CHAPTER IV

Materials and Methods

4.1 Isolation and characterization of *sakA* and *atfA* genes from *Penicillium marneffei*

4.1.1 Strains and growth conditions

P. marneffei F4 (CBS no. 119456) was isolated from an AIDs patient admitted to Maharaj Nakorn Chiang Mai Hospital, Chiang Mai in 1999. The fungus was grown on potato dextrose agar (Difco Becton, Dickinson, and Company, NJ USA) or malt extract agar (Oxoid Hampshire, England) and incubated for 7 days at 25ºC. For long-term storage, the fungus was kept in 30% glycerol at -80 ºC. To prepare conidial suspension for inoculating broth cultures, fungal conidia were collected using cotton swab scrapping in sterile 0.01% Tween 80 and filtrating through sterile glass wool (Corning, Acton, MA, USA). The conidia were washed and resuspended with sterile distilled water and counted with hemocytometer. Approximately 10^7 conidia were inoculated into 50 ml brain-heart infusion (BHI) broth cultures (Oxoid Hampshire, England) and incubated at 25ºC for 72 h and 37ºC for 96 h to produce mycelial and yeast forms, respectively. For heat shock condition, conidia, mycelia, and yeast cells were incubated at 39ºC for 1 h. For H_2O_2-treated condition, hydrogen peroxide was added to flask containing conidia, mycelia and yeast cells to a final concentration 1 mM and incubated at 25ºC or 37ºC for 1 h. In all conditions, the cultures were maintained in shaking incubator with continuous shaking at 150 rpm. For bacterial transformation, *Escherichia coli* DH5α were grown on Luria-Bertani (LB) agar (Difco Becton, Dickinson, and Company, NJ USA). For plasmid propagation, transformants were selected on LB medium containing 100 µg/ml ampicillin.

4.1.2 Genomic DNA preparation

Fungal genomic DNA was isolated using a modified method described in Kummasook's thesis (2010). *P. marneffei* was cultured on potato dextrose agar (PDA) or malt extract agar (MEA) at 25ºC for 7-10 days. One loopful of fungal colony including conidia and hyphae was inoculated to 10 ml of Sabouraud dextrose broth (SDB) in a 50 ml centrifuge tube and incubated at 25 ºC in shaking incubator at 150 rpm for 24 h. The culture tubes were then transferred to 37ºC-shaking incubator and incubated for 40 h. Fungal cells were collected by centrifugation at 4,500 rpm for 10 min at 4ºC. The pellets were washed once with 15 ml of osmotic buffer and resuspended in 10 ml of chilled osmotic buffer. One milliliter of solution including filter-sterilized bovine serum albumin (BSA) solution and lysing enzyme (Sigma-Aldrich, USA) was added to the cell suspension, to a final concentration of 1.2 mg/ml and 10 mg/ml, respectively. The mixture was transferred to volumetric flask and incubated in shaking incubator at 37ºC, 150 rpm for 3 h. The protoplasts were collected, washed twice with ST buffer and resuspended in 5 ml lysis buffer containing 50 µg/ml RNaseA. The suspension was mixed thoroughly and incubated at 65ºC for 1 h. To pellet the protein and cell debris, 1.5 ml of 5 M potassium acetate was added to the suspension and mixed by inversion before incubating on ice for 1 h. After incubation, the suspension was centrifuged at 4ºC, 4,500 rpm for 15 min and the supernatant was transferred to a new 15 ml centrifuged tube. The DNA purification was done twice by adding equal volume of phenol-chloroform-isoamyl alcohol (AMRESCO, Solon, Ohio, USA) and vortexing for 2 min. The mixture was centrifuged at 4,500 rpm for 15 min and the aqueous phase was transferred into a new 15-ml centrifuge tube. To precipitate DNA, two volumes of absolute ethanol were added into the aqueous phase, mixed by inversion and incubated at -20ºC overnight. After incubation, the tube was centrifuged at 4ºC, 4,500 rpm for 30 min and the pellet was washed twice with 70% ethanol. The pellet was dried at room temperature and resuspended with distilled water. The concentration of DNA was measured using spectrophotometer by the optical density (OD) at 260 nm absorbance.

The ratio of OD values at 260 and 280 nm was used to indicate the purity of the DNA. The DNA quality was evaluated by agarose gel electrophoresis and the DNA was kept at -20ºC.

4.1.3 Amplification of the *sakA* and *atfA* genes of *P. marneffei*

Primers *SakA*-WF and *SakA*-WR (Table 1) and primers *AtfA*-WF and *AtfA*-WR (Table 2) were designed based on the genome nucleotide sequences of *P. marneffei* ATCC 18224 from NCBI database (http://www.ncbi.nlm.nih.gov/nuccore/XM_002146211.1 and http://www.ncbi.nlm.nih.gov/nuccore/XM_002143920.1). The 1,637 bp and 1,632 bp PCR products covering the open reading frame of *sakA* and *atfA* genes were amplified. PCR amplification was performed using 100 ng of *P. marneffei* genomic DNA, 1X phusion® HF buffer, 1.5 mM $MgCl_2$, 0.5 µM of forward and reverse primers, 0.2 mM dNTPs, and 1.5 units of Phusion™ Hot Start High Fidelity DNA polymerase (New England Biolabs, Finnzyme). The reaction was started at 98ºC for 30 seconds; followed by 35 cycles of 98ºC for 10 s, 65ºC for 30s, 72ºC for 1 min and a final extension at 72ºC for 7 min. PCR products were subjected for nucleotide sequencing in both directions.

Table 4.1 PCR primers used for *P. marneffei sakA* gene study.

Primer name	sequence	Reference
Pm-S1	5’-GCC AGG CGA ATA TCC ATA TGA-3’	This study
Pm-S2	5’-TCC TGT GTG AAA TTG TTA TCC GCT CGA GTA ACG CGA CGA CAA TC-3’	This study and Gravelat *et al.*, 2010
Pm-S3	5’-CGT TAC CCA ACT TAA TCG CCT TG AAG CCA CTA CAC GGT GTT CA-3’	This study and Gravelat *et al*., 2010
Pm-S4	5’-CAT AGA CGT TCG CAA CGA GA-3’	This study
HY	5’-GGA TGC CTC CGC TCG AAG TA-3’	Gravelat *et al*., 2010
YG	5’-CGT TGC AAG ACC TGC CTG AA-3’	Gravelat *et al*., 2010
*SakA*F	5’-TGG TCT AGC TCG AAT CCA AG-3’	This study
*SakA*344-R	5’-GTC GAC GTA GGC CTC AGT TA-3’	This study
*SakA*355-R	5’-GCG GAT TGG TCA ATG TTA TG-3’	This study

Table 4.1 PCR primers used for *P. marneffei sakA* gene study (Cont.).

Primer name	sequence	Reference
SakA-WF	5’-ATT GTC GTC GCG TTA CTC G-3’	This study
SakA-WR	5’-ACC TGA ACA CCG TGT AGT GG-3’	This study
SakA-ComF	*Cla*I 5’-CAT GA ATC GAT GCC AGG CGA ATA TCC ATA TGA-3’	This study
SakA-ComR	ApaI 5’-CAT GAG GGC CC GT CAT GAT CTT CAC CGC A-3’	This study
Pm1	5’-ATG GGC CTT TCT TTC TGG G-3’	Vanittanakom *et al*., 2002
Pm2	5’-GCG GGT CAT CAT AGA AAC C-3’	Vanittanakom *et al*., 2002
LPW21406_GAPDH	5’-TGG TCT AGC TCG AAT CCA AG-3’	Lau *et al*. 2013
LPW21407_GAPDH	5’-GTC GAC GTA GGC CTC AGT TA-3’	Lau *et al*. 2013
sakA_exon3	5’-AAC GCA CAT ACC GAG AAC TC -3’	This study
sakA_exon5	5’-CAA GTC AGT GCC TAG GAG CT -3’	This study

Table 4.2 PCR primers used for *P. marneffei atfA* gene study.

Primer name	Sequence	Reference
AtfA-A1	5’-AGG AAC GTA CCA CCA CTG AA-3’	This study
5’atfARev500	5’-CCA GCA TAG CAG GAC TCA GC-3’	This study
AtfA-A3	5’-CGT TAC CCA ACT TAA TCG CCT TG CGT ACA ACC TCG CAA CCA AT-3’	This study and Gravelat *et al*., 2010
AtfA-A4	5’-GTG TCA TGT CCA GTC GAG TCC-3’	This study
*AtfA*F-RT	5’-CGC TGA GTC CTG CTA TGC TG-3’	This study
*AtfA*R-RT	5’-GCT CGA CCT TGG CTT GGA GA-3’	This study
AtfA-WF	5’-GCC ATG ACC TCA CAA TTA CC-3’	This study
AtfA-WR	5’-ATT GGT TGC GAG GTT GTA CG-3’	This study

Table 4.2 PCR primers used for *P. marneffei atfA* gene study (Cont.).

Primer name	Sequence	Reference
AtfA-ComF	*Hin*dIII 5'- GTG CG AAG CTT GTG TCG AAT TGG CCA TGT TG-3'	This study
AtfA-ComR	*Kpn*I 5'- CTA TA GGT ACC GAC AAG GCA TCG TCG ACA C-3'	This study
atfA_exon2	5'- CCG AAG AAG ATG ACT GAT GAA G -3'	This study
atfA_exon3	5'- TTG CAA GCC ACT GCT TCT TA -3'	This study

4.1.4 DNA sequencing and sequence analysis

DNA sequencings of the PCR products were performed using the dideoxynucleotide chain termination method (Sanger *et al*., 1977). The NCBI BLAST program (http://www.ncbi.nlm.nih.gov) was used to search for nucleotide and protein sequence similarities. The programs 'nucleic acid translation' of BioEdit Sequence Alignment Editor Software was used to predict an open reading frame and deduced amino acid sequences from the nucleotide sequences. Conserved domains of SakA putative protein was predicted using ScanProsite tool (http://prosite.expasy.org/scanprosite/). Deduced amino acid sequences of the *sakA*/*hog1* or *atfA*/*atf1*genes of other fungal homologous sequences that were obtained from GenBank databases (http://www.ncbi.nlm.nih.gov) were used for multiple alignments. Multiple sequence alignment was generated with the ClustalW program (http://www.ebi.ac.uk/clustalw/index.html).

4.2 Analysis of *sakA* and *atfA* gene expressions during phase transition and stress condition

4.2.1 Total RNA extraction

Total RNA from *P. marneffei* cells in different phases (conidia, hyphae and yeast) and in different stress conditions (control, heat stress and oxidative stress) was isolated by using total RNA isolation Nucleospin® RNA II kit (NucleoSpin, MACHEREY-NAGEL). Fungal cells were harvested by centrifugation at 12,000 rpm for 2 min. The pellets were resuspended in 350 µl Buffer RA1 and 3.5 µl β-mercaptoethanol (β-ME) and the suspensions were transferred to screw-cap microtube containing sterilized glass bead. Cells were lysed by agitating the microtube using a bead beater (Biospec, Bartlesville, OK, USA) 5 times at 4,600 rpm for 30 seconds with cooling on ice. The lysates were centrifuged at 13,200 rpm for 2 min. The supernatant was filtrated through Nucleospin®II Filter by placing the Filter in the collection tube, applying the supernatant and centrifuging at 11,000 x g for 1 min. The flow-thorough (homogenized lysate) was transferred to a new 1.5 ml microcentrifuge tube and 350 µl of 70% ethanol was added and

mixed by pipetting up and down. The ethanolic lysate was loaded into Nucleospin® RNA II column placed on collection tube and centrifuged for 30 s at 11,000 x g. To desalt the silica membrane, 350 µl of membrane desalting buffer (MDB) was added to the column and centrifuged at 11,000 x g for 1 min. The membrane was firstly washed with 200 µl of Buffer RA2 and centrifuged at 11,000 x g for 30 s followed by 600 µl of Buffer RA3 at 11,000 x g for 30 s and 250 µl of Buffer RA3 at 11,000 x g for 2 min, respectively. RNA was eluted from the membrane using 60 µl of nuclease-free H_2O with centrifugation at 11,000 x g for 1 min. The contaminated DNA was digested by rDNase digestion. The mixture of 6 µl of reaction buffer for rDNase and 0.6 µl of rDNase was added to eluted RNA and the reaction tube was incubated at 37ºC for 10 min. RNA was repurified by ethanol precipitation using 0.1 volumes of 3 M sodium acetate pH 5.2 and 2.5 volumes of absolute ethanol. After thoroughly mixing, precipitated RNA was incubated at -20ºC for several hours before centrifugation at 13,200 rpm for 15 min. RNA pellet was washed twice with 70% ethanol and dried. RNA pellet was resuspended in nuclease-free H_2O. The concentration of RNA was measured and PCR amplification was done using extracted RNA as a template to detect DNA contamination in RNA samples.

4.2.2 Reverse transcriptase (RT) PCR

All RNA samples were converted to cDNA using RevertAid First Strand cDNA synthesis Kit (Fermentas, Burlington, Canada). Two micrograms of each sample were added to the reaction containing 1X reaction buffer, 20 U of RiboLock RNase Inhibitor, 5 µM random hexamer primer, 1 mM dNTP mix and 200 U of reverse transcriptase. The reverse transcription was performed at 25ºC for 5 min, followed by 60 min at 42ºC. The reaction was terminated by incubating at 70ºC for 5 min. cDNA products were used as template for PCR amplification using specific primers for *sakA* (*Sak*AF and *Sak*A344R; Table 1) and *atfA* genes (*Atf*AF-RT and *Atf*AR-RT; Table 2). 18S rRNA was amplified using the primers Pm1 and Pm2 (Table1) and used as a loading control. The reaction was initiated at 94ºC for 3 min; followed

by 25 cycles of 94ºC for 45 s, 65ºC for 30 s, 72ºC for 1 min and a final extension at 72ºC for 7 min. The RT-PCR products were loaded into agarose gel electrophoresis and the band intensities were analyzed using Gel Doc 1000 Fluorescent Imaging System (BIO-RAD, Hercules, CA, USA). To identify differential gene expression, relative expression level of *sakA* or *atfA* gene was calculated from the ratio of band intensities between *sakA* or *atfA* PCR product and 18S rRNA loading control.

4.2.3 Real-time quantitative PCR

Real-time PCR was performed using primers sakA_exon3 and sakA_exon5 (Table 1) and primers atfA_exon2 and atfA_exon3 (Table 2) for *sakA* and *atfA* genes, respectively. Glycerladehyde-3-phosphate dehydrogenase (GAPDH) gene was amplified using primers LPW21406 and LPW21407 (Table 1; Lau *et al*., 2013) and used for normalization. The reactions were carried out in 20 µl reaction mixtures containing 10 µl FastStart DNA Master SYBR Green I Mix reagent kit (Roche, Switzerland), 1 µl cDNA and 0.5 µM primers. cDNA was amplified in an ABI Prism 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) at 95ºC for 10 min; followed by 40 cycles of 95ºC for 15 s and 60ºC for 1 min. At the end of each cycle, a melting curve analysis was performed from 60ºC to 95ºC for monitoring primer dimers or formation of non-specific product. The reactions were performed in triplicate.

4.3 Functional analysis of *sakA* and *atfA* genes

4.3.1 Generation of *P. marneffei sakA* and *atfA* mutant strains using split marker method

To disrupt *sakA* and *atfA* genes, target gene deletion method was performed using split marker recombination modified from previously described (Catlett *et al*., 2003; Sheppard *et al*., 2005; Gravelat *et al*., 2010). For *sakA* gene, in first round PCR, two pairs of primers (Pm-S1+Pm-S2 and Pm-S3+Pm-S4) were used to amplify 5’ and 3’ flanking regions of *sakA* gene, respectively (Table1). Approximately 2 kb and 1.9 kb PCR products were

amplified separately using 100 ng of *P. marneffei* genomic DNA, 1X Phusion® HF buffer, 1.5 mM $MgCl_2$, 0.5 µM of forward and reverse primers, 0.2 mM dNTPs, and 1.5 units of Phusion™ Hot Start High Fidelity DNA polymerase (New England Biolabs, Finnzyme). The reaction was performed at 98ºC for 30 seconds; followed by 35 cycles of 98ºC for 10 s, 65ºC for 30 s, 72ºC for 1 min and a final extension at 72ºC for 7 min. For second round PCR, PCR product from Pm-S1 and Pm-S2 was mixed with pAN7-1 plasmid (Cardoza *et al*., 2006) containing hygromycin resistant gene (*hph*) and used as templates to generate DNA fragment including 5' flanking region of *sakA* gene and around two thirds of *hph* gene cassette using primers Pm-S1 and HY (Table1). While PCR product from Pm-S3 and PmS4 was also mixed with pAN7-1 and used as templates for generating DNA fragment using primers YG and Pm-S4 (Table1). This DNA fragment contains 3'flanking region with incomplete fragment of *hph* gene that have overlapping region with the other DNA fragment. Two DNA fragments from second round PCR were transformed into *P. marneffei* wild type strain using protoplast transformation method to generate *P. marneffei sakA* mutant strain (Figure 4.1).

In protoplast transformation, conidia from *P. marneffei* wild type was inoculated in 100 ml SDB and was incubated with shaking at 25ºC, 150 rpm for 24 h and then shift to 37ºC for 40 h. After incubation, culture were transferred to 50 ml centrifuge tube and centrifuged at 4,500 rpm at 4ºC for 10 min. Cell pellet was washed twice with 20 ml 0.6 M $MgSO_4$ and centrifuged at 4,500 rpm at 4ºC for 10 min. Pellet was washed again with 25 ml of ice-cold osmotic buffer (see appendix) and centrifuged at 4,500 rpm at 4ºC for 5 min. After centrifugation, pellet was resuspended with 10 ml of ice-cold osmotic buffer and kept on ice. Lysing enzyme from *Trichoderma harzianum* (Sigma-Aldrich, St. Louis, MO, USA) and bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) solution were added to fungal cell suspension to final concentration 10 mg/ml and 1.2 mg/ml, respectively. The mixture was then transferred to sterile Erlenmeyer flask and incubated at 37ºC for 1 h with gentle agitation. After incubation,

the mixture was transferred to 50 ml centrifuge tube, overlaid on the top with equal volume of trapping buffer (see appendix) and centrifuged at 4,500 rpm at 4ºC for 20 min. Protoplasts were taken from protoplast band formed at the interface between osmotic buffer and trapping buffer and resuspended with ice-cold STC buffer (see appendix). The suspension was centrifuged at 4,500 rpm at 4ºC for 10 min and protoplasts were washed and resuspended with STC buffer. Two to five micrograms of two DNA fragments from second round PCR were mixed with 100 µl protoplast in 15 ml centrifuge tube and 25 µl of 60% polyethylene glycol (PEG) was added to the mixture followed by gentle mixing (tube containing only protoplast was used as a control). The mixture was incubated on ice for 30 min. After 30 min, 1 ml of 60% PEG was added to the mixture and mixed by gentle rolling the reaction tube. Tube was incubated at room temperature for 30 min followed by adding 10 ml of STC buffer and mixed by inversing the reaction tube. The mixture was centrifuged at 4,500 rpm at 4ºC for 30 min, and pellet was resuspended with 300 µl STC buffer. Transformants were plated on BHA containing 1.2 M sorbitol and supplemented with 200 µg/ml hygromycin to select hygromycin resistance transformants. Plates were incubated at 25ºC for 5-7 days and then turned to 37ºC.

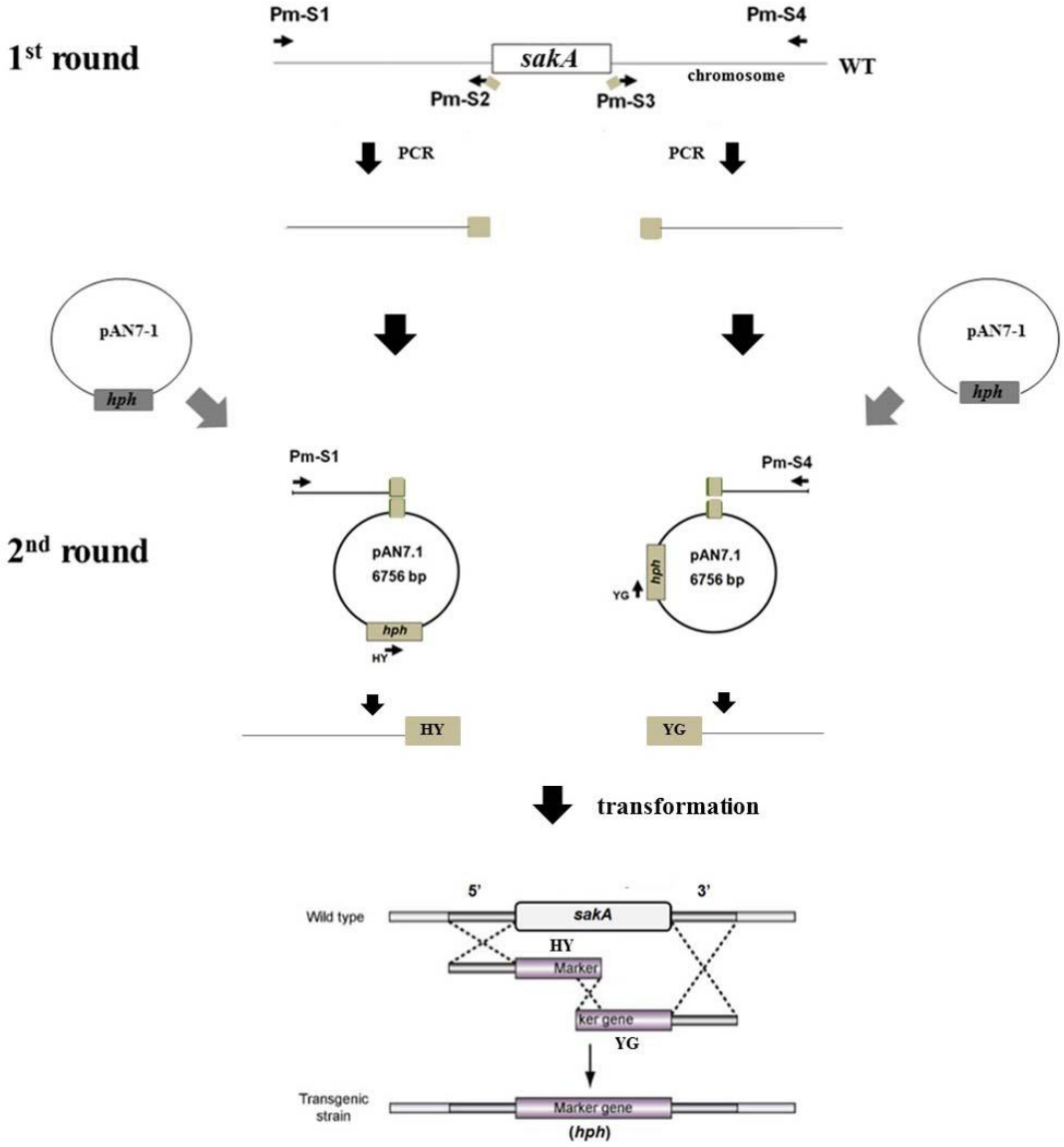

Figure 4.1 Strategy for generation of *P. marneffei sakA* mutant by split marker recombination method. Two DNA fragments containing 5' and 3' *sakA* flanking regions and truncated sequence of the *hph* gene (HY and YG) that were overlapped were transformed to *P. marneffei* wild type. The predicted results of three homologous recombinations of 5' and 3' *sakA* flanking regions and *hph* gene at the *P. marneffei sakA* locus in the mutant strain are shown.

For *atfA* gene, primers *AtfA*-A1 and 5'atfARev500 (Table 2) were designed to amplify 5'flanking region with first 500 nucleotide of *atfA* gene. The 1.9-kilobase blunt end amplified product was cloned into pAN7-1 digested with *Sfo*I restriction enzyme to generate pANatfAflank. Primers *AtfA*-A3 and *AtfA*-A4 (Table 2) were used for first round PCR to amplify 3'flanking region of *atfA* gene. The 1.5-kilobase amplified product and pAN7-1 were used as templates for second round PCR using primers AtfA-A4 and YG to generate the 3.1-kilobase DNA fragment. The 7.9-kilobase DNA fragment containing 5'flanking region with 500-bp of *atfA* gene and *hph* gene from pANatfAflank digested with *Bam*HI and *Hin*dIII restriction enzymes and the 3.1-kilobase DNA fragment carrying 3'flanking region and truncated sequence of *hph* gene were transformed to *P. marneffei* wild type strain. The generation of *P. marneffei atfA* mutant strain is shown in Figure 4.2.

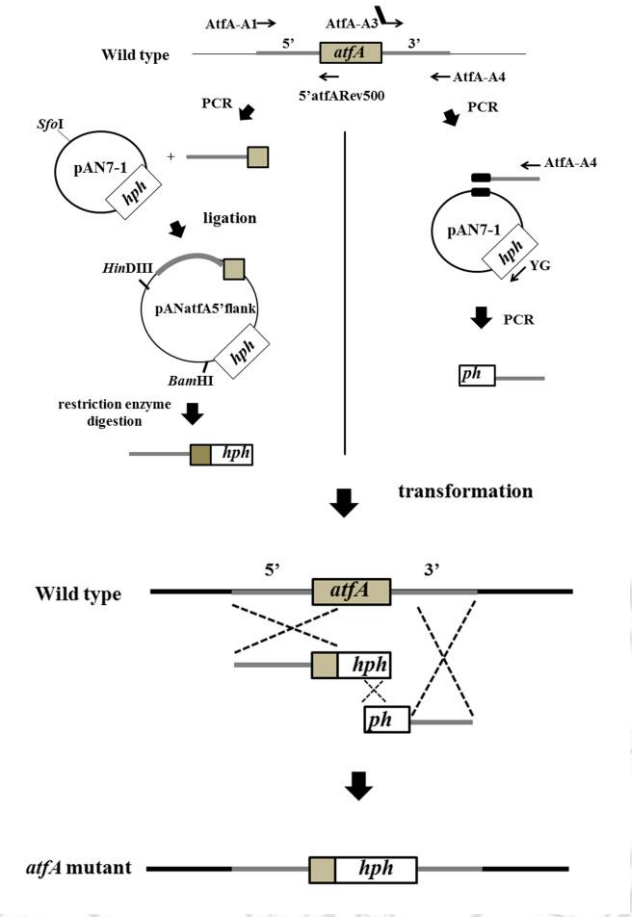

Figure 4.2 Strategy for deletion of the *atfA* gene by replacing the entire *atfA* ORF with two DNA fragments using modified split marker method. For the first DNA fragment, PCR amplification of 500 nucleotides of the *atfA* gene with 5' flanking region from genomic DNA of wild type is performed. The PCR product is ligated to pAN7-1 containing the *hph* gene. The DNA fragment containing 500 nucleotides of the *atfA* gene with 5' flanking region and the *hph* gene without terminator (*hph*) is obtained by digestion of recombinant plasmid with *Hin*dIII and *Bam*HI. For the second fragment, 3' *atfA* flanking region of *atfA* is amplified and PCR product is used as a template with pAN7-1 in the second round PCR. Product from this PCR step consists of 3' *atfA* flanking region and the truncated sequence of the *hph* gene (*ph*) with terminator. Two DNA fragments are then transformed into *P. marneffei* wild type to generate *atfA* mutant strain. The primers used for mutant construction and the predicted results of three homologous recombinations of 5' and 3' *atfA* flanking regions and *hph* gene at the *P. marneffei atfA* locus in split marker recombination method are shown.

4.3.2 Construction of *P. marneffei sakA* and *atfA* complemented strains

For *sakA* gene, DNA fragment containing *sakA* open reading frame (ORF) plus 2 kb of promoter and 0.5 kb of 3'flanking region was amplified using primers *SakA*-ComF and *SakA*-ComR including recognition sites of enzymes *Cla*I and *Apa*I at 5' end, respectively (Table 1). The 4,072 bp amplified product was cloned into pJL43B1 plasmid digested with *Cla*I and *Apa*I resulting pJLsakA. This plasmid includes bleomycin-phleomycin resistant gene (*ble*) used as second selectable marker. For *atfA* gene, primers *AtfA*-ComF and *AtfA*-ComR were used to amplify *atfA* ORF plus 2.5 kb of promoter and 1.7 kb of 3'flanking region. The 5.7 kb amplified product containing recognition sites of *Hin*dIII and *Kpn*I at 5' and 3' end, respectively, was cloned into pJL43B1 to generate pJLatfA.

Plasmids pJLsakA and pJLatfA were transformed to *P. marneffei sakA* and *atfA* mutant strains, respectively using protoplast transformation. For *sakA* gene, transformants were inoculated in 5 ml SDB supplemented with 1.2 M sorbitol and incubated with shaking at 25ºC, 150 rpm for 2 days. After recovery, transformants were plated on PDA and incubated at 25ºC for 5 days. The transformants that reveal colonies similar to wild type strain were collected and selected on selective agar (SDA containing 200 µg/ml hygromycin and 2 µg/ml bleomycin) and incubated at 37ºC for 5 days. For *atfA* gene, after transformation, transformants were plated on selective agar and incubated at 25ºC for 7 days and changed to 37ºC.

4.3.3 Southern blot hybridization analysis

To determine the number of *hph* copies in *sakA* and *atfA* mutants and the number of *sakA* and *atfA* copies in complemented strains, Southern blot hybridization was performed. Southern blot was done using alkaline transfer of TurboBlotter™ System (Whatman® Schleicher & Schuell). Fifteen to twenty micrograms of genomic DNA from wild type, mutant or complemented strains was digested with selected restriction enzymes and subjected to electrophoresis on 0.8% agarose gel. Digested DNA was then transferred onto Nytran®SuperCharge Nylon Membranes and fixed on the

membrane by UV cross linking in GS Gene Linker^{TM} UV chamber (Bio-rad, Hercules, California) for a total dose of 125 mJ/cm^2. After cross linking, hybridization was performed using AmerSham Gene Images AlkPhos Direct Labelling and Detection system (GE Healthcare Biosciences, Piscataway, New Jersey). Fragment of *hph*, *sakA* or *atfA* gene was used as a hybridization probe and hybridization was done at 55ºC for overnight. After detection reagent was added, membrane was exposed to hyperfilm ^{TM} ECL. Hybridization signals were observed after film was removed and developed.

4.3.4 Characterization of *sakA* and *atfA* mutants

1). Morphologies and Growth

To observe colony morphologies at 25°C, conidia of *P. marneffei* wild type, *sakA* and *atfA* mutants and *sakA* and *atfA* complemented strains were collected and inoculated on PDA and MEA and incubated at 25°C for 7-10 days. For morphologies under a microscope, the slide culture technique was used. All strains were inoculated on PDA and incubated at 25°C for 4, 7 and 10 days. To visualize cell wall and chitin deposition, fungi on slide culture were stained with calcofluor white and observed under the fluorescence microscope (Nikon Eclipse 50i Tokyo, Japan).

To evaluate the effect of *sakA* gene on growth at 25°C, growth curves of wild type and *sakA* mutant were done. Approximately 10 conidia of wild type and *sakA* mutant were collected and plated on PDA and incubated at 25°C for 7 days. Colony diameters of five colonies were measured on day 3 to day 7. Three independent experiments were done and the results were analyzed using standard *t*-tests (http://www.graphpad.com/quickcalcs/ttest1.cfm?Format=SD).

For yeast cell induction at 37°C, conidia of *P. marneffei* wild type, *sakA* and *atfA* mutant and *sakA* and *atfA* complemented strains were collected and inoculated on SDA and incubated at 37°C for 14 days or were inoculated into tested media including 1% peptone, BHI and

SDB and incubated at 37°C for 2 days (1% peptone) and 7 days (BHI and SDB). Cell morphologies were visualized under a light microscope (Nikon Tokyo, Japan).

To study effect of *sakA* gene on intracellular growth in macrophages, THP1 human monocytes (THP1, American type culture collection, ATCC) were cultured in RPMI 1640-HEPES medium (GIBCO-BRL, Life Technologies) supplemented with 10% fetal bovine serum (FBS) at 37ºC and 5% CO_2. For macrophage differentiation, THP1 cells were seeded into 24-well tissue culture plate at 5×10^5 cells per well and 100 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma) were added to each well and incubated at 37ºC with 5% CO_2 for 48 h (Adapted from Marr et al., 2001). J774 mouse macrophage cell line (Sigma-Aldrich, St. Louis USA) were grown in Dulbecco's Modified Eagle Medium (DMEM, GIBCO-BRL, Life Technologies) and were seeded to 24-well tissue culture plate at 4×10^5 cells per well (Adapted from Woo et al., 2012). After incubation, the culture medium was removed and 1 ml culture medium containing 5×10^6 conidia (wild type, *sakA* mutant and complemented strains) for THP1 or 1×10^6 conidia for J774 was added to each well (MOI = 10 for THP1 and MOI = 2.5 for J774) and incubated for 48 or 72 hours. At specific time point, macrophages were washed twice with phosphate buffer saline (PBS) and were trypsinized with 0.25% trypsin-EDTA (GIBCO-BRL, Life Technologies) to remove infected macrophages. Macrophages were collected by centrifugation and were observed under a microscope (Nikon Eclipse 50i Tokyo, Japan).

2). Asexual development

For quantification of conidia (asexual development), 3 microliters of 10^6 spores/ml of *P. marneffei* wild type strain F4, the *sakA* mutant and *sakA* complemented strains were inoculated in the center of PDA and MEA plates. Conidia of each strain were harvested by cutting the whole colony and suspended in 0.01% Tween 80 and mixed by

vortexing for 10 minutes. The conidia were counted using a hemocytometer. Tests were performed in triplicate and analyzed using standard *t*-tests (http://www.graphpad.com/quickcalcs/ttest1.cfm?Format=SD).

3). Susceptibility of conidia to stresses

For osmotic, oxidative, heat at 39°C and cell wall stress susceptibility studies of conidia, the drop dilution assay was used. Conidia of *P. marneffei* wild type, *sakA* and *atfA* mutant and *sakA* and *atfA* complemented strains were counted using a hemocytometer. Series of ten-fold dilutions derived from a starting solution of 1×10^7 conidia/ml to 1×10^3 conidia/ml were spotted in aliquots of 5 microliters onto minimal medium (MM) pH 5.0 plates containing 0.2% NH_4Cl, 0.1% $(NH_4)_2SO_4$, 0.05% KCl, 0.05% NaCl, 0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.002% $FeSO_4.7H_2O$, 1% glucose and 1.5% agar (Jin *et al*., 2004) supplemented with/without NaCl, sorbitol, H_2O_2, *tert*-butylhydroperoxide (*t*-BOOH), menadione (Md), calcofluor white and sodium dodecyl sulphate (SDS) and incubated at 25°C or 37°C for 5 days. For heat stress at 39°C, plates were put in 39°C incubator for 5 days.

In heat stress at 42°C, conidia of *P. marneffei* wild type, *atfA* mutant and complemented strains were inoculated into BHI and incubated at 25°C or 42°C, 250 rpm. After one hour, conidia of each strain were diluted and plated on SDA for colony forming unit count. A number of colonies on control plate at 25°C was used as the baseline for calculation of % viability at 42°C.

For UV stress, the experiment was done following the method described previously (Woo *et al*., 2010). Approximately one hundred conidia of wild type, *sakA* mutant and *atfA* mutant were spread on SDA plates and were exposed under different doses of UV light (254 nm) including 0, 2000, 4000, 6000 and 8000 microjoules/cm^2 using

CL-1000 Ultraviolet crosslinker (UVP, Upland, CA, USA). Plates were incubated at 25°C for 3-4 days and colony forming units (CFUs) on plate 0 microjoules/cm^2 were used as the baseline values for calculating % survival of conidia at different UV doses.

4). Susceptibility of mycelia to oxidative stresses

For oxidative stress susceptibility of mycelia, mycelial plugs (diameter 5 mm) cut from the growing edge of 5-day-old colonies of the wild type and *sakA* mutant strains were inoculated on MM plates containing10 mM H_2O_2 and 3 mM *t*-BOOH and incubated for 5 days at room temperature (Adapted from Lara-Rojas *et al.*, 2011).

5). Fungal survival inside macrophages

To evaluate the involvement of *sakA* and *atfA* genes in survival of *P. marneffei* conidia inside macrophages, the intracellular survival assays were done following the method described previously (Woo *et al*., 2012). J774 mouse monocyte macrophages (Sigma-Aldrich, St. Louis USA) were maintained in Dulbecco's Modified Eagle Medium: DMEM (Gibco-life technologies, New York USA) supplemented with 10% fetal bovine serum and THP-1 human monocytes (American type culture collection, ATCC) were grown as suspension in RPMI-1640 medium (Gibco-life technologies, New York USA) containing 10% fetal bovine serum at 37ºC, 5% CO_2.

For infection, J774 macrophages were seeded into 24-well tissue culture plate (TPP, Trasadingen, Switzerland) at concentration 4 x 10^5 cells per well and incubated at 37ºC, 5% CO_2 for 24 h before infection. THP-1 monocytes were diluted in RPMI supplemented with 100 nM (or 100 ng/ml from stock 10^6 ng/ml in DMSO) phorbol 12-myristate 13-acetate (PMA) and seeded to 24-well tissue culture plate at concentration 1 x 10^6 cells/well and incubated at 37ºC, 5% CO_2 for 72 h to allow monocytes to differentiate to macrophages. After incubation, culture media were replaced with fresh culture

media containing conidia of *P. marneffei* wild type, *sakA* and *atfA* mutant and *sakA* and *atfA* complemented strains at concentration 1 x 10^6 cells/well (multiplicity of infection : MOI of 2.5 for J774 and MOI of 1 for THP-1). Cells were incubated for 2 h to allow adhesion and phagocytosis of the conidia. After 2 h, each well was washed with media containing 240 U/ml (50 µg/ml from stock 100 mg/ml in DMSO) of nystatin (Sigma-Aldrich, St. Louis USA) to kill extracellular conidia. Nystatin was replaced by fresh media and incubated for 24 h. After incubation, infected macrophages were lysed with 1% Triton X-100 (Sigma-Aldrich, St. Louis USA). Cell lysates were diluted, plated on SDA and incubated at 25ºC for colony forming unit (CFU) count. The CFUs harvested from cell lysates at 2 h were used as the initial inocula and were acted as the baseline values for intracellular survival analysis. CFUs harvested at 24 h were used for calculation % recovery of fungal conidia inside macrophages. The experiments were performed in triplicates and analyzed using standard *t*-tests (http://www.graphpad.com/quickcalcs/ttest1.cfm?Format=SD).