no.	Length	Identity
Penicillium marneffei	GenBank:	337 aa	-
Ajellomyces capsulatus	GenBank: AAG33368	337 aa	87%
Neurospora crassa	GenBank: XP956977	338 aa	86%
Paracoccidioides brasiliensis	GenBank: AAL34975	338 aa	85%
Coccidioides immitis	GenBank: Q1DTF9	337 aa	84%
Aspergillus fumigatus	GenBank: XP748145	338 aa	83%
Cryptococcus neoformans	GenBank: XP571627	339 aa	73%

Figure 14. The alignment of fungal GAPDH amino acid sequences. Shaded residues indicate ≥ 75% homology (black) or ≥ 50% homology (gray). Sequences were aligned using ClustalW and shaded by BOXSHADE. The percent identity between *P. marneffei* and other sequences was determined by pairwise comparison using DIALIGN.

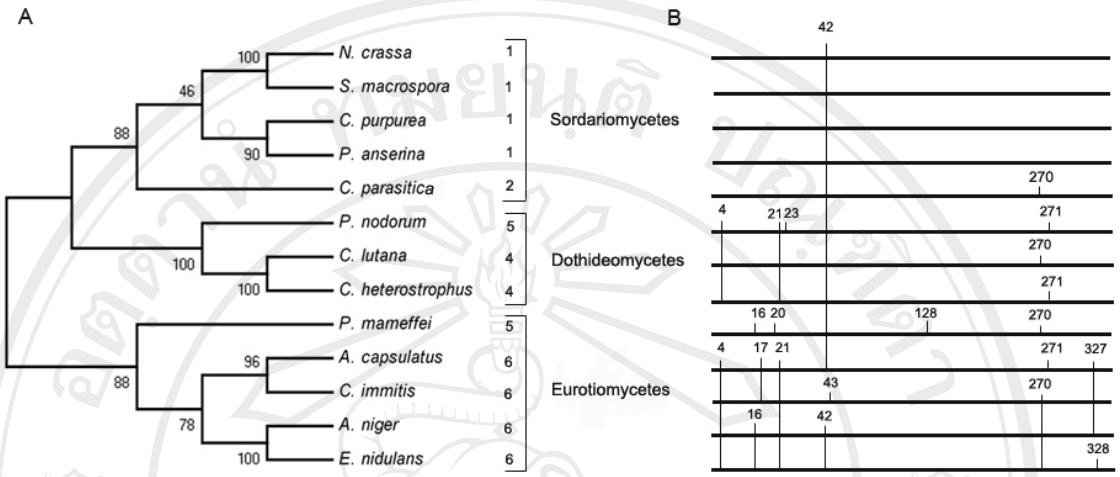


Figure 15. Phylogeny of the *P. marneffei* GAPDH and analysis of the intron positions. (A) The Neighbor-Joining tree derived from MEGA3 analysis of 13 species inferred from the protein sequences of GAPDH is shown. The bootstrapping values are shown above the branch. Organism sources and NCBI database accession numbers for sequences are: *Cryphonectria parasitica* (X53996), *Sordaria macrospora* (AJ313527), *Neurospora crassa* (U67457), *Claviceps purpurea* (X73282), *Podospora anserina* (X62824), *Phaeosphaeria nodorum* (AJ271155), *Curvalaria lunata* (X58718), *Cochliobolus heterostrophus* (X63516), *Emericella nidulans* (M19694), *Aspergillus niger* (X99652), *Penicillium marneffei* (EF987476), *Ajellomyces capsulatus* (AF273703) and *Coccidioides immitis* (Q1DTF9). On the right side are the numbers of introns in the *gapdh* cognate genes. (B) Structural diagram of *gapdh* genes. The corresponding amino acid residues indicate the position of introns. The sequences utilized were those described above.

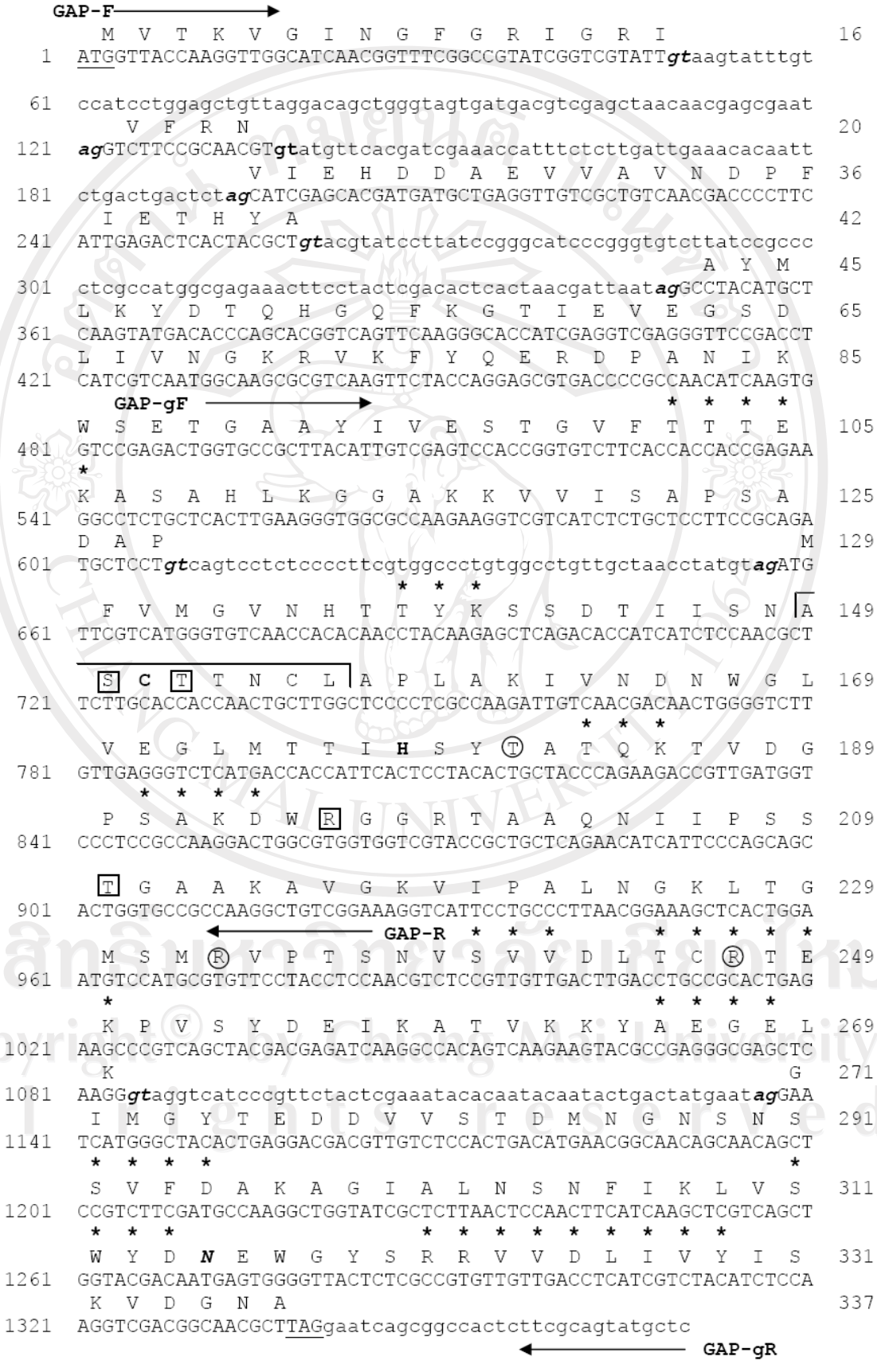


Figure 16. Nucleotide and deduced amino acid sequences of the *P. marneffei* gDNA encoding the GADPH. The oligonucleotide primers are marked by arrows. Primers GAP-F (sense) and GAP-R (anti-sense) were designed on basis of the alignment of *gapdh* gene from several fungi. The GAP-gF and GAP-gR primers were used in PCR amplification of a 1.4-kb fragment of *P. marneffei* genomic DNA. Uppercase letters of nucleotides indicate the exon, while lowercase letters indicate the intron. Nucleotides in bold italics represent the conserved 5' and 3' consensus of the intron. Start and stop codons are underlined. The deduced amino acid sequence is shown above the nucleotide sequence (single letter code). The sites putatively related to the inorganic phosphate binding are marked by squares. Amino acids putatively related to the NAD^+ binding are in bold italics. The substrate-binding site is marked with a bracket. Amino acids potentially associated with catalysis are in bold. Circles mark the residues related to the binding to the phosphate from the substrate. Asterisks indicate the putative phosphorylation sites.

5.5.3) Calmodulin gene (*cam*)

Isolation of a specific probe for *cam* gene. Calmodulin genes were identified in PCR experiment using genomic DNA from *P. marneffei* and *H. capsulatum* as a control. The gradient annealing temperature was performed to optimize the PCR conditions. The amplified product of about 570 bp was detected from gDNA of *H. capsulatum* and about 540 bp from *P. marneffei*. These products were cloned and sequenced (Figure 17 and 18). The nucleotide sequencing of the fragment produced from *H. capsulatum* showed 96% homology to *Ajellomyces capsulatus* calmodulin (CAM1) gene (AF072882). By comparison, the PCR product from *P. marneffei* showed 87% homology to calmodulin gene of *Talaromyces flavus* (AY678609), *Penicillium pinophilum* (AY678604) and *Penicillium aculeatum* (AY678603). Subsequently, this PCR product of *P. marneffei* was used as a probe to screen the cDNA and gDNA libraries.

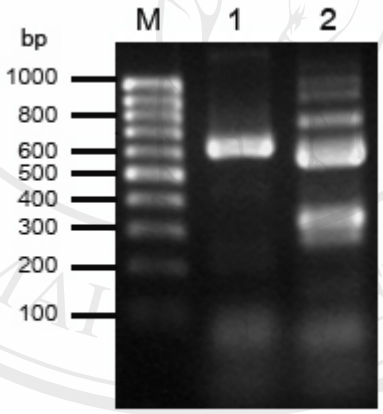


Figure 17. The amplification products of *P. marneffei cam* gene. The degenerate primers (CAM-F and CAM-R) were used to amplify *cam* from *H. capsulatum* with expected band 570 bp (lane 1) and more than 6 products from *P. marneffei* (lane 2).

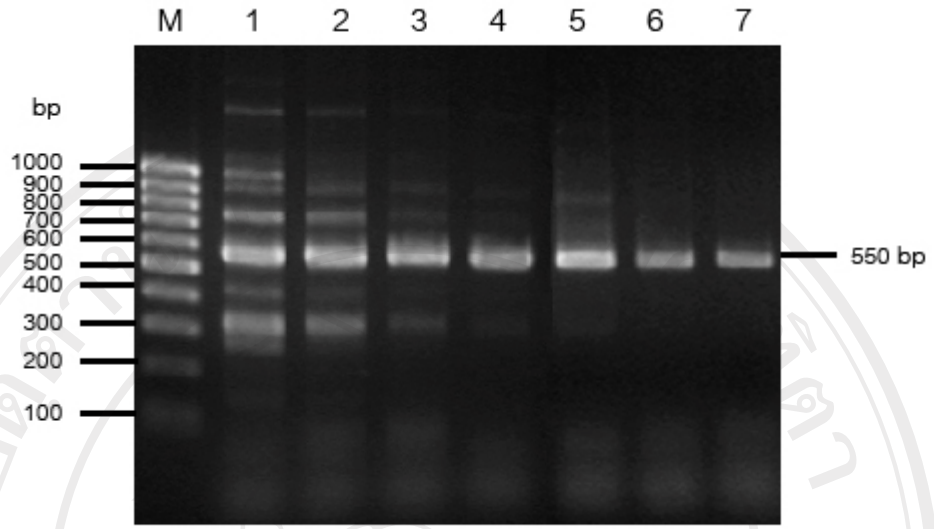


Figure 18. The PCR amplification results of *P. marneffei cam* gene with gradient annealing temperatures. The CAM-F and CAM-R degenerate primers were used in gradient annealing temperature at 52, 54, 56, 58, 61, 63 and 65^{o}C (lane 1-7, respectively). The 550 bp expected product was purified and cloned for sequencing.

Screening for full-length gene from cDNA and gDNA libraries. The 550 bp calmodulin probe was labeled and used in a plaque hybridization procedure. There was no positive clone from screening of 10^5 recombinant plaques of the *P. marneffei* cDNA or gDNA library. However, the *P. marneffei* calmodulin gene fragment was characterized and the specific primers were designed for gene expression study.

Characterization of partial *cam* gene. The coding frame of 550 bp fragment was readily identified from the six-frame translation. The forward frame 3 revealed an open reading frame of 84 amino acids without start and stop codons. In addition, the result from BLASTX search showed that the polypeptide from the forward frame 3 was functionally identical to the calmodulin. A motif scan search found a significant match to an EF hand, a known calcium binding motif. This match thus confirmed the possible calmodulin function of this gene fragment (Figure 19).

Calmodulin

DNA sequence of *cam* fragment

```
  1 CTCTCTATTT GTAAGTTTGG ATTTCTGGTT GTCGCAATGT TGTGGTGGGT GGTTCGCTGA
 61 CTAGCCGTTT GGATGAATAG GACAAGGATG GTGATGGTGA GTGACGCCAC GAACACCAGA
121 CATATAGTCT TCGAACAAAA AGTTATTACT GCGAACAGAT ATTAATAACA TCAATAGGTC
181 AAATTACAAC CAAGGAACTG GGCACCGTCA TGCGTTCCCT CGGCCAGAAC CCTTCCGAAT
241 CCGAATTGCA GGACATGATC AACGAGGTCG ACGCTGACAA CAACGGCACA ATCGATTTCC
301 CTGGTATGAT GCAGCCTCTA TTTATCGCAG CCGTTTCCGA TCATAAGGGC AGATACTGAC
361 TGCCTTAGAA TTCTTGACAA TGATGGCCCG CAAAATGAAG GATACCGACT CCGAGGAAGA
421 GATCCGCGAG GCTTTCAAGG TGTTTGATCG TGACAACAAT GGATTCATCT CTGCTGCTGA
481 ATTGCGCCAC GTTATGACCT CGATTGGCGA AAAGTTGACC GACGACGAAG TTGACGAGAT
541 GATTCGCGAG
```

Sequence analysis

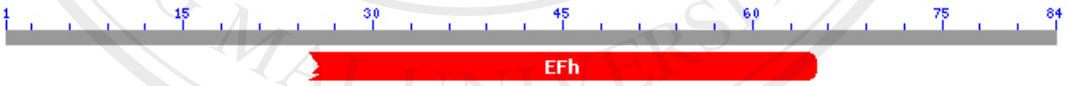
A. Similarity search

Types	Organism	Description	GenBank acc. no.	E-value	Identity (%)
BLASTN	*Talaromyces flavus*	calmodulin (cmd) gene	AY678609	7e-166	469/537 (87%)
	Penicillium pinophilum	calmodulin (cmd) gene	AY678604	7e-166	469/537 (87%)
	Penicillium aculeatum	calmodulin (cmd) gene	AY678603	7e-166	468/536 (87%)
BLASTX	*Aspergillus heteromorphus*	Calmodulin	CAM12277	6e-47	62/62 (100%)
	Fusarium proliferatum	Calmodulin	AAL04428	1e-46	61/61 (100%)
	Coccidioides immitis	Calmodulin (CaM)	XP1248642	2e-46	61/61 (100%)

B. Recognition of coding frame

```
  3  CTCTATTTGTAAGTTTGGATTTCTGGTTGTCGCAATGTTGTGGTGGGTGGTTCGCTGACT

  1                                                R  H  E  H  Q  T
 63  AGCCGTTTGGATGAATAGGACAAGGATGGTGATGGTGAGTGACGCCACGAACACCAGACA

  7   Y  S  L  R  T  K  S  Y  Y  C  E  Q  I  L  I  T  S  I  G  Q
123  TATAGTCTTCGAACAAAAAGTTATTACTGCGAACAGATATTAATAACATCAATAGGTCAA

 27   I  T  T  K  E  L  G  T  V  M  R  S  L  G  Q  N  P  S  E  S
183  ATTACAACCAAGGAACTGGGCACCGTCATGCGTTCCCTCGGCCAGAACCCTTCCGAATCC

 47   E  L  Q  D  M  I  N  E  V  D  A  D  N  N  G  T  I  D  F  P
243  GAATTGCAGGACATGATCAACGAGGTCGACGCTGACAACAACGGCACAATCGATTTCCCT


 67   G  M  M  Q  P  L  F  I  A  A  V  S  D  H  K  G  R  Y
303  GGTATGATGCAGCCTCTATTTATCGCAGCCGTTTCCGATCATAAGGGCAGATACTGACTG


363  CCTTAGAATTCTTGACAATGATGGCCCGCAAAATGAAGGATACCGACTCCGAGGAAGAGA


423  TCCGCGAGGCTTTCAAGGTGTTTGATCGTGACAACAATGGATTCATCTCTGCTGCTGAAT


483  TGCGCCACGTTATGACCTCGATTGGCGAAAAGTTGACCGACGACGAAGTTGACGAGATGA


543  TTCGCGAG
```

C. Function analysis



Descriptions

EFh (cd00051) or EF-hand, calcium binding motif; a diverse superfamily of calcium sensors and calcium signal modulators; most examples in this alignment model has 2 active canonical EF hands. Ca_2^+ binding induces a conformational change in the EF-hand motif, leading to the activation or inactivation of target proteins.

Figure 19. Characterization of *P. marneffei* calmodulin (*cam*) gene.

5.5.4) Catalase gene (*cat*)

Isolation of a specific probe for *cat* gene. In a previous study by Yuen *et al.* (2003), a gDNA library was constructed from the mycelial phase of *P. marneffei* in ZAP Express® vector. The catalase specific primers were designed and used to amplified gene fragment from gDNA of *P. marneffei*. The amplified product of about 550 bp was sequenced and used as a probe to screen the cDNA and gDNA libraries.

Screening for full-length gene from cDNA and gDNA libraries. There was no positive clone from screening of 10^5 recombinant plaques from the *P. marneffei* cDNA library. However, 13 positive clones were obtained from screening of gDNA library (3 x 10^4 pfu) screening. These clones were characterized for the largest insert size by PCR using T7 and T3 primers (Figure 20).

Characterization of partial *cat* gene. The selected positive clone 3.1, 6.2 and 8.1 were sequenced and characterized. The BLASTN analysis of clone 3.1 nucleotide sequences demonstrated homology to the *catA* gene, which encodes for spore-specific catalase (*CatA*) of the fungus *Aspergillus fumigatus*. BLASTX analysis also gave significant hits to the catalase A. This gene was functionally identified. This clone did not contain a full-length gene. The deduced amino acid showed a reading frame of 329-amino acids. The motif scan search found the conserved domain for catalase. This match thus confirmed the possible catalase function of this clone (Figure 21).

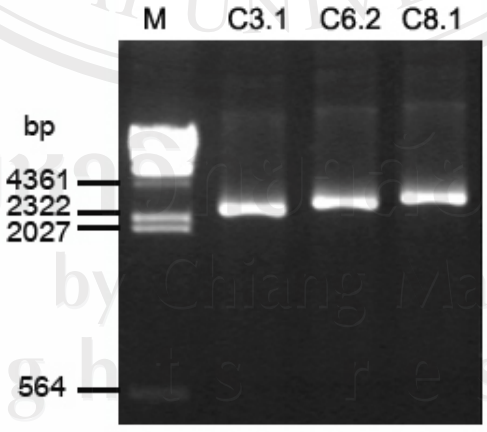


Figure 20. The PCR amplification of *cat* gene form gDNA library positive clones. The λ-phage positive clones were checked for insert of *P. marneffei cat* gene fragment by using T3 and T7 franking primers. These clones were amplified and sequenced.

Catalase

DNA sequence of *P. marneffei cat* fragment

```
   1 GATCACTAAG GGAACAGTCA ACTACTGGCC GAATCGGTTT GATGCGGTAC CACCAATCAA
  61 GCCTGAGGAC GGTGGATTCG TTTCTTACCC CGAGAAGATC CAGGCAATCA AAAAGCGTAG
 121 CCAGGGACCC AAGTTCCGCG AACATCACAA CCAGGCGCAG TTATTCTACA ACTCTCTTAC
 181 TGAATACGAG CAACACCACG TCGCCAAAGC GTTTAGCTTT GAGCTTGACC ATTGCGACGA
 241 CCCTGTGGTC TACCAGCGCA TGGCCTTGCG TATTGCAGAG ATTGATCTTT CCCTCGCCCA
 301 GAAAGTCGCC GTAATGGTCG GCGCCCCAAC ACCTGAAACT CCCGGAAAGC AGAACCACGG
 361 CAAAAAGACT CGCGGTCTTT CACAAATTGA CTTCAAGCCT CGTGTACCAA CTGTGGCCAG
 421 CAGACGTATC GCCATAATCA TTGGCGACGG CTTCGACTCT GTCGCTTTTA ACGGAGTGTA
 481 TACTGCCATC AAAGCTGCTG GCGCTCTGCC ATTTGTCATC GGCACAAAGA GACAACCCAT
 541 CTTCGCAGAT GGCGTTGACC CCCAGACCGG AAATGGCGTA ACACCCGAAC ACCAATACGA
 601 GGGTGTGCGT TCTACAATGT TCGACGCTAC TTTCATCCCC GGTGGACCAC ACGTCAAGAC
 661 CCTTGCCAGG ATCGGACGGA TTCGCCACTG GATAACAGAA ACATTCGGTC ACCTCAAAGC
 721 GCTCGGCGCG ACAGGTGAGG CCGTCGATTT TGTCAAGCAG GCGCTTCTAG GTGTGGAGGC
 781 TGTACAATTT GCTAGTCAGA GTAGCACTGG TGTCGTTGAG TCGTATGGCG TGGTTACTGC
 841 AGCTGGCCCT CAGAAACCGG AGAGTTTCAA GGAAGGATGG AAGATGATTA AGGGTGCGAC
 901 TGATTTCCTA GGCAAGTTCT TCTTTGAGGT TGGTGAACAT AGGAATTATC GACGTGAGCT
 961 TGATGGGTTG GCTGATACCC TCGCCTTCTA AATGGCATTG ATATGATGAC
```

Sequence analysis

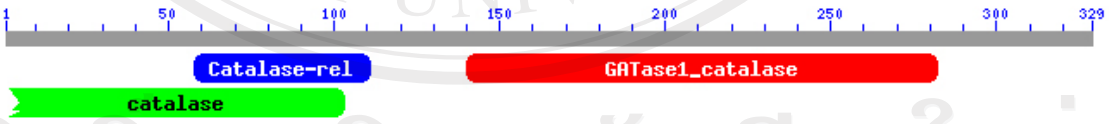
A. Similarity search

Types	Organism	Description	GenBank acc. no.	E-value	Identity (%)
BLASTN	*Aspergillus fumigatus*	spore-specific catalase (CatA)	XM742595	3e-75	683/1014 (67%)
	Aspergillus clavatus	catalase A mRNA	XM127163	1e-73	671/1000 (67%)
	Aspergillus fumigatus	catalase gene	AFU87630	7e-71	681/1013 (67%)
BLASTX	*Aspergillus fumigatus*	spore-specific catalase CatA	XP747688	6e-125	220/334 (65%)
	Aspergillus oryzae	catalase	BAC56946	9e-117	208/330 (63%)
	Aspergillus nidulans	catalase A	EAA60671	1e-113	206/330 (62%)

B. Recognition of coding frame

```
  1   I  T  K  G  T  V  N  Y  W  P  N  R  F  D  A  V  P  P  I  K
  2  ATCACTAAGGGAACAGTCAACTACTGGCCGAATCGGTTTGATGCGGTACCACCAATCAAG

 21   P  E  D  G  G  F  V  S  Y  P  E  K  I  Q  A  I  K  K  R  S
 62  CCTGAGGACGGTGGATTCGTTTCTTACCCCGAGAAGATCCAGGCAATCAAAAAGCGTAGC

 41   Q  G  P  K  F  R  E  H  H  N  Q  A  Q  L  F  Y  N  S  L  T
122  CAGGGACCCAAGTTCCGCGAACATCACAACCAGGCGCAGTTATTCTACAACTCTCTTACT

 61   E  Y  E  Q  H  H  V  A  K  A  F  S  F  E  L  D  H  C  D  D
182  GAATACGAGCAACACCACGTCGCCAAAGCGTTTAGCTTTGAGCTTGACCATTGCGACGAC

 81   P  V  V  Y  Q  R  M  A  L  R  I  A  E  I  D  L  S  L  A  Q
242  CCTGTGGTCTACCAGCGCATGGCCTTGCGTATTGCAGAGATTGATCTTTCCCTCGCCCAG

101   K  V  A  V  M  V  G  A  P  T  P  E  T  P  G  K  Q  N  H  G
302  AAAGTCGCCGTAATGGTCGGCGCCCCAACACCTGAAACTCCCGGAAAGCAGAACCACGGC

121   K  K  T  R  G  L  S  Q  I  D  F  K  P  R  V  P  T  V  A  S
362  AAAAAGACTCGCGGTCTTTCACAAATTGACTTCAAGCCTCGTGTACCAACTGTGGCCAGC

141   R  R  I  A  I  I  I  G  D  G  F  D  S  V  A  F  N  G  V  Y
422  AGACGTATCGCCATAATCATTGGCGACGGCTTCGACTCTGTCGCTTTTAACGGAGTGTAT

161   T  A  I  K  A  A  G  A  L  P  F  V  I  G  T  K  R  Q  P  I
482  ACTGCCATCAAAGCTGCTGGCGCTCTGCCATTTGTCATCGGCACAAAGAGACAACCCATC

181   F  A  D  G  V  D  P  Q  T  G  N  G  V  T  P  E  H  Q  Y  E
542  TTCGCAGATGGCGTTGACCCCCAGACCGGAAATGGCGTAACACCCGAACACCAATACGAG

201   G  V  R  S  T  M  F  D  A  T  F  I  P  G  G  P  H  V  K  T
602  GGTGTGCGTTCTACAATGTTCGACGCTACTTTCATCCCCGGTGGACCACACGTCAAGACC

221   L  A  R  I  G  R  I  R  H  W  I  T  E  T  F  G  H  L  K  A
662  CTTGCCAGGATCGGACGGATTCGCCACTGGATAACAGAAACATTCGGTCACCTCAAAGCG

241   L  G  A  T  G  E  A  V  D  F  V  K  Q  A  L  L  G  V  E  A
722  CTCGGCGCGACAGGTGAGGCCGTCGATTTTGTCAAGCAGGCGCTTCTAGGTGTGGAGGCT

261   V  Q  F  A  S  Q  S  S  T  G  V  V  E  S  Y  G  V  V  T  A
782  GTACAATTTGCTAGTCAGAGTAGCACTGGTGTCGTTGAGTCGTATGGCGTGGTTACTGCA

281   A  G  P  Q  K  P  E  S  F  K  E  G  W  K  M  I  K  G  A  T
842  GCTGGCCCTCAGAAACCGGAGAGTTTCAAGGAAGGATGGAAGATGATTAAGGGTGCGACT

301   D  F  L  G  K  F  F  F  E  V  G  E  H  R  N  Y  R  R  E  L
902  GATTTCCTAGGCAAGTTCTTCTTTGAGGTTGGTGAACATAGGAATTATCGACGTGAGCTT

321   D  G  L  A  D  T  L  A  F
962  GATGGGTTGGCTGATACCCTCGCCTTCTAAATGGCATTGATATGATGAC
```

C. Function analysis



Descriptions

- GATase1_catalase (cd03132); type 1 glutamine amidotransferase (GATase1)-like domain.
- Catalase-rel (pfam06628); Catalase-related, this family represents a small conserved region within catalase enzymes.
- Catalase (cd00328); catalase is an ubiquitous enzyme found in both prokaryotes and eukaryotes involved in the protection of cells from the toxic effects of peroxides.

Figure 21. Characterization of *P. marneffei* catalase (*cat*) gene.

5.6. Specific primers for RT-PCR analysis

Specific primers for RT-PCR analysis were indicated in Table 1. The primers were 19-22 nucleotides in length, GC content 50-65% and *T*m was 60-80^{o}C. All of primers were checked for non-dimers and hairpin formation. The desired product size was about 400-450 bp which was smaller than 630 bp of internal control. Specific primers for *sodA* and *gapdh* could amplify the mRNA products in an expected size while amplified products with genomic DNA-containing intron were larger (Appendix C). The expected cDNA product sizes were successfully amplified with *hsp70*, *acuD cat*, *cam* and *cpeA* primers (Appendix C). However, these primers could amplify genomic DNA in the same product size as well (data not shown). Because these primers cannot differentiate between mRNA and gDNA amplification, all RNA samples were treated with DNaseI and checked for DNA contamination with PCR before performing the RT-PCR analysis. In addition, to confirm the specificity of primers, the RT-PCR amplified products of gene of interest were sequenced (data not shown).

5.7. RNA qualitative and quantitative

The RNA samples should be purified and should not be contaminated with any mouse DNA or RNA. Thus the aliquot samples were checked for the absence of mouse GAPDH by RT-PCR amplification with mouse GAPDH primers (Figure 22). Direct PCR of the DNaseI-treated RNA samples failed to be amplified with the primers for the 18S rRNA fragment. This result indicated no DNA contamination in the RNA samples used in RT-PCR.

5.8. RT-PCR analysis

Semi-quantitative RT-PCR was used to examine expression of genes of interest in phase transition and during macrophage infection. The one-tube reactions included 18S rRNA primers as an internal control to normalize the amount of total RNA. The experiments were performed in duplicate.

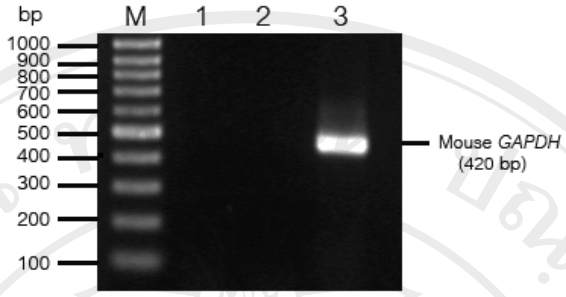
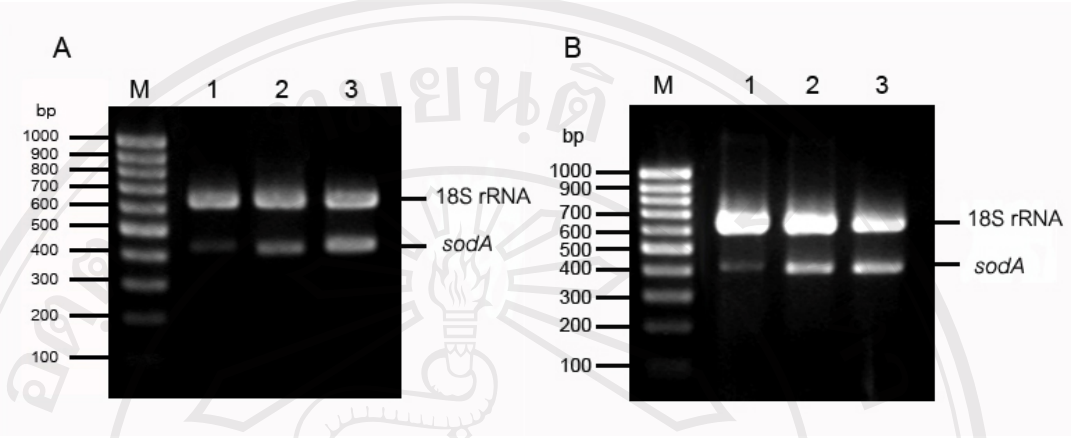


Figure 22. Detection of mouse RNA and DNA contamination in RNA samples. The aliquot samples were checked for the absence of mouse RNA by RT-PCR amplification with mouse G3PDH primers. The product of 420 bp of mouse *GAPDH* is shown in lane 3, the absence of product in RNA and DNA of *P. marneffei* is shown in lane 1 and 2, respectively.

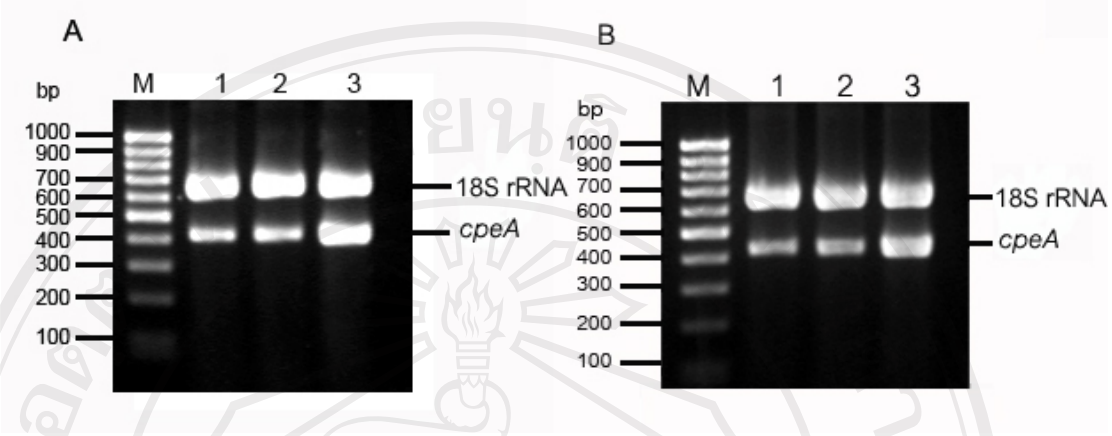
5.8.1) Differential gene expression in different phases of *P. marneffei*

Transcripts of *sodA* and *cpeA* appeared to be deposited in dormant conidia. However, the expression of both genes markedly increased when conidia underwent conversion to the yeast phase by incubation at 37°C for 96 h. Expression of the *sodA* and *cpeA* in filamentous phase at 25°C was significantly lower than that at 37°C ($P<0.05$) (Figure 23, 24 and 30). Transcripts of *hsp70*, *acuD* and *gapdh* were constitutively expressed in mycelial, conidia and yeast phases. Although the slight upregulation of these genes could be observed in yeast phase, the statistical comparison of relative genes expression revealed no significant differences ($P>0.05$) (Figure 25, 26, 27 and 30). As shown in Figure 28 and 29, the *cat* and *cam* were differentially regulated according to cell types, respectively. The transcripts of both genes were abundant in conidia and extremely low in mycelium and yeast cells.



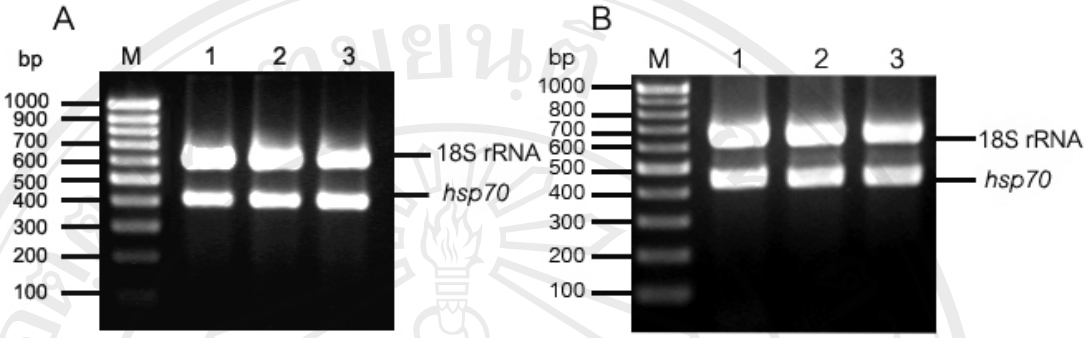
Phases	Relative *sodA* expression levels		
	A	**B**	**Mean**
Mycelium	0.25	0.13	0.19
Conidia	0.48	0.36	0.42
Yeast	0.72	0.55	0.64

Figure 23. Differential expression of *P. marneffei* Cu, Zn SOD gene (*sodA*) in different phases. Total RNA from mycelium, conidia and yeast was extracted and used in RT-PCR experiments (lanes 1-3, respectively). Panel A and B shows the results of agarose gel electrophoresis analysis, where the amplified RT-PCR products resulting from duplicate samples. The molecular marker is indicated on the left (M). The *sodA* expression levels were calculated in relation of the internal control 18S rRNA as shown in a table.



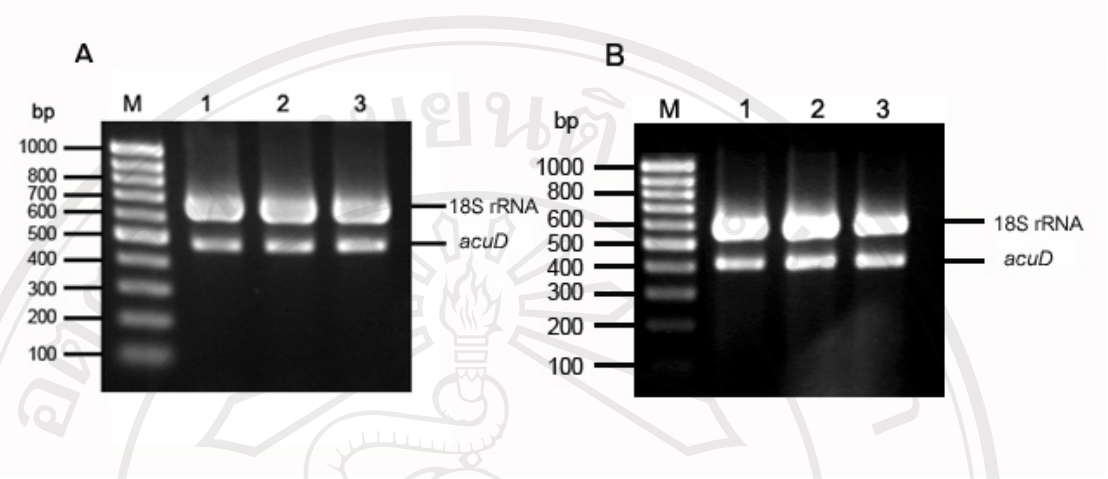
Phases	Relative *cpeA* expression levels		
	A	**B**	**Mean**
Mycelium	0.22	0.21	0.22
Conidia	0.51	0.56	0.54
Yeast	0.90	0.93	0.92

Figure 24. Differential expression of *P. marneffei* catalase peroxidase gene (*cpeA*) in different phases. Total RNA from mycelium, conidia and yeast was extracted and used in RT-PCR experiments (lanes 1-3, respectively). Panel A and B shows the results of agarose gel electrophoresis analysis, where the amplified RT-PCR products resulting from duplicate samples. The molecular marker is indicated on the left (M). The *cpeA* expression levels were calculated in relation of the internal control 18S rRNA as shown in a table.



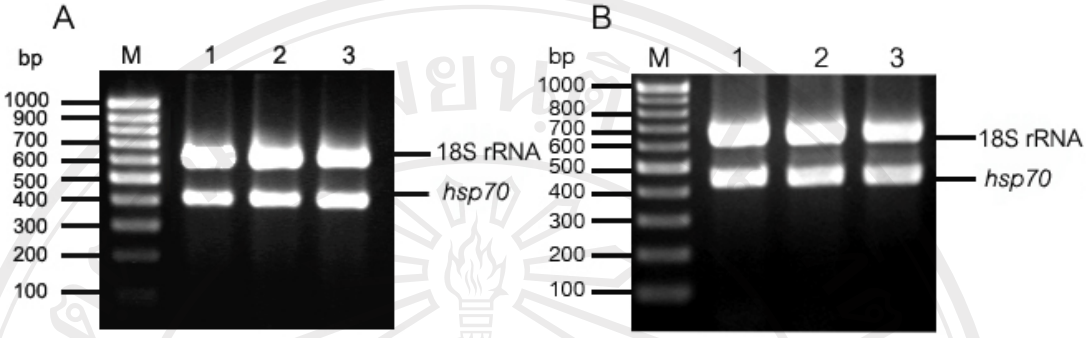
Phases	Relative *hsp70* expression levels		
	A	**B**	**Mean**
Mycelium	0.68	0.67	0.68
Conidia	0.71	0.68	0.70
Yeast	0.76	0.75	0.76

Figure 25. Differential expression of *P. marneffei* heat shock protein 70 gene (*hsp70*) in different phases. Total RNA from mycelium, conidia and yeast was extracted and used in RT-PCR experiments (lanes 1-3, respectively). Panel A and B shows the results of agarose gel electrophoresis analysis, where the amplified RT-PCR products resulting from duplicate samples. The molecular marker is indicated on the left (M). The *hsp70* expression levels were calculated in relation of the internal control 18S rRNA as shown in a table.



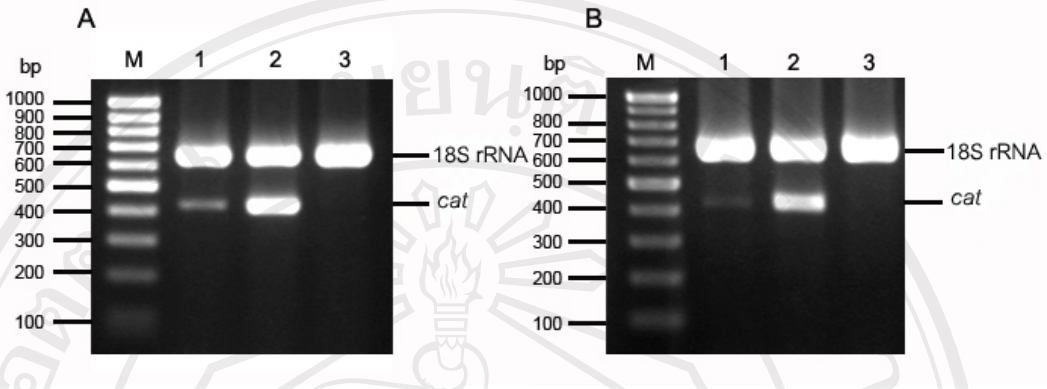
Phases	Relative *acuD* expression levels		
	A	**B**	**Mean**
Mycelium	0.50	0.37	0.44
Conidia	0.48	0.42	0.45
Yeast	0.53	0.45	0.49

Figure 26. Differential expression of *P. marneffei* isocitrate lyase gene (*acuD*) in different phases. Total RNA from mycelium, conidia and yeast was extracted and used in RT-PCR experiments (lanes 1-3, respectively). Panel A and B shows the results of agarose gel electrophoresis analysis, where the amplified RT-PCR products resulting from duplicate samples. The molecular marker is indicated on the left (M). The *acuD* expression levels were calculated in relation of the internal control 18S rRNA as shown in a table.



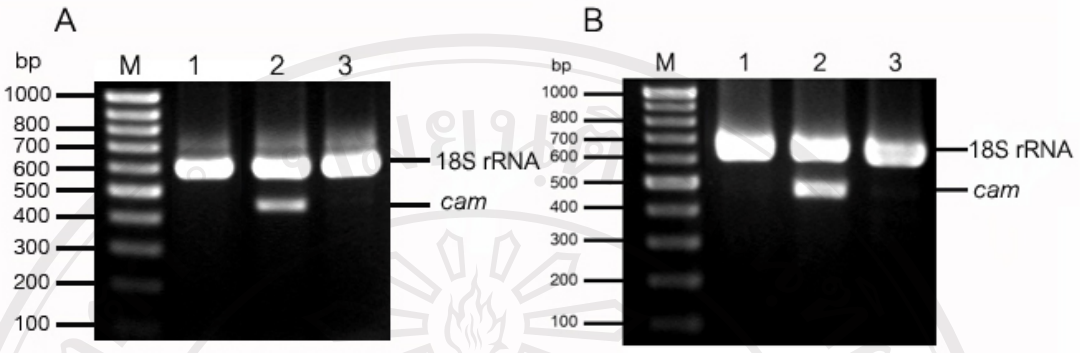
Phases	Relative *gapdh* expression levels		
	A	**B**	**Mean**
Mycelium	0.60	0.63	0.62
Conidia	0.76	0.75	0.76
Yeast	0.47	0.58	0.53

Figure 27. Differential expression of *P. marneffei gapdh* gene in different phases. Total RNA from mycelium, conidia and yeast was extracted and used in RT-PCR experiments (lanes 1-3, respectively). Panel A and B shows the results of agarose gel electrophoresis analysis, where the amplified RT-PCR products resulting from duplicate samples. The molecular marker is indicated on the left (M). The *gapdh* expression levels were calculated in relation of the internal control 18S rRNA as shown in a table.



Phases	Relative *cat* expression levels		
	A	**B**	**Mean**
Mycelium	0.25	0.17	0.21
Conidia	0.71	0.61	0.66
Yeast	0	0	0

Figure 28. Differential expression of *P. marneffei* catalase gene (*cat*) in different phases. Total RNA from mycelium, conidia and yeast was extracted and 200 ng of total RNA was used in RT-PCR experiments (lanes 1-3, respectively). Panel A and B shows the results of agarose gel electrophoresis analysis, where the amplified RT-PCR products resulting from duplicate samples. The molecular marker is indicated on the left (M). The *cat* expression levels were calculated in relation of the internal control 18S rRNA as shown in a table.



Phases	Relative *cam* expression levels		
	A	**B**	**Mean**
Mycelium	0	0	0
Conidia	0.35	0.47	0.41
Yeast	0	0	0

Figure 29. Differential expression of *P. marneffei* camodulin gene (*cam*) in different phases. Total RNA from mycelium, conidia and yeast was extracted and 200 ng of total RNA was used in RT-PCR experiments (lanes 1-3, respectively). Panel A and B shows the results of agarose gel electrophoresis analysis, where the amplified RT-PCR products resulting from duplicate samples. The molecular marker is indicated on the left (M). The *cam* expression levels were calculated in relation of the internal control 18S rRNA as shown in a table.

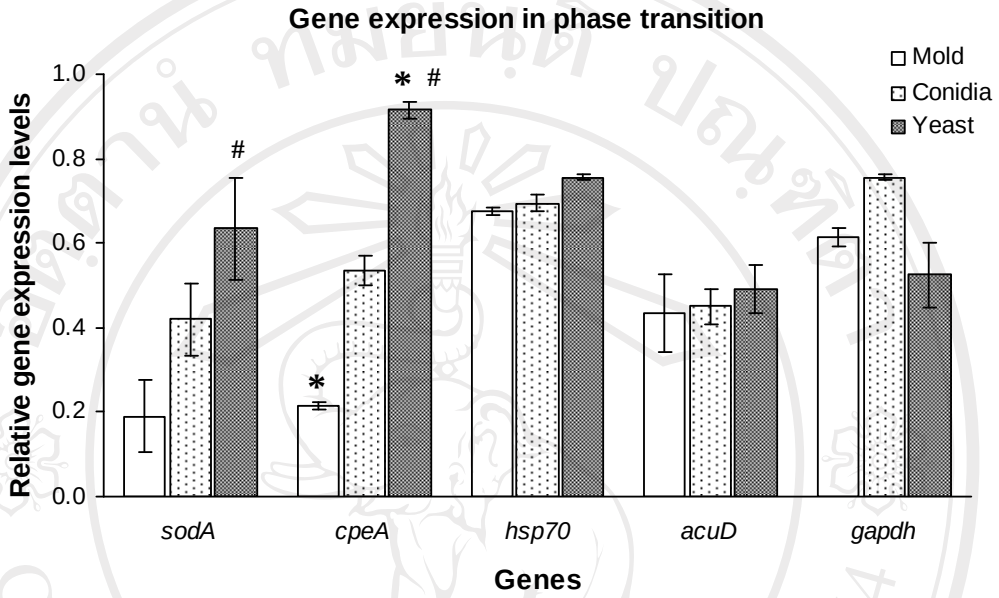


Figure 30. Relative expression levels of genes of interest in phases of *P. marneffei*. The expression of *sodA*, *cpeA*, *hsp70*, *acuD* and *gapdh* in mycelial, conidia and yeast phase were examined by RT-PCR. The product band densities of 18S rRNA internal control and gene of interest were measured and calculated for relative expression levels. The asterisk (*****) indicates the significant different an expression levels from dormant conidia and # indicates a significant different an expression levels from mycelial form ($P<0.05$). The results of *cat* and *cam* expressions were excluded because these genes were not highly expressed in any growth form.

5.8.2) Differential gene expression of *P. marneffei* during macrophage infection

During macrophage infection, the *sodA* transcript was significantly upregulated in comparison with the expression in the control conidia cultured in cell-free medium ($P<0.05$). Also, the relative expression levels slightly increased during prolonged co-incubation of conidia with macrophage cells (Figure 31). The transcript of the *P. marneffei cpeA* was extremely upreglated during macrophage infection at 2 and 4 h of infection ($P<0.05$). Only slight upregulation of *cpeA* transcript was observed at 8 h of infection ($P>0.05$) (Figure 32). The expression of *hsp70*, *acuD* and *gapdh* during infection was not significantly different from the conidia control ($P>0.05$) (Figure 33, 34 and 35, respectively). The expression analysis by RT-PCR of *cam* and *cat* was undetectable in both control and intracellular conidia, although a high amount of RNA template (200 ng) was used with the PCR amplification cycles of 30 (data not shown).

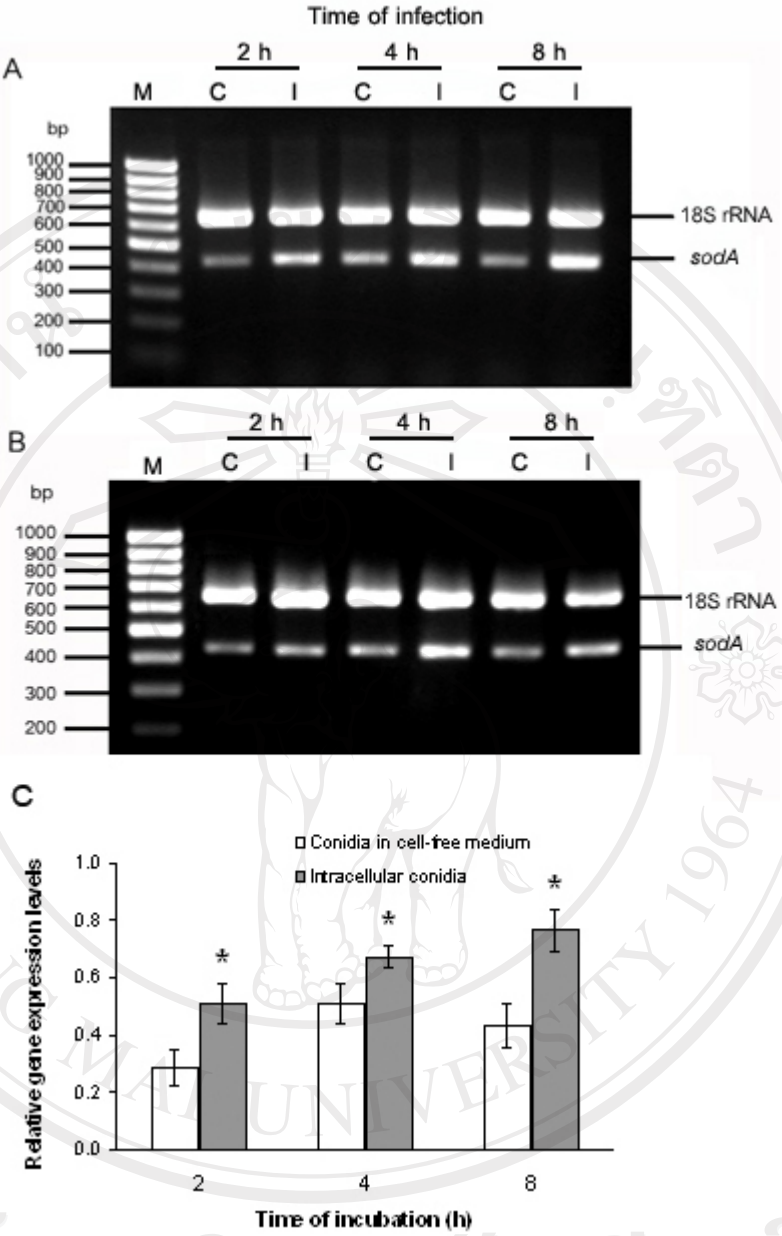


Figure 31. Differential expression of *P. marneffei sodA* during macrophage infection. After co-culture of *P. marneffei* conidia with murine macrophage cells J774.1 for 2, 4 and 8 h, the fungal cells were harvested by centrifugation, after macrophages lysis. The *P. marneffei* total RNA was extracted and used in RT-PCR experiments, as described in material and methods section. As a control condition, total RNA from *P. marneffei* conidia cultivated *in vitro*, in cell-free medium for 2, 4 and 8 h, was employed. Panel A and B show the duplicate results of agarose gel electrophoresis analysis, where the amplified RT-PCR products resulting from the control and macrophage internalization experiments are shown in (C) and (I), respectively. Panel C shows the semi-quantitative analysis of *sodA* expression, performed by densitometry. The relative expression levels were calculated in relation to the internal control 18S rRNA (*P<0.05).

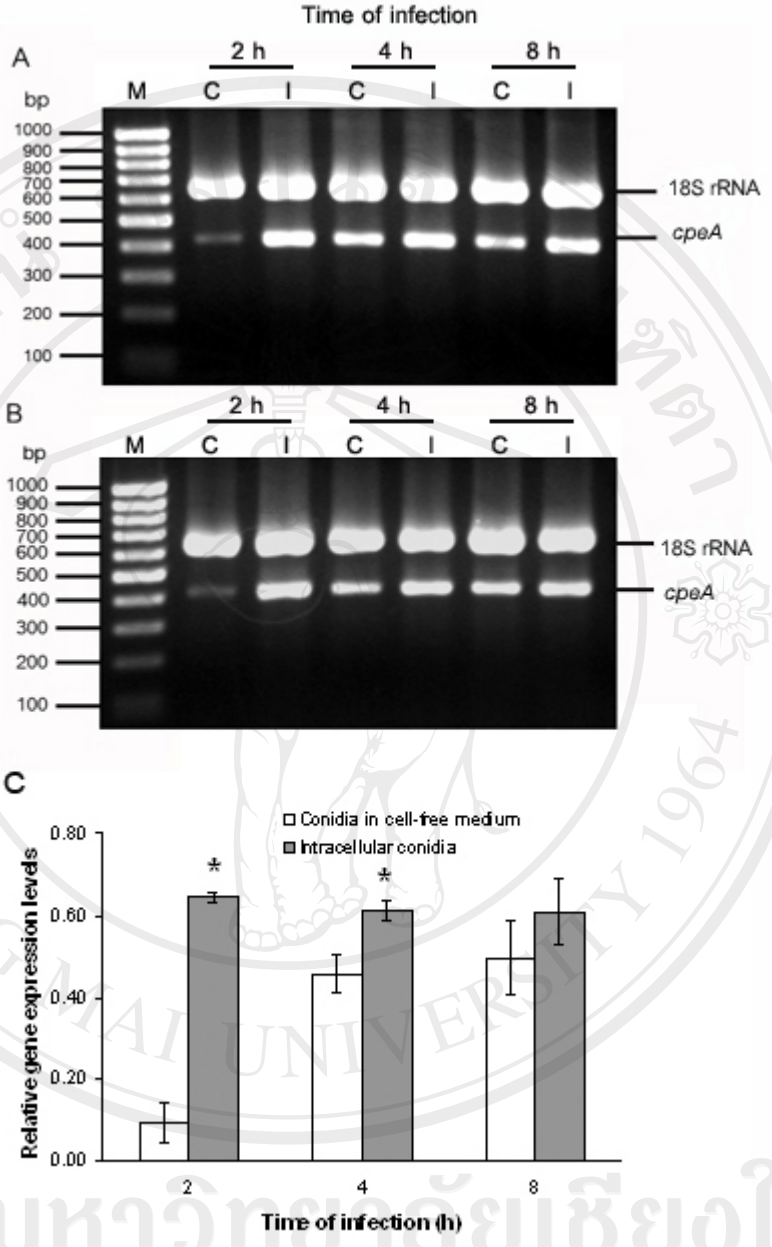


Figure 32. Differential expression of *P. marneffei cpeA* during macrophage infection. Panel A and B show the duplicate results of agarose gel electrophoresis analysis, where the amplified RT-PCR products resulting from the control and macrophage internalization experiments are shown in (C) and (I) at 2, 4 and 8 h after incubation, respectively. Panel C shows the semi-quantitative analysis of *cpeA* expression, performed by densitometry. The relative expression levels were calculated in relation to the internal control 18S rRNA (**P*<0.05).

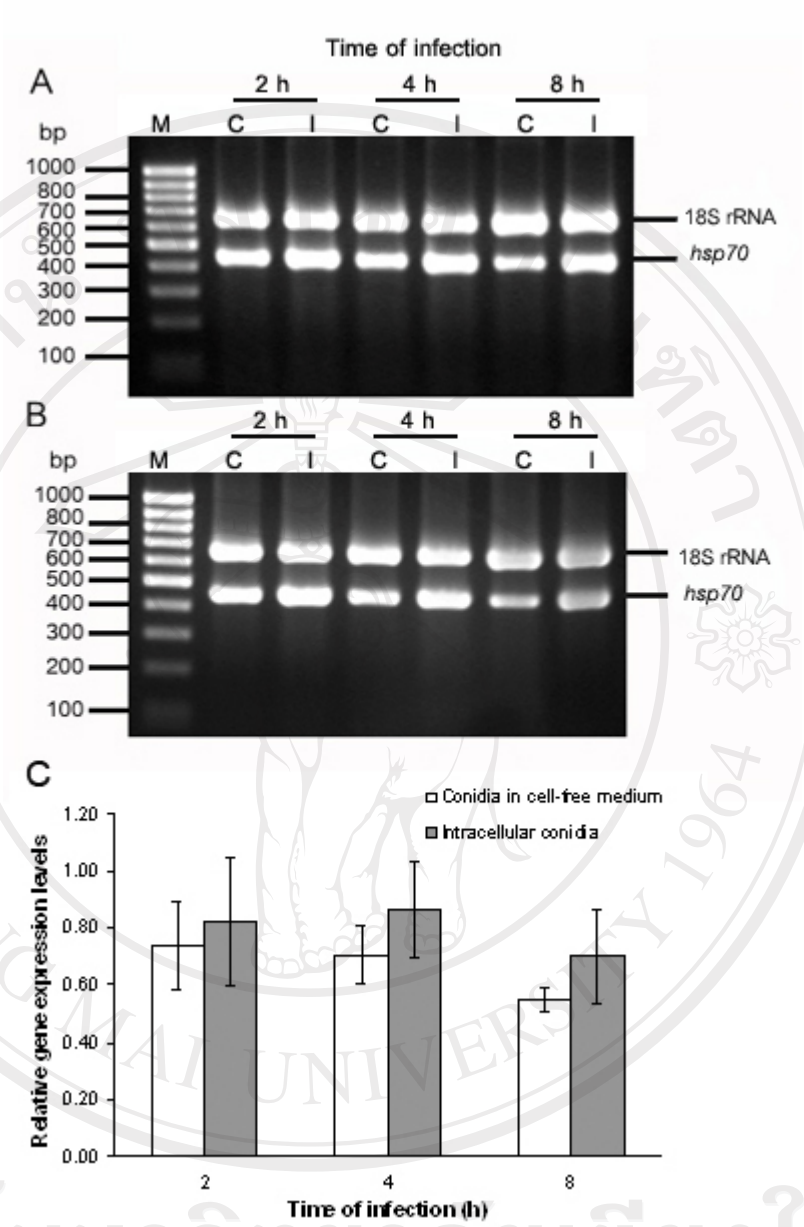


Figure 33. Differential expression of *P. marneffei hsp70* during macrophage infection. Panel A and B show the duplicate results of agarose gel electrophoresis analysis, where the amplified RT-PCR products resulting from the control and macrophage internalization experiments are shown in (C) and (I) at 2, 4 and 8 h after incubation, respectively. Panel C shows the semi-quantitative analysis of *hsp70* expression, performed by densitometry. The relative expression levels were calculated in relation to the internal control 18S rRNA.

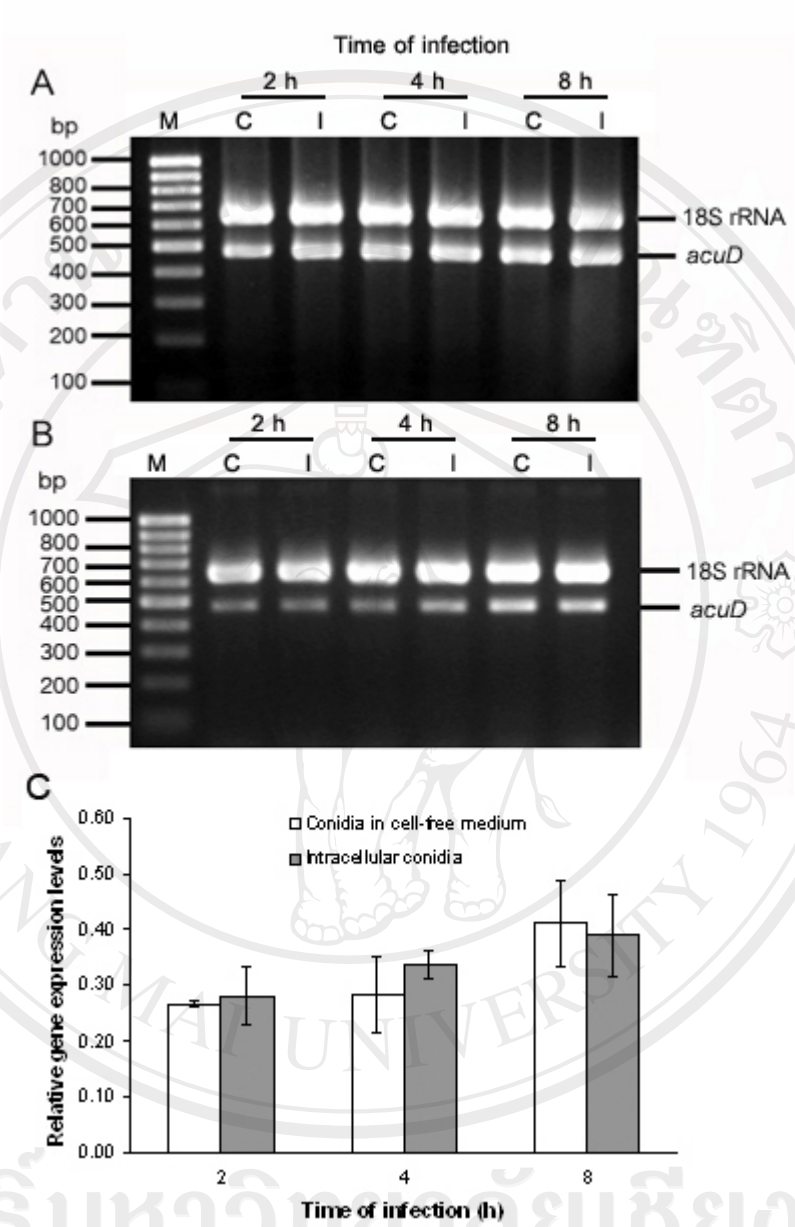


Figure 34. Differential expression of *P. marneffei acuD* during macrophage infection. Panel A and B show the duplicate results of agarose gel electrophoresis analysis, where the amplified RT-PCR products resulting from the control and macrophage internalization experiments are shown in (C) and (I) at 2, 4 and 8 h after incubation, respectively. Panel C shows the semi-quantitative analysis of *acuD* expression, performed by densitometry. The relative expression levels were calculated in relation to the internal control 18S rRNA.

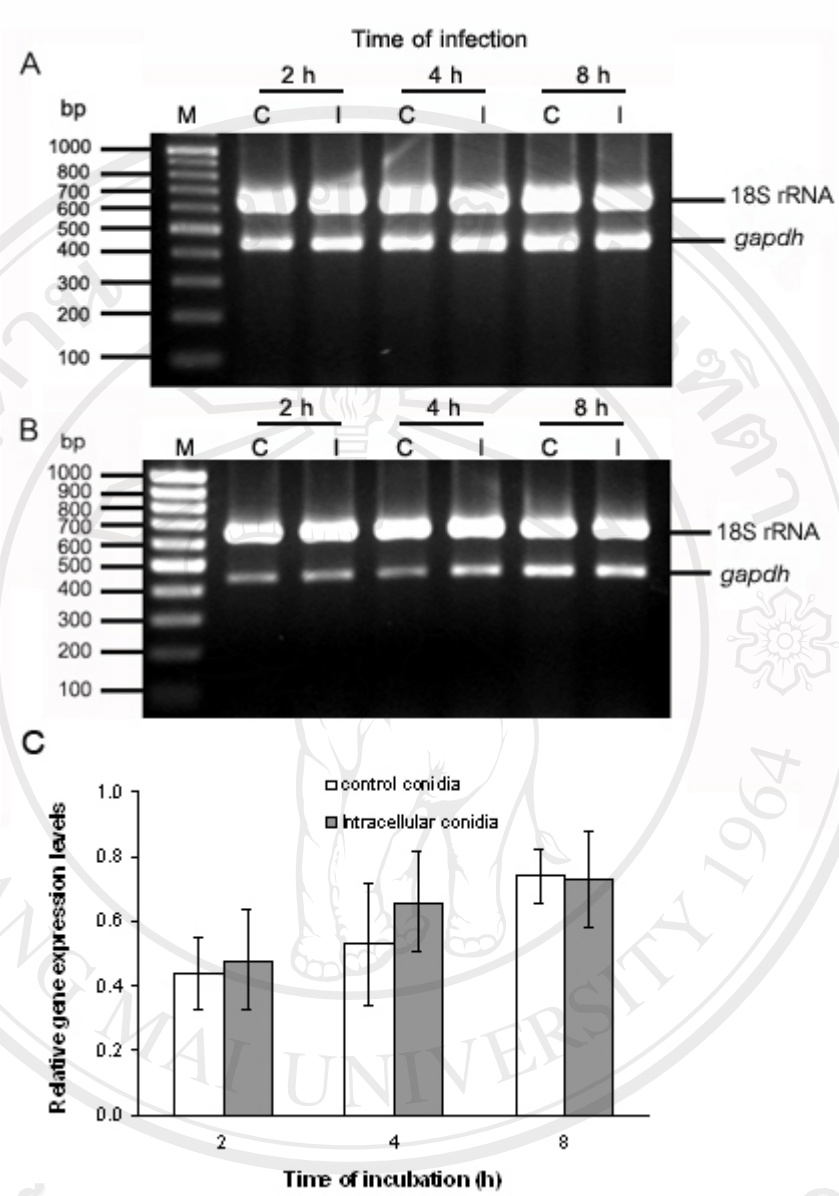


Figure 35. Differential expression of *P. marneffei gapdh* during macrophage infection. Panel A and B show the duplicate results of agarose gel electrophoresis analysis, where the amplified RT-PCR products resulting from the control and macrophage internalization experiments are shown in (C) and (I) at 2, 4 and 8 h after incubation, respectively. Panel C shows the semi-quantitative analysis of *gapdh* expression, performed by densitometry. The relative expression levels were calculated in relation to the internal control 18S rRNA.

5.9. Northern blot analysis of differential *sodA, cpeA, acuD, gapdh* and *hsp70* expression in phase transition and during macrophage infection

The expression of putative virulence genes in phase transition and during macrophage infection of *P. marneffei* was confirmed by Northern blot analysis in which total RNA was probed with a cDNA fragment encoding the whole ORF (*sodA*, *gapdh*, *hsp70* and *cpeA*) or cDNA fragment genes (*acuD*). The *sodA* mRNA transcript was barely detectable in all RNA samples. However, differential expressions could be observed and the results generally supported RT-PCR analysis by showing the *sodA* transcript with more abundant in yeast than in conidia and mycelial phase, respectively. Additionally, during macrophage infection, *sodA* expression was upregulated (Figure 36). Transcription level of *cpeA* was accumulated in yeast and conidial cells but not in mycelial phase. Transcriptional *cpeA* response of *P. marneffei* conidia upon internalization by murine macrophages was extremely upregulated in 2 h of incubation with subsequently extensive upregulation in 4 and 8 h of incubation (Figure 37). In agreement, the increase of expression by the RT-PCR analysis was seen in yeast phase and during 2 h of infection (Figure 24 and 32). Although, there were similar expressions of *acuD*, *hsp70* and *gapdh* by RT-PCR, some differential expression patterns of these genes could be observed by Northern blot analysis. Highly expression of the *acuD* gene was seen in conidia and yeast than in the mycelial cells. In addition, *acuD* was upregulated in intracellular conidia after 2 and 8 h of infection (Figure 38). Otherwise, expressions of *gapdh* and *hsp70* have decreased in the yeast phase of *P. marneffei* and only *gapdh* transcript could be repressed during macrophage infection (Figure 39 and 40). However, the *hsp70* transcript of control conidia was turned over after prolong incubation, whereas the transcript of infective conidia was maintained until 8 h of incubation. At this time point, the expression of *hsp70* was higher than that in the control conidia (Figure 40).

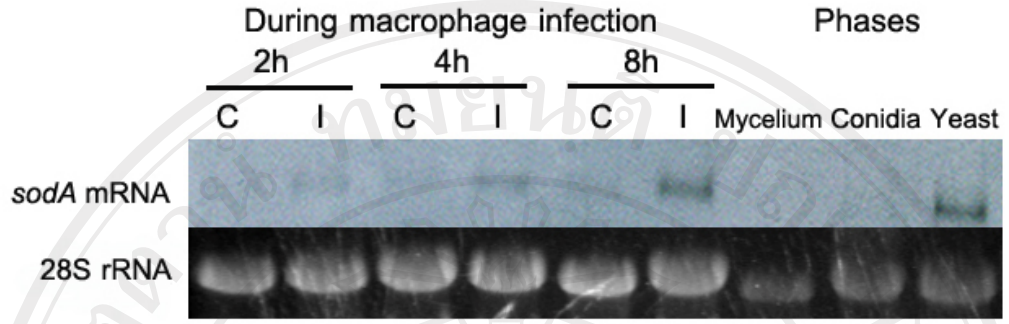


Figure 36. Expression of *sodA* during macrophage infection and different phases of *P. marneffei* by Northern hybridization. Total RNA was extracted from conidia in cell-free medium (C) and intracellular conidia (I) at 2, 4 and 8 h of incubation and also extracted from mycelial (25°C, 96 h), conidia and yeast cells (37°C, 96 h). *P. marneffei sodA* cDNA was used as a probe (upper panel) in Northern hybridization. The 28S rRNA loading control is shown in the lower panel.

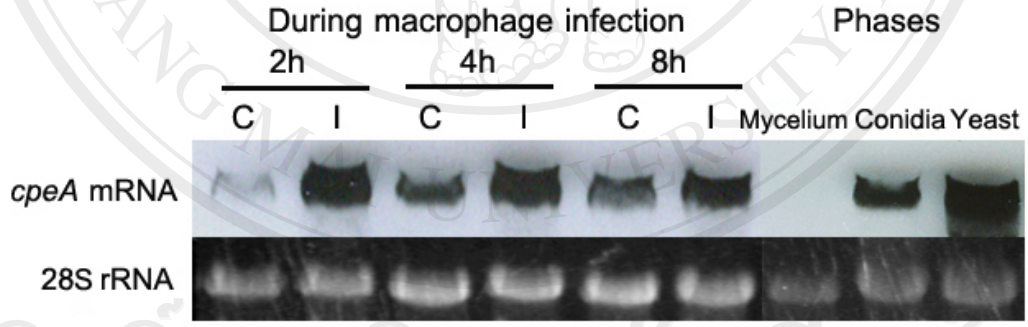


Figure 37. Expression of *cpeA* during macrophage infection and different phases of *P. marneffei* by Northern hybridization. Total RNA was extracted from conidia in cell-free medium (C) and intracellular conidia (I) at 2, 4 and 8 h of incubation and also extracted from mycelial (25°C, 96 h), conidia and yeast cells (37°C, 96 h). *P. marneffei cpeA* cDNA was used as a probe (upper panel) in Northern hybridization. The 28S rRNA loading control is shown in the lower panel.

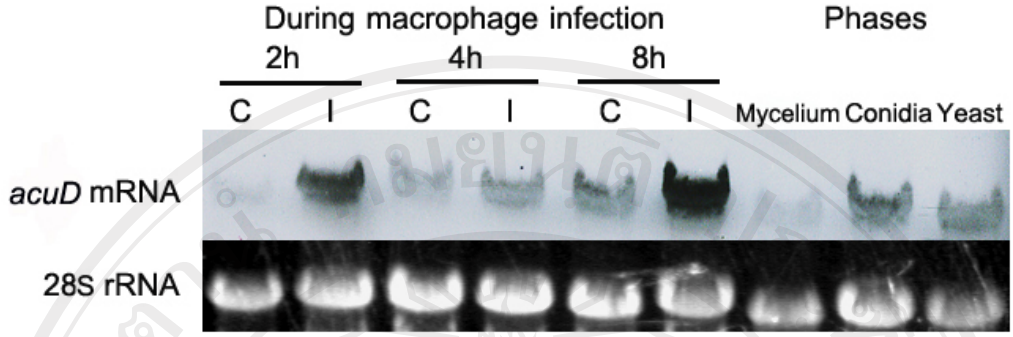


Figure 38. Expression of *acuD* during macrophage infection and different phases of *P. marneffei* by Northern hybridization. Total RNA was extracted from conidia in cell-free medium (C) and intracellular conidia (I) at 2, 4 and 8 h of incubation and also extracted from mycelial (25°C, 96 h), conidia and yeast cells (37°C, 96 h). *P. marneffei acuD* cDNA was used as a probe (upper panel) in Northern hybridization. The 28S rRNA loading control is shown in the lower panel.

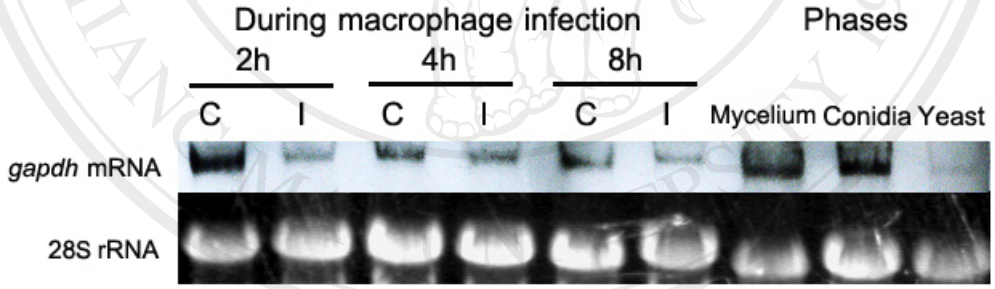


Figure 39. Expression of *gapdh* during macrophage infection and different phases of *P. marneffei* by Northern hybridization. Total RNA was extracted from conidia in cell-free medium (C) and intracellular conidia (I) at 2, 4 and 8 h of incubation and also extracted from mycelial (25°C, 96 h), conidia and yeast cells (37°C, 96 h). *P. marneffei gapdh* cDNA was used as a probe (upper panel) in Northern hybridization. The 28S rRNA loading control is shown in the lower panel.

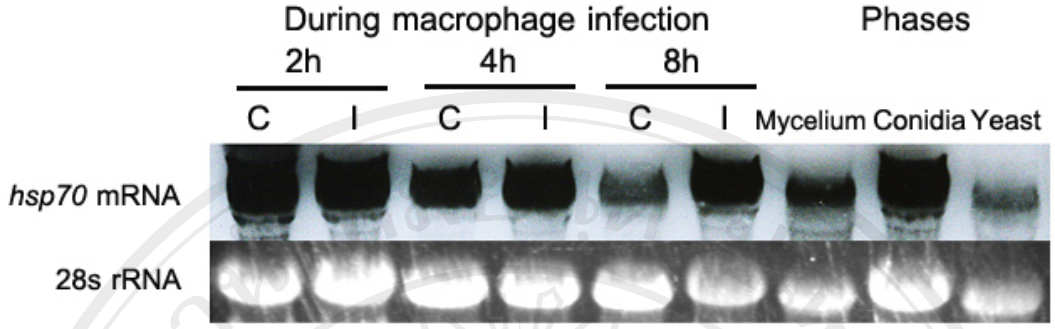


Figure 40. Expression of *hsp70* during macrophage infection and different phases of *P. marneffei* by Northern hybridization. Total RNA was extracted from conidia in cell-free medium (C) and intracellular conidia (I) at 2, 4 and 8 h of incubation and also extracted from mycelial (25°C, 96 h), conidia and yeast cells (37°C, 96 h). *P. marneffei hsp70* cDNA was used as a probe (upper panel) in Northern hybridization. The 28S rRNA loading control is shown in the lower panel.

Table 3. Relative gene expressions by RT-PCR and Northern blot analysis during macrophage infection

Genes	Fold change[a]					
	2 h		4 h		8 h	
	RT[b]	NB	RT	NB	RT	NB
Stress response						
sodA	1.82	2.81	1.31	3.71	1.77	5.04
cpeA	7.11	5.05	1.33	1.45	1.22	1.27
hsp70	1.11	1.01	1.23	1.10	1.27	1.59
Adaptation						
acuD	1.04	7.30	1.21	1.41	0.95	2.79
gapdh	1.03	0.32	1.38	0.87	0.93	0.45

[a] Fold change values were determined after normalization of each gene as the relative amount in the ration to the 18S rRNA and 28S rRNA control expression for RT-PCR (RT) and Northern blot analysis (NB), respectively.

[b] Mean fold change of two RT-PCR independent experiments done in duplicate.