IV. MATERIALS AND METHODS

4.1. Fungal strain and culture condition

Penicillium marneffei F4 strain was obtained from hemoculture of an AIDS-patient from the Central Laboratory, Maharaj Nakorn Chiang Mai Hospital in January 1999. The fungus was maintained in the mold form on SDA slant at 28 °C. The conidia from 5-day-old culture were harvested and 10^{10} conidia were used to inoculate 500 ml of brain-heart infution broth (BHIB) in a 2-liter Erlenmeyer flask ($2x10^7$ conidia/ml). The conidia were cultured at 37 °C in an orbital shaker bath with moderate shaking speed (140 rpm) for 3 days to generate the yeast form.

4.2. Total RNA isolation

Total RNA was isolated from yeast cells of *P. marneffei* by Chomczynski and Sacchi method using TRIzol® reagent (Gibco BRL, Gaithersburg, MD, USA). Three-day-old yeast cells were pelleted and washed once in sterile PBS. The yeast pellet (about 5-g wet weight) was flash-frozen in liquid nitrogen and ground to powder by using a mortar and a pestle. The cell powder was homogenized in TRIzol® reagent (use 1 ml of TRIzol® reagent per 100 mg of pellet). The lysate was transferred to a polypropylene tube and 0.2 volume of chloroform was sequentially added. The mixture was thoroughly shaken for 30 s and the tube was centrifuged at 10,000 x *g* for 20 min at 4 °C. The upper aqueous phase containing total RNA was collected and the RNA was precipitated with equal volume of ice-cold isopropanol. It was kept at room temperature for 5 min and centrifuged at 12,000 x *g* for 10 min at 4 °C. The RNA pellet was washed with 10 ml of ice-cold RNase-free 70% ethanol and dried. Finally, the pellet was resuspended in 600 µl of DEPC-treated water. The RNA concentration and purity was determined by measuring the absorption at 260 and 280 nm (Spectronics Genesys2, Spectronics, London, UK). The suspension was then stored at –80 °C until used.

4.3. Poly $(A)^+$ RNA enrichment

Poly$(A)^+$ RNA was purified from total RNA by using an Oligotex mRNA purification kit (Qiagen, GmbH, Germany). Briefly, 1 mg of the total RNA was mixed with 2X binding buffer (supplied) and the Oligotex suspension. After incubation for 10 min at 37 °C, the mixture was loaded onto a spin column and centrifuged. The column was washed twice with washing buffer (supplied), then the mRNA was eluted twice with 100-µl elution buffer. The Poly $(A)^+$ RNA concentration and purity was determined by spectrophotometry. The enriched poly $(A)^+$ RNA was stored at – 80 °C until used.

4.4. Formaldehyde agarose gel electrophoresis

For each gel, 1.5 g of agarose was melted in 85 ml of DEPC-treated water and allowed to cool down to 50-60 °C. Ten milliliters of 10X MOPS buffer (200 mM morpholinopropanesulfonic acid, 50 mM sodium acetate, 10 mM EDTA, pH 6.5-7.0) and 5.4 ml of 37 % formaldehyde (12.3 M) were added and mixed by swirling (the final concentration of agarose is approximately 1.5 % and 0.66 M for formaldehyde). The gel was poured and allowed to solidify for 30 min in a horizontal 58 gel apparatus (Gibco BRL). A 2.0 µl of sample containing 4 µg of RNA was mixed with 10 µl of formaldehyde gel loading buffer (720 µl of formamide, 160 µl of 10X MOPS buffer, 260 µl of 37 % formaldehyde, 100 µl of DEPC-treated water, 100 µl of 10 mg/ml ethidium bromide, 80 µl of glycerol, 80 µl of saturated bromphenol blue). The mixture was incubated for 15 min at 65 °C and quickly chilled on ice. All 12 µl of each sample was loaded into each well of the gel. Electrophoresis was conducted for 2 h at 75 V in 1X MOPS buffer. The gel was visualized under an UV light and photographed (GelDoc 1000, Hercules, CA, USA).

4.5. cDNA library construction

SuperScript® cDNA synthesis system (Gibco BRL) was used in the *P. marneffei* cDNA library construction. The procedure was carried out following to the Gibco BRL's protocol as described below.

4.5.1) First strand cDNA synthesis. Poly(A)$^+$ RNA (10 μg) was used as template to construct first-strand cDNA. The reaction was carried out in 20 μl of the mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 500 μM each dNTP, 10 μCi of [α-^{32}P] dCTP, 50 μg/ml *Not*I primer adapter, 10 μg of mRNA template, and 10,000 U of SuperScript II reverse transcriptase enzyme. After incubation for 1 h at 37 °C, the reaction was stopped on ice, and 2-μl was removed for first strand yield analysis by 1.4 % alkaline agarose gel electrophoresis.

4.5.2) Second strand cDNA synthesis. For second-strand cDNA synthesis, the following components were added into 18 μl of the first-strand reaction reaction in sequential order: 93 μl of DEPC-treated water, 30 μl of 5X second strand buffer, 3 μl of 10 mM dNTP mix, 1 μl of *E. coli* DNA ligase (10 U/ μl), 4 μl of E. coli DNA polymerase I (10 U/μl), and 1 μl of *E. coli* RNase H (2 U/μl). The mixture was incubated for 2 h at 16 °C.

4.5.3) Blunt ending of the cDNA ends. The cDNA was blunted by incubation with 2 μl (10 U) of T4 DNA polymerase for an additional 5 min at 16 °C. The reaction tube was placed on ice, and 10 μl of 0.5 M EDTA was added to stop the reaction. Then, 150 μl of phenol: chloroform: isoamyalcohol (25:24:1) was added. The mixture was vortexed and centrifuged for 5 min at maximum speed (14,000 rpm) in the microcentrifuge to separate the phases. The aqueous phase was transferred to a new tube and precipitated with 70 μl of 7.5 M ammonium acetate (NH_4OAc) and 500 μl of absolute ethanol for 10 min at -20 °C. After the incubation, the tube was centrifuged at maximum speed for 20 min at 4 °C. The pellet was washed with 500 μl of ice-cold RNase-free 70% ethanol and dried. The pellet was resuspended with 25 μl of DEPC-treated water.

4.5.4) *Sal*I adapter addition. The 25 μl of blunt-ended cDNA in 4.5.3 was then ligated with the *Sal*I adapter by addition of the following components: 10 μl of 5X T4

DNA ligase buffer, 10 µl of *SalI* adapters (1 µg/µl), and 5 µl of T4 DNA ligase (1 U/µl). This 50-µl reaction was incubated for a minimum of 16 h at 16 °C, and followed by the phenol extraction and ethanol precipitation as described above. The *SalI*-end-cDNA was resuspended with 41 µl of DEPC-treated water and digested with 4 µl of *NotI* (15 U/ µl) in the presence of React3 buffer. The reaction was incubated for 2 h at 37 °C. The phenol extraction and ethanol precipitation of the ready-to-clone-cDNA was then performed. The precipitated cDNA was resuspended in 100 µl of TEN buffer (10 mM Tris-HCl, 0.1 mM EDTA, 25 mM NaCl, pH 7.5)

4.5.5) Size fractionation. The double stranded cDNA molecules were size fractionated in Sephacryl S-500 sizing column. Twenty fractions (about 35 µl/fraction) were collected and determined for the amount of cDNA in each fraction by Cerenkov counting. This type of counting was performed without scintillation fluid. The tube containing the solution from each fraction was capped and placed directly in scintillation vial and then counted in the tritium channel of the β-counter (Perkin Elmer). The counts were then converted into ng of cDNA by the following relationship:

Amount of ds cDNA (ng) = [(Cerenkov cpm) x 2 x (4 pmol dNTP/pmol dCTP) x (1,000 ng/µg ds cDNA)]/ SA (cpm/pmol dCTP) x (1,515 pmol dNTP/µg ds cDNA)

The SA is a specific activity of the radioisotope in the reaction. The specific activity is defined as the counts per minute (cpm) of an aliquot of the reaction divided by the quantity of the same nucleotide (pmol) in the aliquot. For [α-^{32}P]dCTP in this study, the SA is given by the relationship:

SA (cpm/pmol dCTP) = [cpm/10 µl]/[200 pmol dCTP/10 µl]

4.5.6) Ligation. Pool-selected fractions containing 50 ng of cDNAs were ligated with the λZipLox, *NotI*/*SalI* Arms by using T4 DNA ligase. The reaction was carried out in 5 μl of the mixture containing 50 ng of the cDNA, 1 μl of 5X T4 DNA ligase buffer, 2 μl of λZipLox, *NotI*/*SalI* Arms (0.25 μg/μl), 1 μl of T4 DNA ligase (1 U/μl). The reaction was incubated overnight at 16 °C.

4.5.7) *In vitro* packaging. The ligation products were packed *in vitro* into λ phage particles. Briefly, 5 μl of the ligated λ DNA was added into the Gigapack III Gold packaging extract (Stratagene). The tube was incubated at 22 °C for 2 h, and 500 μl of SM (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl, 0.01 % gelatin, pH 7.5) buffer was added.

4.6. Analysis of first strand cDNA by alkaline gel electrophoresis

A 1.4%-alkaline agarose gel of 0.4-cm thickness was prepared by melting 0.7 g of agarose in 45-ml of water and the gel solution was allowed to cool down to 50 °C. EDTA and NaCl were added into the molten gel to have the final concentration of 2 mM and 30 mM, respectively. The gel was cast in a model Horizontal 11•14-electrophoresis apparatus (Gibco BRL). The solidified gel was equilibrated for 2-3 h in alkaline electrophoresis buffer (2 mM EDTA, 30 mM NaOH) before loading the samples. Electrophoresis was performed for 6 h at 50 V. The gel was dehydrated for 1 h under vacuum and exposed to an X-ray film overnight at room temperature.

4.7. Amplification of the cDNA Library: a plate lysate method

One hundred microliters of *E. coli* Y1090 ($OD_{600} = 4$) was infected with an 80-µl aliquot of the primary cDNA library to make confluent lysis in amplification plates. After incubation of the mixture for 15 min at 37 °C and mixing with 3-ml top agarose (LB with 0.6 % agarose), the mixture was plated overlayer of the LB agar in a 100-mm plate. The plate was inculated for 6-8 h at 37 °C to allow the growth of bacteriophages. The phages were then eluted from agar surface by covering the plate with SM buffer for 2 h with slowly continuous shaking at room temperature. Titering of the amplified library was performed. The cDNA library was stored in aliquots of 1 ml both in the presence of 7 % dimethysulfoxide (DMSO) at –70 °C and in 0.3 % chloroform at 4-8 °C.

4.8. Titering of the cDNA library

A 100-µl aliquot of 10-fold serial dilutions (10^{-1} to 10^{-10}) of the cDNA library were used to infect the 100 µl of *E. coli* Y1090 host ($OD_{600} = 4$) 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 number of plaques generated on the plate with appropriate dilution was used to calculate the library titer as follows;

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

4.9. Cloning efficiency determination: blue-white plaque assay

The cloning efficiency was determined by infection of the *E. coli* XL-1 blue with the primary or amplified library at a density of 10^3 pfu on 100-mm LB agar plate containing 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 100 μg/ml of X-gal. After overnight incubation at 37 °C, the number of blue and white plaques was counted. The cloning efficiency was determined by calculation of the percentage of white plaques generated on the plate.

4.10. Polymerase chain reaction to determine an insert size

A pair of oligodeoxyribonucleotide primer, T7 and SP6 (Table 1), which their binding sites located in the λZiplox vector, was used to amplify the insert DNA. PCR was performed on 1 μl (approximately 5 x 10^4 phage particles) of SM containing bacteriophage from each plaque. For a 25-μl reaction volume, 0.2 μm of each primer, 0.25 U of Takara *Ex Taq* (Takara Shuzo, Shiga, Japan), *Ex Taq* buffer, and 2 mM of dNTP mixture were added. The condition performed on a Thermal cycler (model 480, Perkin Elmer, Foster City, CA, USA) involved a 5 min denaturation step at 95 °C, followed by 30 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 2 min, and final extension at 72 °C for 10 min. The PCR products were analyzed by electrophoresis in 1% agarose gel. The size of the resulting amplified products were determined by compared to the size of 100 bp DNA ladder (New England Biolabs) and λ *Hin*dIII DNA marker (New England Biolabs).

4.11. Isolation of actin-encoding clones of *P. marneffei* from the cDNA library

To generate an actin probe for screening of actin-encoding clones from the cDNA library by DNA hybridization, ACT1 and ACT2 primers (Table 1) were synthesized (Bioscience unit, National Science and Technology Development Agency (NSTDA), Thailand). The primers were designed based on the consensus sequences of gamma actin from *Emericella nidulans* and *Penicillium chrysogenum* (Genbank accession number of M22869 and AF056975, respectively). An expected size of PCR product was 530-bp. PCR was performed in a 50-μl reaction volume containing 5 μl of the

phage (approximately $2.5x10^7$ particles) from the amplified cDNA library, 0.2 μM of each ACT primer, 0.5 U of *Taq* (Takara), and 2 mM of each dNTP. The program was 95 °C for 5 min, followed with 35 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min, and final extension at 72 °C for 10 min. The PCR product of expected size was purified from the gel by using QIAquick gel extraction kit (Qiagen GmbH, Germany) and sequenced. When needed, this actin fragment was labeled with an alkaline phosphatase and hybridized to the plaque-lifted membrane.

The plaque lifted membrane containing DNAs from the cDNA library was prepared as follows (adapted from cDNA library protocols, Ian & Caroline, 1997). Approximately 10,000 pfu of phage library were mixed with 0.6 ml of *E. coli* Y1090 (OD_{600} = 4) and incubated for 15 min at 37 °C. Infected bacteria were added with 8 ml of molten top agarose (0.5 % agarose). The mixture was poured onto a 150-mm LB agar plate and allowed to solidify at room temperature. The plate was incubated overnight at 37 °C to generate plaques. A 132-mm circular hybond-N^+ membrane (Amersham Pharmacia Biotech) was laid down on the surface of agar for 2 min. The orientation of nylon membrane was marked by piercing 3 locations asymmetrically with a 21-guage needle, and the membrane was carefully lifted from the plate. The filter bound with library DNA was denatured for 5 min with 0.5 M NaOH and neutralized with 2X SSC. UV crosslinking was done at 120 mJ/cm^2 (Fisher Biotech, Foster City, CA, USA).

The performance of probe labeling, high stringent hybridization and detection were carried out by following the manufacturer's protocol (Direct Nucleic Acid Labelling® and Detection kit, Amersham Pharmacia Biotech). After film developing, positive signals on Hyperfilm ECL (Amerham Pharmacia Biotech, London, UK) were aligned with agar plate and plaques corresponding to the positive signals were picked and suspended in SM buffer containing 0.3 % chloroform. Selected positive plaques were subjected to repeated screening to obtain the purified positive clones.

4.12. Antibody screening of the cDNA library for Hsp70-encoding clones

Approximately 10,000 pfu of plaque forming units were plated on 150-mm LB agar plate by the method as described in 4.11. The plate was incubated for 4 h at 42 °C or until small plaques were just visible. Meanwhile, a 132-mm circular nitrocellulose membrane (Amersham Pharmacia Biotech) was wetted with 10 mM IPTG and dried. The IPTG-impregnated nitrocellulose membrane was laid down on the surface of the agar plate. The plate was further incubated for 3 h at 37 °C. After that, the membrane was marked for the orientation and carefully lifted from the plate. The membrane was washed twice with PBS-T and blocked overnight at 4 °C or for 4 h at room temperature in blocking solution (5% non-fat dried milk in PBS-T). The membrane was then incubated with a monoclonal antibody to Hsp70 of *Histoplasma capsulatum* (kindly provided by Hay R.J., London) for 1 h at room temperature. After 4 washes with 0.05% PBS-tween, the membrane was incubated with HRP-conjugated goat anti-mouse IgG (1:5,000 in dilution in blocking buffer) for 1 h at room temperature. After another 6 washes with PBS-T, the membrane was incubated with a substrate solution (SuperSignal® substrate west pico, Pierce) for 5 min at room temperature. The membrane was then removed from the substrate solution and wrapped between 2 sheets of plastic and exposed to Hyperfilm ECL for 5 min before developing. Positive signals were aligned with agar plate and plaques corresponding to positive signals were picked and suspended in SM containing chloroform. Selected positive plaques were subjected to secondary screening to obtain purified positive clones.

4.13. Western blot analysis

Protein antigens were separated on 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a Hybond C extra membrane (Amersham Pharmacia Biotech). The membrane was blocked in 5 % skim milk in PBS for 4 h at room temperature or overnight at 4 °C. The membrane was then washed and cut into 0.4-cm strips. The strips containing protein were incubated with a panel of 1:100 diluted *P. marneffei*-infected patients' sera for 1 h at room temperature followed with 1:2,000 in dilution of HRP conjugated goat anti-human IgG for 1 h at room

temperature. The strips were finally developed for 5-10 min in the substrate solution (90 mg/ml 4-chloro-1-naphthol, 0.003 % H_2O_2 in Tris buffer, pH 7.6).

4.14. Antibody screening of the cDNA library

The constructed cDNA library was screened with pooled sera (n = 5) derived from *P. marneffei*-infected patients. Immunoglobulin G was previously purified from the serum samples using the HiTrap Protein G HP column (Amersham Pharmacia Biotech, Uppsala, Sweden) and was further isolated away from endogenous antibodies directed against *E. coli* determinants by incubation with *E. coli* Y1090 lysate following to the standard protocol (Sambrook & Russell, 2001). Up to 10^5 independent plaques from the amplified library were screened with the previously prepared purified immunoglobulin to obtain immunogenic protein-encoding clones. The screening process was adapted from the standard protocol (Sambrook & Russell, 2001). Briefly, the host *E.coli* Y1090 was infected with the phage library at a density of 10,000 plaque forming unit (pfu) per 150-mm plate and allowed growing for 4 h at 42 °C. A 50 mM IPTG-impregnated nitrocellulose membrane (Hybond C-extra, Amersham Pharmacia Biotech) was overlaid to induce the expression of the phage library for 3 h at 37 °C. After blocking step for 4 h at room temperature in blocking buffer (5 % non-fat dry milk, 0.1 % triton X-100 in Tris buffer saline), the membrane was incubated with the purified IgG (25 μg/ml) and HRP conjugated goat anti-human IgG (20,000-fold dilution) for 1 h at room temperature. The antigen-antibody complex was detected by chemiluminescent substrate (SuperSignal Substrate West Pico; Pierce, Rockford, IL, USA). The positive phage clones were then isolated and purified by repeated screening until the homogeneous positive signal was generated.

4.15. Dot blot hybridization assay

PCR fragments containing the cDNA inserts were amplified from all positive clones using T7 and SP6 primers. The amplified products were spotted onto a Hybond N^+ membrane (Amersham Pharmacia Biotech) and cross-linked by a UV crosslinker (FisherBiotech, Pittsburgh, PA, USA). To prepare a probe, the pZL1 plasmid (see section 4.16 below) from one of the clones was digested with *Not*I and *Sal*I, thus released the DNA insert from the vector. The DNA was labeled with alkaline phosphatase (ECL Direct Nucleic Acid Labelling®; Amersham Pharmacia Biotech). High stringent hybridization and detection were performed according to the manufacturer's protocol.

4.16. DNA sequencing and analysis

Phage-to-plasmid conversion of positive clones was performed by an *in vivo* excision using *E. coli* DH10B strain (Gibco BRL) based on the Cre-*loxP* recombination process. This process generates pZL1 plasmids containing the cDNA inserts of interest (Figure 10). Subsequently, the plasmids were isolated using a plasmid mini kit (Qiagen GmbH, Germany). DNA sequencing of the cDNA was performed by dideoxynucleotide chain termination method (Sanger, Nicklen & Coulson, 1977) using the CEQ Dye terminator cycle sequencing (DTCS) quick start kit or BigDye Terminator sequencing kit (Beckman Coulter, Fullerton, CA, USA). The sequencing products were analyzed on the CEQ2000XL automated sequencer (Beckman Coulter, Fullerton, CA, USA) or a Genetic analyzer Model 310 (Beckman).

The obtained DNA sequences were analyzed by using web-based analysis programs as shown in the Table 2.

4.17. Southern blot analysis of *cpeA*

Genomic DNA was isolated from *P. marneffei* strain F4 as described previously (Cooper *et al.*, 1992; Vanittanakom *et al.*, 1996). Five micrograms of genomic DNA was digested with the restriction enzymes *Xho*I, *Sal*I, *Xba*I, *Eco*RI, *Pst*I, *Hin*dIII, *Bam*HI, *Pvu*II, and *Eco*RV (Promega, Madison, WI, USA). The digested DNA was resolved by gel electrophoresis, and then blotted onto the ZetaProbe GT membrane (Bio-Rad, Melville, NY, USA) using the capillary alkaline transfer conditions described by the manufacturer. A probe was prepared by PCR from the catalase-peroxidase-encoding clone and labeled by using Direct Nucleic acid labelling® kit (Amercham Pharmacia Biotech). Hybridization at 42 °C followed to the low stringent hybridization and detection were performed according to the manufacturer's protocol (ECL Direct Nucleic Acid Labelling Kit, Amersham Pharmacia Biotech).

4.18. Northern blot analysis of *cpeA*

Conidia of *P. marneffei* were inoculated into SDB and incubated for 12, 24, 48, and 72 h at 25°C and 37°C. The cells were collected at times indicated and mechanically disrupted with acid-washed glass beads (0.5 mm in diameter) in a Mini-Bead Beater (Biospec, Bartlesville, OK, USA). Total RNA isolation was extracted from the ruptured cells (RNeasy Mini Kit; Qiagen). Four micrograms of total RNA from the different time points was resolved on denaturing agarose gel using the NorthernMax-Gly system and transferred to the BrightStar-Plus™ positively charged nylon membrane (Ambion, TX, USA). The immobilized RNA was probed with a PCR-generated DNA fragment of the catalase-peroxidase-encoding clone after being labeled with Psoralen-biotin. Labeling probe, hybridization, and detection of the chemiluminescent signal were performed according to manufacturer's protocol (Ambion).

4.19. Reverse transcription-polymerase chain reaction (RT-PCR)

Reverse transcription was carried out on 1.5 μg of DNase-treated total RNA samples with the SuperScript™ III first strand synthesis system for RT-PCR (Invitrogen, MD, USA) and poly(dT) as a first strand primer. The samples were then treated with RNaseH according to the manufacturer's instructions. Two microliters of reverse transcribed product was used for PCR. PCR reactions were performed using specific primers to the *cpeA* (CPE-1F; 5'-TTA ACA TCC TCC GCC AAA AC-3' and CPE-1R; 5'-TCA TCC ACT TCC CAT GTG TC-3') in a 50-μl reaction containing 3 mM $MgCl_2$, 2 mM of each dNTP, 0.2 μM of each primer, 2 U AmpliTaq Gold® DNA polymerase (Applied Biosystems, Foster City, CA, USA). PCR on the DNase-treated total RNA was performed to check the DNA contamination. The PCR conditions were as follows: 94 °C for 2 min; 35 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min; and a final extension at 72 °C for 10 min. The RT-PCR products were electrophoresed on a 1% agarose gel and visualized with ethidium bromide staining.

4.20. Subcloning of *cpeA* gene fragment to pRSET B expression plasmid

The *cpeA* gene fragment was amplified by polymerase chain reaction from the clone P2. The following primers were used: M13 reverse (5'-CAG GAA ACA GCT ATG AC-3'), which has a binding site was in the pZL1 plasmid region prior to the start of P2 insert, and P1-*Eco*RI (5'-ACG CGA ATT CTT AAA GGC GGT GAC TGG-3') which was designed to bind at the stop codon in *cpeA* gene. A 100-μl PCR reaction composed of 50 ng of pZL1-P2 plasmid, 0.2 μM of each primer, 2 mM of each dNTP, and 2 U of *Pfu* DNA polymerase (Invitrogen) in 1X buffer. The PCR condition involved a denaturation step for 9.30 min at 95 °C, followed with 35 cycles of 30 s at 95 °C, 30 s at 50 °C and 1 min at 72 °C, and final extension for 10 min at 72 °C. The amplified product was cut with *Bam*HI and *Eco*RI (New England Biolabs) to generate a directional fragment for cloning into pRSET B vector (Gibco BRL). The pRSET B was cut with the same enzymes and gel purified. Ligation reaction was performed in a 10-μl reaction containing 100 ng of cut vector and 100 ng of cut insert (insert to vector molar ratio of 3:1) and 1 U of ligase (Invitrogen). Then 5-μl of the

ligation reaction was used to transform a 50 µl of $CaCl_2$-treated TOP10 *E. coli* competent cell (genotype in Appendix D, $CaCl_2$-treated competent cells were prepared following the protocol in Molecular cloning edited by Sambrook & Russell, 2003). The mixture was transformed at 42 °C for 45 s and chilled on ice. The transformed bacteria were cultured in 0.95-ml SOC medium for 1 h. The bacteria were then pelletted by centrifugation for 2 min in microcentrifuge. The pellet was resuspended in 50 µl of SOC medium and plated on a 25 µg/ml of ampicillin-LB plate. Colonies grew on the selection plate were screened for the presence of recombinant plasmids by alkaline lysis method for plasmid isolation (Sambrook & Russell, 2001) and agarose gel analysis. *Eco*RI and *Bam*HI digestion was then performed to check for the presence of insert. Sequencing was performed using T7 and T7 reverse primers whose binding sites are on the pRSET B adjacent to the multiple cloning sites.

Subsequently, the *cpeA*-pRSET B recombinant plasmid was digested with *Bam*HI. A 1,100-bp of *cpeA* gene fragment, which was released from the recombinant plasmid, was then ligated to a dephosphorylated *Bam*HI-cut pRSET B (Calf intestinal alkaline phosphatase and *Bam*HI from New England Biolabs, Inc.). The ligated plasmid was transformed into the $CaCl_2$-treated TOP10 competent cells. The transformed bacteria were selected on ampicillin-LB plate and the colony of transformants was screened for clones containing recombinant with the 1,100-bp insert. *Not*I and *Hin*dIII digestion of the recombinant plasmid containing the 1,100-bp *cpeA*-gene fragment was carried out to check an orientation of the insert. The transformants, which contain an insert in the proper direction for expression of the fusion protein, were chosen for further expression experiments.

4.21. Expression of the CpeA-His_6 tag fusion protein

The purified construct containing a *cpeA* gene insertion in the proper direction from 4.20 was purified and transformed into *E. coli* BL21(DE3)pLysS expression strain (Appendix D). The desired transformants were selected on a 25 µg/ml ampicillin-LB agar plate and maintained on a LB agar plate containing 35 µg/ml chloramphenicol and 50 µg/ml ampicillin. Initially, pilot expression experiments were

performed to determine optimal conditions, including time, temperature, and IPTG concentration, for expression of CpeA-His tag fusion protein. First, an optimal induction time was determined using a fixed IPTG concentration of 1 mM. The experimental procedures started with inoculation a colony of transformant into 2 ml of LB containing ampicillin (50-μg/ml) and chloramphenicol (35-μg/ml). The bacteria were grown overnight at 25 °C. The overnight culture was diluted in 25 ml of LB broth to an OD_{600} of 0.1. The culture was grown at 37 °C with vigorous shaking to an OD_{600} of 0.4-0.6. An aliquot of a cell density 10^8/ml (about 2 ml of the culture) was removed. The bacteria were centrifuged for 2 min at maximum speed at 4 °C in a microcentrifuge. The culture supernatant was removed and the bacterial pellet at time zero was frozen at –20 °C. Meanwhile, the IPTG was added to the culture to a final concentration of 1 mM from a 100 mM IPTG stock solution. The culture was continued to grow and the bacterial cells were collected at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, and 6 hours after addition of the IPTG. When all samples had been collected, the cell pellets were resuspended in a 100-μl of 20 mM phosphate buffer, pH 7.0. Five to ten times of repeated freeze-thaw cycles were performed to lyse the cells from each sample. The cell lysates were centrifuged for 10 min at maximum speed at 4 °C in microcentrifuge to separate the soluble and insoluble fractions. Each fraction was then analyzed for the presence of the fusion protein using a 10 % SDS-PAGE and stained with 0.1 % Coomassie blue. Twenty-five microliters of the soluble samples were mixed with 6 μl of 5X SDS-PAGE sample buffer. All 31-μl of each soluble sample was loaded into the well of the gel. For the insoluble sample, the cell pellets were resuspended with a 100-μl 1X SDS-PAGE sample buffer (312.5 mM Tris-HCl, pH 6.8, 2.5 % SDS, 5 % glycerol, 2.5 % β-mercaptoethanol, 0.005 % bromphenol blue). The 30 μl of each insoluble sample was loaded into separate wells of the gel.

Second, the optimal temperature and IPTG concentration were determined. The final IPTG concentration of 0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, and 1 mM were compared for the induction at either 25 °C or 37 °C. The experimental procedures were performed as described above for determination of induction time.

These pilot experiments determined optimal conditions for expression, including the IPTG concentration, induction-time, and temperature. Further expression experiments were performed using the experimentally determined optimal condition.

4.22. Purification of the CpeA-His_6 tag fusion protein

The CpeA-His_6 tag protein was purified from the mixture of *E. coli* protein by using a Probond™ resin (Gibco-BRL). The experimental procedures were performed according to the manufacturer's protocol. Briefly, a transformant containing the *cpeA* gene fused to pRSET B vector in the proper direction for expression was inoculated 2 ml of LB and grown overnight at 25 °C. The overnight culture was diluted to an OD_{600} of 0.1 with 50 ml LB. The bacteria were grown to an OD_{600} of 0.5 and induced with 0.05 mM IPTG for 2 h. The cells were collected by centrifugation for 10 min at 4 °C. The bacterial pellet was resuspended in 10 ml of guanidinium lysis buffer, pH 7.8. The suspension was sonicated by three 10 s-bursts at maximal power in a sonicator (Branson Sonifier 250, American Laoratory Trading, Niantic, CT, USA). The cell debris was removed by centrifugation at 10,000 x g for 10 min. The clear lysate was loaded into the Probond™ resin column and incubated for 10 min. After removal of the cell lysate, low and high stringent washing were performed. The resin was washed 2 times for 2 min with 4 ml of denaturing wash buffer, pH 6.0, and 2 times for 2 min washing with 4 ml of denaturing wash buffer, pH 5.3. Elution was performed by applying 10 ml of denaturing elution buffer, pH 4.0 onto the column. Twelve fractions of 1-ml eluate were collected. Ten microliters from each fraction were analyzed on SDS-PAGE. The protein concentration from each fraction was determined by Bradford protein assay (Protein Assay Dye reagent; BIO-RAD). The eluates were dialyzed versus 10 mM Tris, pH 8.0, 0.1 % triton X-100 overnight at 4 °C to remove urea from the purified fusion protein.

4.23. Accession number

The *cpeA* open reading frame plus 68 nucleotides of an upstream sequence and 175 nucleotides of a downstream sequence was submitted to the GenBank database under the accession number AF537129.

Table 1. Sequence of primers used in this study.

Primers	Sequence	Total bases
ACT1	GGT GAT GAG GCA CAG TC	17
ACT2	GAA GCG GTC TGG ATC TC	17
T7	TAA TAC GAC TCA CTA TAG GG	20
SP6	ATT TAG GTG ACA CTA TAG	18
M13 reverse	CAG GAA ACA GCT ATG AC	17
M13 forward	GTA AAA VGA CGG CCA G	16

Table 2. Web-based analysis tools used in this study.

Type of Analysis	Program(s)	Web Site(s)
Similarity searching	BLASTN, BLASTX, BLASTP	http://www.ncbi.nlm.nih.gov/BLAST
Multiple sequence alignment	Clustal W	http://www. ebi.ac.uk/clustalw/index.html
Motif scan searching	Motif Scan	http://hits.isb-sib.ch/cgi-bin/PFSCAN
Translation	Translate	http://arbl.cvmbs.colostate.edu/molkit/translate
	Translate	http://us.expasy.org/tools/translate.html
Molecular weight and pI prediction	Protparafam	http://us.expasy.org/tools/protparafam.html
Restriction mapping	NEBcutter	http://tools.neb.com/NEBcutter2/index.php
	Web Map Preferences	http://pga.mgh.harvard.edu/web_apps/web_map/start

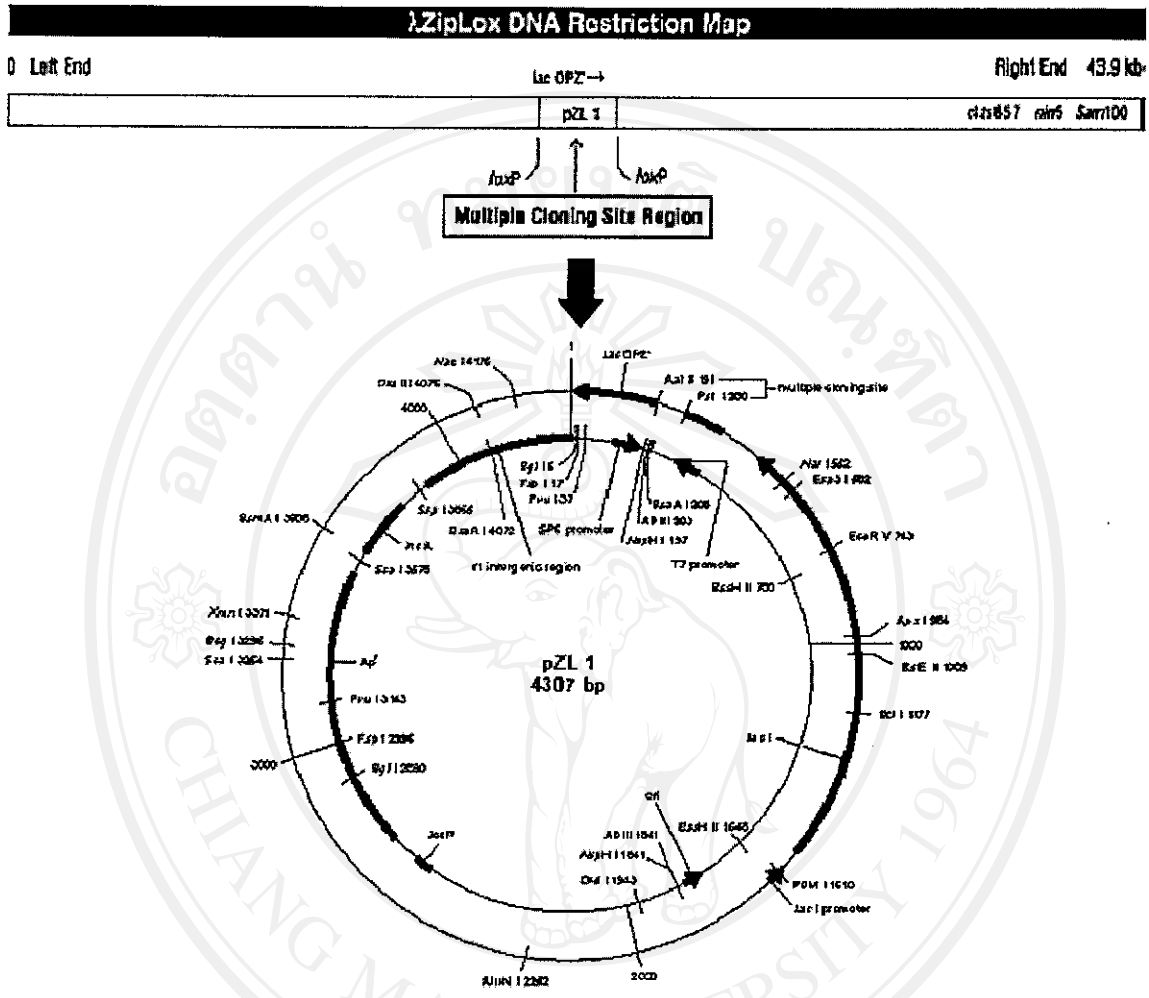

Figure 10. ***In vivo*** **excision of the lambda ZipLox vector and the resulting pZL1 plasmid.** The λZipLox vector contains two of *loxP* sites flanking to the multiple cloning sites. The *loxP* sites mediate the site-specific recombination and excision of the pZL1 plasmid from the λZipLox vector.