III. Materials and methods

III.1 Organisms

Seven isolates of *P.marneffei* were used in all experiments. Five were human isolates (no.496, 518, 233, 500 and 444), the one was a bamboo rat isolate and one was a soil isolate (no. LUB 0601 and no. SB 0501, respectively). All were cultured on Sabouraud Dextrose Agar (SDA) slants at 25 °C for 10 days.

III.2 Inoculum

Conidial suspensions were prepared by adding 10 ml of sterile saline solution to each of 10 day old mycelial slants. Cultures were gently scraped and the suspensions were filtered through sterile glass wool. The filtrates were shaken with sterile glass beads (3 mm. in diameter) and centrifuged 2500 x g for 20 minutes. The pelltes were washed twice and resuspended in sterile normal saline solution. The numbers of viable conidia were determined by dilution spread plate on Sabouraud Dextrose Agar (SDA). The suspensions used in all experiments had a concentration of approximately 10^5-10^6 CFU/ml.

III.3 Sequential morphogenesis of *P.marneffei* conidia on Brain Heart Infusion Agar (BHIA).

Conidial suspensions of *P.marneffei* isolates no.496 and no.LUB 0601 were used in this study. Sequential observations of *P.marneffei* conidia were done using microculture system as the method described by Salazar *et. al.* (1988). Slide cultures were set up using 1 cm^2 blocks of BHIA. A 0.005 ml suspension containing 10^4 to 10^5 viable conidia were applied to the blocks and allowed to dry for 5 minutes. A sterile cover slip was placed on the block.

The slide culture was then incubated in a humid chamber at 37°C. Microscopic examinations were done throughout a period of 10 days to determine stepwise conversion of *P.marneffei* from conidium to yeast-like cells.

III.4 Sequential morphogenesis of *P.marneffei* conidia in human blood

Conidial suspensions of *P.marneffei* no. 496 and no.LUB 0601 were used in this study. Human peripheral blood of healthy individual was obtained from Blood Bank Unit, Maharaj Nakorn Chiangmai Hospital. A 0.1 ml suspension of each isolate was mixed with a 2 ml human blood in sterile cotton plugged tubes. The tubes were shaked to mix well and incubated at 37°C. The blood in the tubes were smeared for Wright's stain on Conidial suspensions of in the tubes were smeared for Wright's stain on glass slides at 2 hours, 1, 2, 3 and 5 days after inoculation, respectively.

III.5 Effects of nitrogen sources on transformation of *P. marneffei*

The basal salts medium was prepared according to the method of Philpot (1977). This medium consisted of the following composition per litre : KH_2PO_4 1.0 g ; $MgSO_4$ 0.5 g ; glucose 10 g and agar 16.0 g. Various nitrogen sources were used in this study : $(NH_4)_2 SO_4$, $NaNO_3$, $NaNO_2$ L-glutamine, L-arginine, L-asparagine, L-cysteine, L-cystine, L-phenylalanine, L-tyrosine. Each nitrogen source was supplemented into the medium to give the final concentration of 1 gram per litre. The medium was used to make agar slants (20 ml per tube). Two sets of the medium were inoculated with 0.1 ml conidial suspensions. One set was incubated at 37°C , another set was incubated at 25°C . Brain Heart Infusion agar was used as the control media for transformation of *P.marneffei* at 37°C . Estimation of yeast-like cells were made by microscopic observation at

12 to 15 days after inoculation. The growth of yeast-like cells was recorded as follows : +4 = heavy yeast-like growth (mycelium virtually absent) ; +3 = heavy yeast-like growth (small amount of mycelium) ; +2 = moderate yeast-like growth (moderate mycelium) ; +1 = predominantly mycelium (slight amount of conversion) ; 0 = no conversion.

III.6 Growth on glutamine gradient plates

The basal salts medium was prepared as the method described above. The upper agar layer contained 0.3 g. of L-glutamine (per 100 ml medium), giving a concentration gradient of L-glutamine from 0 to 0.3 g (per 100 ml medium). Conidial suspensions of *P. marneffei* were inoculated on gradient plates. The plates were incubated at 37°C for 7-10 days.

III.7 Effects of glutamine concentrations on transformation of *P.marneffei*

The basal salts medium agar was prepared and L-glutamine was added to the medium to give final concentrations of 0.001 g, 0.005 g , 0.01 g, 0.05 g, 0.1 g and 0.2 g (per 100 ml medium), respectively. The medium was used to make agar slants (20 ml per tube). A 0.1 ml conidial suspension of each isolate was inoculated on the surface of agar slants. The cultures were incubated at 37°C . Microscopic morphology of each isolate was observed.

III.8 Assimilation reactions of yeast-like forms of *P.marneffei*

Assimilation reactions of yeast-like forms of *P.marneffei* were tested by Dye Pour Plate Auxanography (Kern, 1985). Seven yeast-like isolates of *P.marneffei* were subcultured on Yeast Extract Malt Extract (YM) agar and

incubated at 37°C. Suspensions of yeast-like cells were prepared to give the turbidity to a McFarland standard no. 5 The basal medium was prepared according to the method of Kerns (1985). This medium consisted of Yeast nitrogen base 0.67 g ; agar 20.0 g ; distilled water 1 L ; stock bromcresol purple solution (1 g/L) 20 ml ; 0.1 N Sodium hydroxide 4 ml. This medium was sterilized by autoclaving (15 psi for 15 minutes) and poured into screw-capped tubes (20 ml aliquots per tube). A 1.0 ml suspension of *P.marneffei* was mixed with each tube of melted agar and poured into sterile petri dishes. Carbohydrate discs (glucose 30 mg/ml, maltose 50 mg/ml, sucrose 30 mg/ml, lactose 30 mg/ml and trehalose 30 mg/ml) were placed on the agar surfaces. The plates were incubated at 35-37°C. A negative reaction is a purple color and no growth around the discs. In this procedured, color change is correlated with carbohydrate metabolism.

III.9 Effects of carbon source concentrations on transformation of *P.marneffei*

The basal medium contained KH_2PO_4 1.0 (g/L), $MgSO_4$ 0.5 (g/L), L-glutamine 2.0 (g/L) and Agar 15.0 (g/L) was prepared. D-glucose or D-maltose (sterilized by filtration) was added to the medium at various concentrations (0.5% , 1.0%, 2.0%, 3.0% and 4.0% w/v). The medium was used to make agar slants (20 ml per tube). All slants were inoculated with 0.1 ml of conidial suspensions. The cultures were incubated at 37°C , Microscopic examination of the cultures were made at 12 to 15 days after inoculation using wet mounts under coverslips.

III.10 The effect of temperature on transformation of *P.marneffei*

The medium used in this experiment was Brain Heart Infusion Agar (BHIA) slants. The slants were inoculated with 0.1 ml of conidial suspensions. The cultures were incubated at 20°C, 22.5°C, 25°C, 27.5°C, 30°C, 33°C, 35°C, 37°C, 39°C and 41°C ($\pm$ 0.5°C). Microscopic observations were made at 12 to 15 days after inoculation using wet mounts under coverslips.

III.11 The effect of pH on transformation of *P.marneffei*

Brain Heart Infusion Agar (BHIA) slants were prepared and adjusted the final pH to 3.5, 4.5, 5.5, 6.5 7.5, 8.5 and 9.5. The slants were incubated with 0.1 ml of conidial suspensions and incubated at 37°C. Microscopic examinations were made at 12 to 15 day after inoculation as described above.

III.12 The effect of CO_2 on transformation of *P.marneffei*

The media used in this experiments were SDA and BHIA slants. The slants were inoculated with 0.1 ml of conidial suspensions of *P.marneffei.* One set of the agar slants was incubated at 37°C under aerobic conditons and the other set was incubated at 37°C 5-10% CO_2 atmosphere. Microscopic examinations were made at 12 to 15 days after inoculation.

III.13 The effects of sex steroid hormones on transformation of *P.marneffei.*

Conidial suspensions of seven isolates of *P.marneffei* (isolates no.500, 518, 444, 496, 233, LUB 0601 and SB 0501) were used in this study. Testosterone and 17-beta-estradiol used in this experiment were purchased from Sigma Chemical Co. (St. Louis, Mo). Each hormone was diluted in ethanol (Merck) to yield a final concentration of 10^{-3} M. Subsequent dilutions were prepared using ethanol (0.6% v/v) as the diluent. Each hormone was mixed at 56°C with Brain Heart Infusion Agar (BHIA) to give final concentrations of 10^{-6}M, 10^{-8}M, 10^{-10}M and 10^{-12}M, respectively. Slants of agar mixtures were prepared. Controls included BHIA alone and BHIA with ethanol (0.6%. v/v) A 0.1 ml suspension containing 10^4 to 10^5 viable conidia was inoculated on each slant. All slants were incubated at 37°C. Microscopic observation were done after 12 to 15 days of incubation using wet mounts under coverslips.

III.14 Study on ultrastructure of *P.marneffei* using transmission electron microscopy (TEM)

P.marneffei (isolate no.496) was used in this study. The yeast-like phase of *P.marneffei* was obtained by culturing the conidial suspension on Brain Heart Infusion agar (BHIA) and on basal salts medium agar plus L-glutamine (0.2 g%) at 37°C. The mycelial phase was obtained by culturing the conidial suspension on Sabouraud Dextrose Agar (SDA) at 25°C.

Chitin localization in *P.marneffei*

Yeast-like cells of *P.marneffei* were fixed in 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 4 hours at 4°C and rinsed in 4% glycine in 0.1 M phosphate buffer (3 x 10 minutes). The cells were suspended in 0.5% agarose held at 42 °C. Small blocks (1 mm^3) were cut from the agarose, dehydrated in a graded ethanol series and embedded in L.R. White resin in gelatin capsules. The resin was polymerized at 50°C for 48 hours. Sections approximately 90 nm. thick were cut with a diamond knife and mounted on formvar-coated 200 mesh copper grids. Sections were then incubated at 37 °C for 1 hour with wheat germ agglutinin (WGA) conjugated to 10 nm gold particles (Calbiochem) (dilution 1 : 50) and rinsed with distilled water, then stained for 4 minutes in 1% uranyl acetate followed by 1 minute lead citrate. Control sections were treated with chitinase (Sigma, St louis) (40 μg/ml) for 2 hours at room temperature before incubation with labelled wheat germ agglutinin. Samples were examined with transmission electron microscope (JEM-1200 ExII) at 80 KV. Mycelial form of *P.-marneffei* which obtained from cultures on SDA at 25°C for 7-10 days was processed by the same procedure.

Addition to these studies, numbers and sizes of the mitochondria of cells in mycelial phase and yeast-like phase were recorded.