CHAPTER 2

Literature reviews

2.1 History and epidemiology of *Penicillium marneffei*

P. marneffei is the only dimorphic pathogenic fungus of the genus *Penicillium.* It was first isolated in 1956 from the hepatic lesions of a bamboo rat (*Rhizomys sinensis*) at the Pasteur Institute of Indochina, Vietnam (Capponi *et al*., 1956). This bamboo rats died from a spontaneous disseminated mycosis involving the reticuloendothelial system. Later, mice were experimentally infected with this fungus and sent to the Pasteur Institute in Paris for further studies. The fungus was identified by Dr. Gabriel Segretain of a new species called *P. marneffei*, in honor of Dr. Hubert Marneffe, the director of the Pasteur Institute in Indochina (Segretain 1959). Human penicilliosis marneffei was first described as a laboratory-acquired infection when Segretain accidentally pricked his finger with a needle used to inoculate hamsters with the yeast cells of *P. marneffei*. He developed a small nodule at the site of inoculation followed by lymphangitis and axillary lymph nodes hypertrophy (Drouhet 1992).

The first natural human infection was described in 1973 by Di Salvo and collaborators in a patient with Hodgkin's disease who lived in Southeast Asia (Disalvo *et al*., 1973). In 1988, the first case was reported in AIDS patient who traveled in Southeast Asia (Piehl *et al*, 1988). After that, the majority of infections by *P. marneffei* were diagnosed in AIDS patients in endemic area (Li *et al*., 1992; Supparatpinyo *et al*, 1994; Maniar *et al*. 2005; Devi *et al*., 2007). Countries of endemicity include Thailand,

Cambodia, Southern China, Hong Kong, India, Malaysia, Taiwan, Indonesia, Myanmar, Laos, and Vietnam (Ko *et al*., 1994; Chang *et al*., 1995; Rokiah *et al*., 1995; Chang *et al*., 1998; Chiang *et al*., 1998; Hung *et al*., 1998; Hsueh *et al*., 2000; Hien *et al*., 2001; Bailloud *et al*., 2002; Liao *et al*., 2002; Ranjana *et al*., 2002; Huynh *et al*., 2003). However, the incidences of infection among AIDS patients who had visited throughout endemic areas were reported, including patients from Australia, Belgium, France, Germany, Japan, Sweden, Switzerland, the Netherlands, the United Kingdom, and the United States (Hulshof *et al*., 1990; Borradori *et al*., 1994; Heath *et al*., 1995; Grise *et al*., 1997; Julander *et al*., 1997; Depraetere *et al*., 1998; Rimek *et al*., 1999; Mohri *et al*., 2000; Bateman *et al*., 2002). All reported cases of *P. marneffei* infected in AIDS patients showed the relationship with the endemic areas except for one case that was reported in an African AIDS patient from Ghana who had never been to Southeast Asia (Lo *et al*., 2000). In the endemic area, the greatest number of cases was reported in Northern Thailand during the uprising of HIV-infected patients (Supparatpinyo *et al*., 1994; Sirisanthana and Supparatpinyo, 1998; Subsai *et al*., 2004). For the data of patients with *P. marneffei* infection, from 1991 to 1994, 550 cases of *P. marneffei* infections were diagnosed at Chiang Mai University Hospital in Northern Thailand. A numbers of AIDS patients with *P. marneffei* infection increased each year leading up to1994 (Chariyalertsak *et al*., 1996a).

2.2 Mycology

P. marneffei was classified in the section Asymmetrica, subsection *Divaricata*, in the classification of Raper and Thoms (Raper and Thoms, 1949). Later, Pitt assigned *P. marneffei* to subgenus *Biverticillium* which was confirmed by Frisvad and Filtenborg (Frisvad and Filtenorg, 1990) on the basis of similar physiology and secondary

metabolite profiles. A phylogenetic analysis of *P. marneffei* as assessed by the nucleotide sequences of nuclear and mitochondrial rRNA gene regions were used in classification (LoBuglio *et al*., 1995). The results demonstrated that *P. marneffei* is closely related to the species of the *Penicillium* subgenus *Biverticillium* and sexual *Talaromyces* species with asexual biverticillate *Penicillium* states (LoBuglio *et al*., 1995). Subsequently, the concepts of nomenclatural priority and single name nomenclature, all accepted species of *Penicillium* subgenus *Biverticillium* were transfer to *Talaromyces*. Recently, *P. marneffei* has been renamed as *Talaromyces marneffei* based on new molecular phylogenetic analyses (Samson *et al*., 2011).

P. marneffei is the only identified thermal dimorphic fungus within the genus *Penicillium* (Segretain 1959). The fungus grows rapidly as a mold at 25ºC on Sabouraud dextrose agar (SDA), Potato dextrose agar (PDA), and Malt extract agar (MEA). Mycelial colonies appear within 3-5 days. The colonies are granular with shade of greenish-yellow colour and one of the most characteristic features is the production of a soluble red carotenoid pigment that diffuses into the medium (Chiewchanvit *et al*., 1991) (Fig. 2.1A). Microscopically, the mold form is typical of other *Penicillium* species with hyaline septate branching hyphae with lateral and terminal conidiophores. The conidiophores contain basal stripes with terminal verticils of 3 to 5 metulae, each metula bearing 3-7 phialides with spherical conidia in chains (Fig. 2.1B). At 37ºC, the fungus grows rapidly as yeast on Brain heart infusion agar (BHA). The colonies are yeast like and white to brownish white in colour (Fig. 2.1C). Microscopically, the growth consists of many ovate to cylindrical yeast like cells dividing by binary fission (Fig. 2.1D).

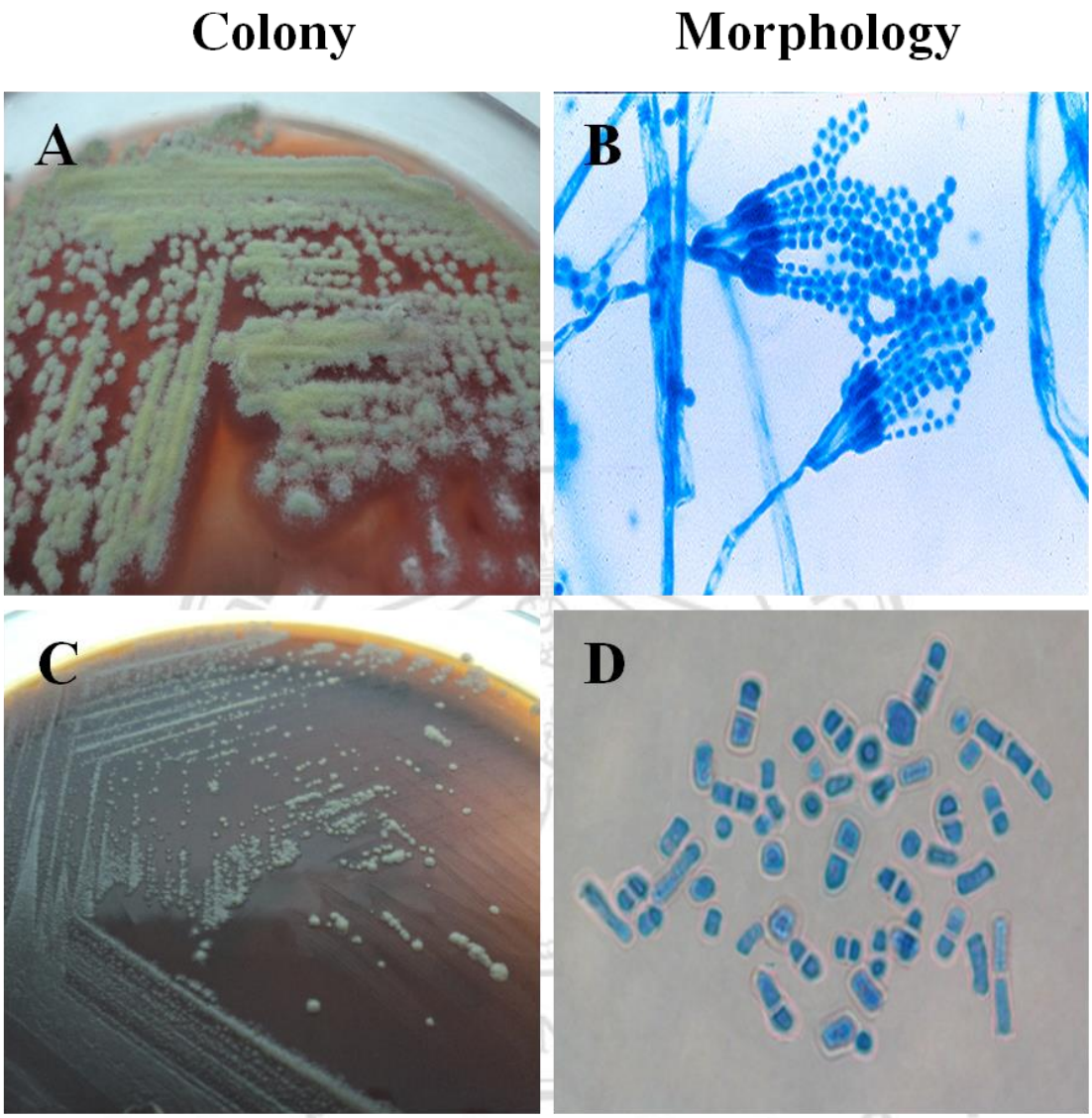

Figure 2.1 Colony appearance and microscopic morphology of *P. marnffei*. *P. marnffei* was cultured on PDA (A) and BHA (C) for 5 days at 25ºC and 37ºC, respectively. Microscopic morphology of mold (B) and yeast (D) of *P. marneffei* was observed under light microscope (magnification, x100).

2.3 Ecology

At present, the basic ecology of *P. marneffei* remains enigmatic. The main issue is whether the human disease, penicilliosis marneffei, occurs as a consequence of animal or environmental transmission. In other words, the ecological reservoirs of human penicilliosis marneffei remain unclear (Vanittanakom *et al*., 2006). Since *P. marneffei* was isolated from bamboo rats, *Rhizomys sinensis* by Capponi and collaborators in 1956, several studies in rodent species have become a focus. In 1986, *P. marneffei* was found in internal organs of 18 out of 19 bamboo rats belonging to the species *R. pruinosus* (Deng *et al*, 1986). After that, *P. marneffei* was isolated from the various species of bamboo rats, including the reddish-brown subspecies of *Cannomys badius, R. sumatrensis,* and *R. pruinosus* (Ajello *et al*., 1995; Chariyalertsak *et al*., 1996b). Deng and collaborators (1988) isolated *P. marneffei* from three soil samples collected from the burrows of *R. pruinosus*. Moreover, Chariyalertsak and collaborators (1996b) also reported that *P. marneffei* was isolated from one out of 28 soil samples collected from the burrows of *R. sumatrensis*.

It is still unclear whether bamboo rats are important natural reservoirs for transmission of the infection to humans in areas of endemicity or whether the organism resides in soil and bamboo rats are only another natural host infected coincidentally with *P. marneffei* (Chariyalertsak *et al*., 1997). Available information seems to suggest the latter. A study compared between 80 cases of disseminated *P. marneffei* infection in patients with AIDS and 160 control patients with AIDS who did not have *P. marneffei* infection showed that exposure to or consumption of bamboo rats was not a risk factor for *P. marneffei* infection. Furthermore, occupational history and exposure to soil,

especially during rainy season, were found to be the critical risk factors associated with infection by *P. marneffei* (Chariyalertsak *et al*., 1997).

2.4 Pathogenesis and clinical features

The route of transmission of *P. marneffei* is likewise unclear. The infection from *P. marneffei* is presumably via inhalation of conidia, and subsequent dissemination to other internal organs by hematogenous spread (Jayanetra *et al*., 1984; Deng *et al*., 1988). Pathology is varied, depending on immunological status of the host. In AIDS patients with *P. marneffei* infection, $CD4^+$ count is less than 100 cells/μl and most of cases have the CD4+ count below 50 cells/μl (Supparatpinyo *et al*., 1994; Antinori *et al*., 2006; Wu *et al*., 2008). Kawila and collaborators (2013) demonstrated that HIV-uninfected penicilliosis patients were less likely to have fever, splenomegaly, and umbilicated skin lesions; more likely to have bone and joint infections; had higher white blood cell count, platelet count, and CD4 cell count; had lower alanine transaminase (ALT); and less likely to have positive fungal blood cultures when compared with HIV-infected penicilliosis patients. Infections in non-HIV-infected patients have also been observed, primarily among immunocompromised patients and less commonly in patients without any known underlying diseases (Duong 1996; Chim *et al*., 1998). Reported cases of non-HIV-associated penicilliosis had occurred in patients with alcoholism, tuberculosis, systemic lupus erythematosus, patients receiving corticosteroid or other forms of immunosuppressive therapy, and even patients with no any apparent underlying disease (So *et al*., 1985; Louthrenoo *et al*., 1994; Lo *et al*., 1995; Lam *et al*., 1997; Chim *et al*., 1998).

2.5 Immunity to *P. marneffei*

The route of entry of *P. marneffei* into the body and mechanisms of host immune response are poorly understood. However, microbiologists have proposed that the infection seems to be acquired from the inhalation of conidia from the contaminated environment (Vanittanakom *et al*., 2006; Cooper and Vanittanakom, 2008). The attachment of *P. marneffei* conidia to host cells and tissues is the first step in the establishment of an infection. The conidia-host interaction may occur via adhesion to the extracellular matrix glycoproteins, laminin and fibronectin via a sialic acid-dependent process (Hamilton *et al*., 1999). To clear this fungal infection, macrophages and T lymphocytes are important for protection against the *P. marneffei* infection (Kudeken *et al*., 1996). The conidia are normally eliminated in an immunocompetent host by macrophages with T-cell-derived cytokines supporting. Conidia were eliminated within 2 to 3 weeks depending on the number of the inhaled conidia, whereas an impairment of cell-mediated immunity in immunocompromised host leads to deprive of T-cell-derived cytokines necessary for macrophage activation. Macrophages are able to control *P. marneffei* growth and to kill intracellular yeast cells when activated *in vitro* by T-cell-derived cytokines, especially IFN-γ. It has been demonstrated that intracellular *P. marneffei* was damaged via the L-arginine-dependent nitric oxide (NO) pathway in IFN-γ-stimulated murine macrophages (Cogliati *et al*., 1997). On the other hand, *P. marneffei* possesses abilities to adapt and defense itself to trigger infection. The interaction between human leukocytes and heat-killed yeast-phase *P. marneffei* has been studied by Rongrungruang and Levitz (1999). Their data suggested that monocyte-derived macrophages bind and phagocytose *P. marneffei* even in the absence of opsonisation and the major receptor(s) recognizing *P. marneffei* could be glycoprotein

with exposed *N*-acetyl-b-D-glucosaminyl groups. *P. marneffei* stimulates the respiratory burst of macrophages regardless of whether opsonins are present, but TNF-α secretion is stimulated only in the presence of opsonins (Rongrungruang and Levitz 1999). The authors thus speculated that the ability of unopsonized *P. marneffei* to parasitize mononuclear phagocytes without stimulating the production of TNF-α may be critical for the virulence of this intracellular parasite. Kudeken and collaborators (1999) investigated the fungicidal activity of human polymorphonuclear leukocytes (PMN) against *P. marneffei*. They found that proinflammatory cytokines, especially granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) and IFN-γ enhanced PMN activity from being fungistatic to fungicidal. In addition to GM-CSF, G-CSF and IFN-γ, other cytokines such as TNF-α and IL-8 are capable of increasing the inhibitory effects of PMN on germination and morphological conversion of *P. marneffei* (Kudeken *et al*., 1999). GM-CSF was reported to enhance phagocytosis and oxidative metabolism of both rabbit pulmonary alveolar macrophages and human monocytes suggesting a potential role for this cytokine in host defense against pulmonary and disseminated *P. marneffei* infection (Roilides *et al*., 2003). In addition, Koguchi and collaborators (2002) demonstrated that osteopontin secreted from monocytes could be involved in the production of IL-12 from PBMCs during infection by *P. marneffei.* The production of osteopontin was also regulated by GM-CSF. It was also indicated the possible involvement of the mannose receptor as a signal-transducing for triggering the secretion of osteopontin by *P. marneffei*-stimulated PBMCs (Koguchi *et al*., 2002). Additionally, Srinoulprasert and collaborators (2009) demonstrated that *P. marneffei* conidia were able to induce TNF-α and IL-1β production in monocytes.

2.6 The putative virulence factors of *P. marneffei*

2.6.1 Adhesins

In *P. marneffei*, a Y674 clone that shows 46% similarity to *Pneumocystis carinii* with major Surface Glycoprotein type II (MSR) showed approximately 364-fold higher expression in yeast compared to mold (Liu *et al*., 2007). MSR has been thought to play a crucial role in the host–parasite interaction during *P. carinii* pneumonia. The attachment of the organism via interaction between MSGs and fibronectin on alveolar macrophages is the key step to the uptake of *P. carinii* by alveolar macrophages mediated by the mannose receptor (Wada and Nakamura, 1999). Liu and collaborators suggested that the protein product of Y674 might be a specific ligand or adhesin on the surface of the yeast of *P. marneffei* that is involved in the specific binding of *P. marneffei* to the host cell. However, the exact role this gene played in host–fungus interaction warrants further investigation.

Extracellular matrix proteins including laminins and fibronectin have been described in an attachment of a variety of pathogens to both host tissues and cells. Hamilton and collaborators (1998, 1999) investigated the interaction of *P. marneffei* conidia with laminin and fibronectin. The authors demonstrated that laminin and fibronectin bound to the surface of *P. marneffei* conidia and phialides and the interaction was mediated through a sialic acid-dependent process.

2.6.2 Dimorphic switching

Dimorphism is a virulence factor, because the morphologic conversion from mold to yeast is thought to be an important in the pathogenesis of dimorphic pathogenic fungi such as *Paracoccidioides brasiliensis*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *P. marneffei* (Sternberg 1994). Conversion to the yeast form may offer

protection against killing by neutrophils, monocytes, and macrophages. Thermal dimorphism of *P. marneffei* plays an important role for survival in host cells. It is responsible for the host body temperature change. Thus, the dimorphic switching is one of the virulence factors of *P. marneffei*. Although it has long been believed that phase transition from mold to yeast is obligatory for pathogenicity, but the mechanism that regulates this switch has remained a mystery. Since molecular genetic studies in *P. marneffei* has become frequent, many investigators have studied the genes controlling dimorphism steps, as they are thought to be potential virulence factors that control the pathogenic growth of *P. marneffei.* There are two reports regarding yeast development. In 2000, Borneman and collaborators showed that the *abaA* deletion mutant displays aberrant yeast morphology as both the developing transitory-state arthroconidial filaments and the yeast cells fail to couple nuclear and cellular division, where multiple nuclei were seen within both the arthroconidial compartments and yeast cells (Borneman *et al*. 2000). Recently, Boyce and collaborators demonstrated that deletion of *pakB* gene leads to a failure to produce yeast cells inside macrophages but no effect *in vitro* at 37^{o}C. Loss of *pakB* gene also leads to an inappropriate production of yeast cells at 25^{o}C *in vitro* (Boyce *et al*. 2009). Identifying these host cell specific signals is the important next step in understanding how pathogens sense and respond to their hosts.

2.6.3 Stress response and adaptation proteins

Oxidative stress is one of innate defenses produced by the host immune cells, including monocyte, macrophage and polymorphonuclear cells, to eliminate parasite. These immune cells play an essential role in killing fungal pathogens by producing reactive oxygen species (ROS), including hydroxyl peroxide (H_2O_2), superoxide radical

anion (O_2^-), and hydroxyl radicals (OH^-) (Fridovich 1998). These ROS can harm pathogens by readily altering or inactivating proteins, lipid membranes, and DNA, and they have potent immunomodulatory effects on the immune system that affect the efficacy of the host response (Missall *et al*., 2004). To protect the cells from oxidative stress, fungi possess superoxide dismutase enzyme (SOD) to detoxify oxygen radicals through the conversion of superoxide to hydrogen peroxide and oxygen (Fridovich 1998). Subsequently, catalases break down hydrogen peroxide into H_2O and O_2 (Miller and Britigan, 1997). In *P. marneffei*, a catalase-peroxidase encoding gene (*cpeA*) and a superoxide dismutase (*sodA*), were previously isolated and characterized (Pongpom *et al*., 2005; Thirach *et al*., 2007)

A high level expression of catalase-peroxidase was observed in *P. marneffei* yeast phase (Pongpom *et al*., 2005) and during macrophage infection (Thirach 2008). The upregulation of *sodA* transcript occurred during yeast growth of *P. marneffei* and during macrophage infection (Thirach *et al*., 2007; Thirach 2008). Pongpom and collaborators demonstrated that the *P. marneffei CpeA* contributed to the fungus' tolerance to hydrogen peroxide but not to heat stress. The hydrogen peroxide treatments caused high expression of this gene in both mycelia and yeast forms. The results suggested that the *CpeA* of *P. marneffei* is used to protect the conidia and yeast cells from oxidative stress in host macrophages (Pongpom *et al*., 2013). Both catalase-peroxidase and superoxide dismutase may be important *in vivo* as they facilitate the intracellular survival of the fungus by providing a nontoxic environment within the macrophage phagosome (Thirach *et al*., 2007).

Since the parasitic phase of *P. marneffei* is closely linked with the higher temperature for normal growth in environment, heat shock proteins (HSPs) are

considered as virulence factors, as they could be upregulated upon infection to prevent misfolding and aggregation of damaged proteins (Lindquist and Craig, 1988). The role of heat shock proteins 70 (Hsps70) of *P. marneffei* was isolated and characterized (Kummasook *et al*., 2007). Their data demonstrated that the upregulation of *hsp70* expression during the mycelium to yeast phase transition. Upregulation was also observed when yeast or mycelial cells encountered a heat shock condition at 39ºC. It has been suggested that Hsp70 may play an important role in environmental stress response and adaptation in *P. marneffei*. Then, Vanittanakom and collaborators described a *hsps30* of *P. marneffei* that showed high level in yeast cells grown at 37ºC, whereas a very low or undetectable transcript level was observed in mycelia cells at 25ºC. The authors speculated that *hsp30* may play a role in heat stress response and cell adaptation (Vanittanakom *et al*., 2009).

2.6.4 Secreted enzymes from *P. marneffei*

Analysis of the enzymatic activity of mycelial and yeast phases of *P. marneffei* was reported by Youngchim and collaborators (Youngchim *et al*., 1999). The authors demonstrated that both mycelia and yeast expressed alkaline phosphatase, acid phosphatase and naphthol-AS-BI-phosphohydrolase activity, whereas a variety of other enzyme activities, including trypsin, chymotrypsin and α-fucosidase were absent. The results suggested that production of acid phosphatase is considered to be one of the virulence factors for survival of *P. marneffei* in phagocytes. Later, Moon and collaborators purified and characterized two protinases (PMAP-1 and PMAP-2) and serine protease (PMNP) produced in culture supernatant by mold and yeast form of *P. marneffei*, respectively. The degradation of elastin, fibronectin and fibrinogen by PMNP *in vitro* suggested that this enzyme may play a significant role in the virulence of

P. marneffei, especially considering its proposed route of infection (inhalation). However, roles for PMAP-1 and the putative enzyme PMAP-2 remains to be investigated (Moon *et al*., 2006).

2.6.5 Melanin

Melanins are pigments of high molecular weight formed by oxidative polymerization of phenolic or indolic compounds and usually are dark brown or black. These pigments are conjugated bipolymer of *ortho*-dihydroxyphenols. (Piattelli *et al*., 1965). These biopolymers are negatively charged, hydrophobic and insolubility in aqueous and organic solvents (Piattelli *et al*., 1965; Hamilton and Gomez, 2002; Gomez and Nosanchuk, 2003). Melanin are produced by a remarkable variety of organisms, including bacteria, fungi, plants, and animals. In dimorphic fungi such as *H. capsulatum, P. brasiliensis, Sporothrix schenckii, B. dermatitidis* and *Coccodioides posadasii,* melanin production may promote fungal survival in different environments, augment their resistance to immune effector responses in the infected host, and reduce their susceptibility to antifungal drugs (Taborda *et al*., 2008). In *P. marneffei,* melanization of *P. marneffei in vitro* and during infection was investigated by Youngchim and collaborators (2005). The authors found melanin in both conidia and yeast cells *in vitro*. Further investigation *in vivo*, they found melanin in yeast cells inside skin tissue from penicilliosis marneffei patients. Moreover, sera from *P. marneffei*-infected mice developed a significant antibody response (both IgG and IgM) against melanin. An enzyme phenoloxidase activity capable of synthesizing melanin from L-DOPA was detected in cytoplasmic yeast cell extracts. The data indicated that *P. marneffei* conidia and yeast cells can produce melanin or melanin-like

compounds *in vitro* and that yeast cells can synthesize pigment *in vivo*. The authors speculated that pigment may play some role in the virulence of *P. marneffei*.

Melanin can be classified according to precursor molecules; the two major ones are DHN-melanin and DOPA-melanin. Pentaketide or DHN and DOPA pathways are shown in Figure 2.2 and 2.3, respectively (Langfelder *et al*., 2003; Pihet *et al*., 2009).

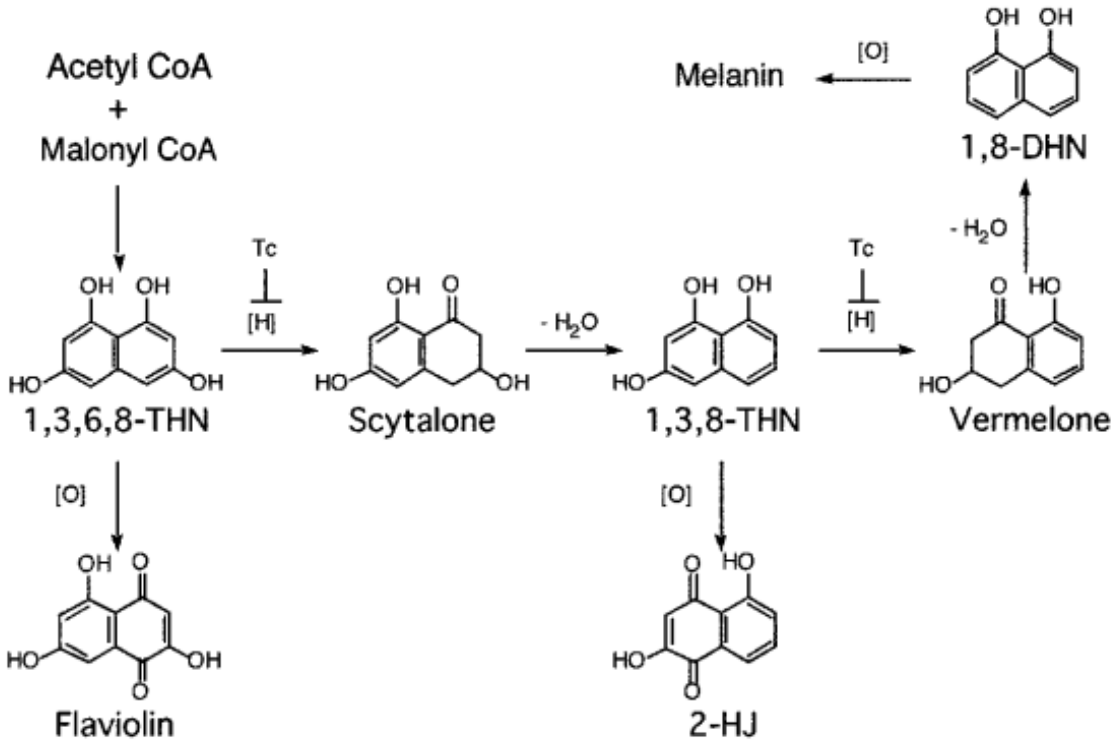

Figure 2.2 Dihydroxynaphthalene (DHN) melanin synthesis pathway in *A. fumigatus*. Acetyl-CoA and malonyl-CoA are the starter and extender of polyketide synthases for production of the precursor 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN). The pentaketide, 1,3,6,8-THN, is then reduced by 1,3,6,8-THN reductase to scytalone, which is subsequently converted to DHN following the dehydration and reduction steps. Finally, DHN is polymerized to form DHN-melanin (Tsai *et al*., 2001).

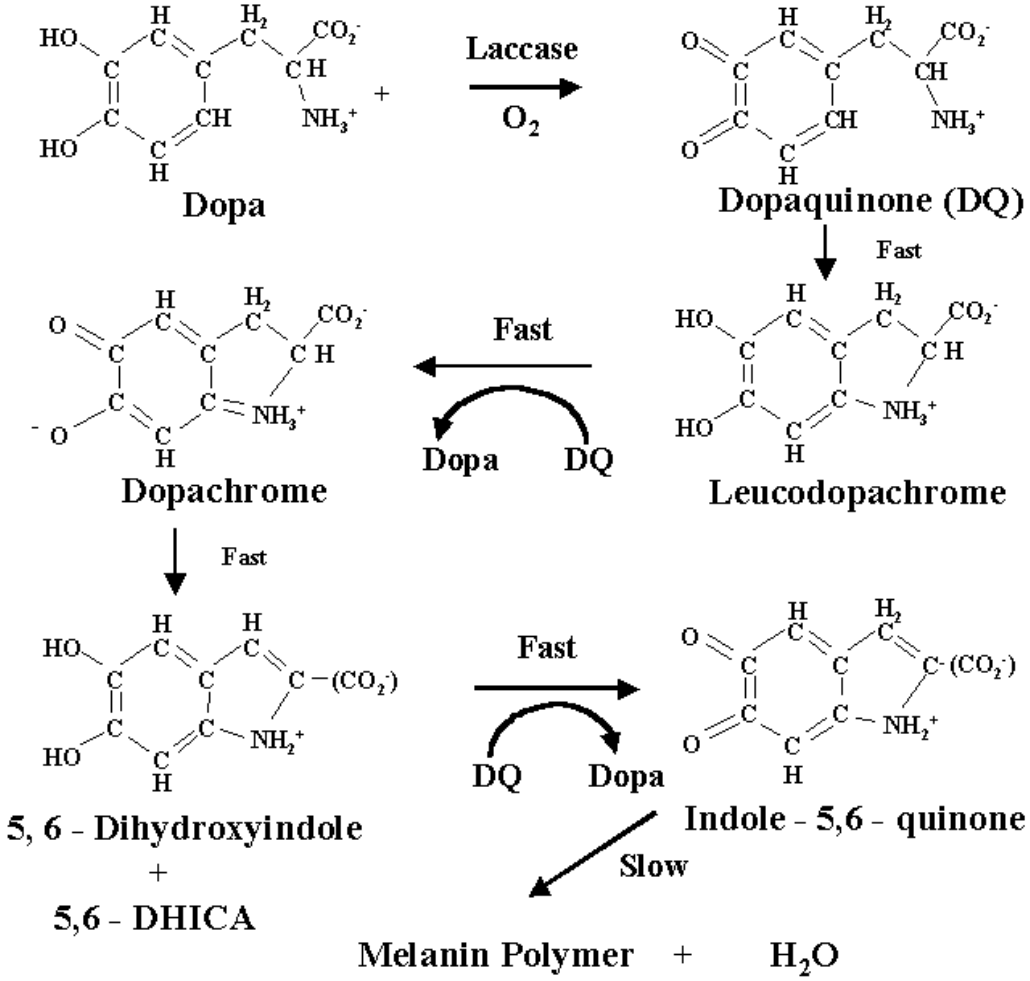

Figure 2.3 DOPA melanin synthesis pathway in *C. neoformans.* DOPA undergoes a four electron oxidation by a phenoloxidase enzyme (laccase) through a series of steps to form the somewhat stable intermediate, dopachrome. This intermediate then non-enzymatically decarboxylates to form 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole carboxylic acid (DHCI), which then undergoes first a two electron oxidation to indole-5,6-quinones followed by a sequential polymerization to melanochrome and then to melanin (Williamson 1997).

Moreover, the pyomelanin production via the tyrosine degradation pathway was demonstrated in *A. fumigatus* (Figure 2.4) (Schmaler-Ripcke *et al*., 2009).

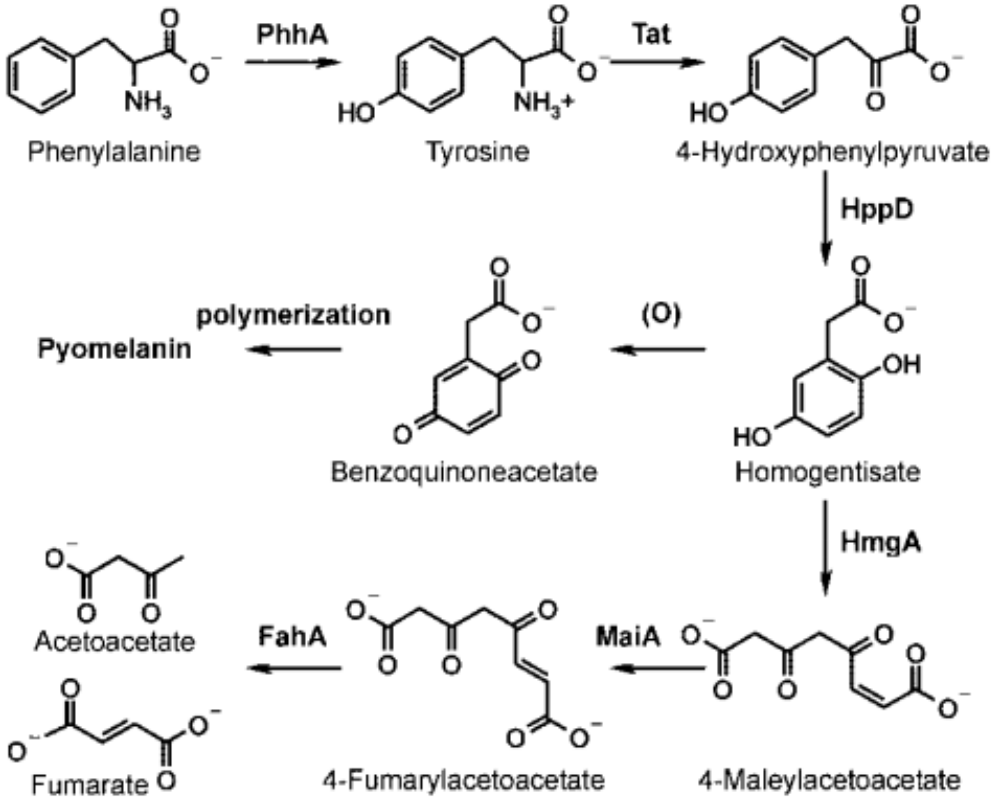

Figure 2.4 Pyomelanin production through tyrosine degradation pathway. The enzymes function in pyomelanin synthesis are phenylalanine hydroxylase (PhhA); tyrosine aminotransferase (Tat); 4-hydroxyphenylpyruvate dioxygenase (HppD); homogentisate dioxygenase (HmgA); maleylacetoacetate isomerase (MaiA); fumarylacetoacetate hydrolase (FahA). Oxidation of homogentisate to be benzoquinone acetic acid which will be polymerized to produce pyomelanin (Schmaler-Ripcke *et al*., 2009).

Dimorphic fungi appear to produce DHN-melanin in conidia/arthroconidia in nature primarily via a polyketide synthase pathway, but there is an absence of this pigment in hyphae. In addition, the yeast form produces DOPA-melanin (Taborda *et al*., 2008). It has been reported that laccase is one of enzymes for DOPA-melanin

production using L-DOPA as a substrate (Nosanchuk and Casadevall, 2006). The pathogenic fungi were investigated in the production of DOPA-melanins via a laccase include many model organisms like *C. neoformans* (Staib 1962; Wang *et al*., 1996), *P. brasiliensis* (Gómez *et al*., 2001, da Silva *et al*., 2006), *H. capsulatum* (Nosanchuk *et al*., 2002), *B. dermatitidis* (Nosanchuk *et al*., 2004), *Candida albicans* (Morris-Jones *et al*., 2005), and *C. posadasii* (Nosanchuk *et al*., 2007). Melanin production and its effects on parasitism by yeast cells or sperules/endospores require specific substrates as shown *in vitro* by the addition of L-DOPA or other suitable phenolic compounds in a process mediated by laccase-like enzymes (Nosanchuk *et al*., 2004; Taborda *et al*., 2008).

2.7 Laccase and their contribution to fungal pathogenesis

2.7.1 Laccase

Laccase (p-diphenol:dioxygen oxidoreductase, EC 1.10.3.2) has been studied since the nineteenth century. Yoshida first described laccase in 1883 from the exudates of *Rhus vernicifera*, the Japanese lacquer tree (Levine 1965; Thruston 1994). Laccase are multi-copper-containing oxidoreductase enzymes that catalyze the oxidation of various organic and inorganic substrate, including amino phenols, mono-, di-, and polyphenols, methoxy phenols, aromatic amines and ascorbate, with the concomitant four electrons reduction of oxygen to water (Galhaup *et al*., 2002). Laccase is most widely distributed in a wide range of higher plants and fungi (Benfield *et al*., 1964), and has been more recently identified in bacteria (Beloqui *et al*., 2006; Kellner *et al*., 2008; Ferrer *et al*., 2010; Ausec *et al*., 2011). It is a glycosylated monomer or homodimer protein generally having fewer saccharide compounds (10-25%) in fungi and bacteria than in the plant enzymes. The carbohydrate compound contains monosaccharides such

as hexoamines, glucose, mannose, galactose, fucose, and arabinose. Mannose is one of the major components of the carbohydrate attached to laccase. Glycosylation in laccase is responsible for secretion, proteolytic susceptibility, activity, copper retention, and thermal stability (Xu 1999). Laccase activity has been described in many fungi and the enzyme has been purified from many species of ascomycetes, deuteromycetes, and mainly basidiomycetes fungi (Assavanig *et al*., 1992).

The role of fungal laccases have been documented that they involve in fungal development, morphogenesis, detoxification process, stress defences and pathogenicity (Thurston 1994). The localization of laccase is presumably connected with its physiological function and determines the range of substrates available to the enzyme. It is possible that the intracellular laccases of fungi could participate in the transformation of low molecular weight phenolic compounds in the cell. The cell wall and spores-associated laccases were linked to the possible melanin synthesis and other protective cell wall compounds (Eggert *et al*., 1996; Galhaup *et al*, 2002).

In *A. fumigatus, Abr2* is laccase functioning in polymerization of dihydroxynaphthalene (DHN)-like melanin (Sugareva *et al*., 2006), whereas *C. neoformans* laccase (*Lac1*) functions in DOPA melanin synthesis (Eisenman *et al*., 2007). In *P. marneffei*, laccase-encoding genes were characterized and functions of their translational protein products, which investigated by Sapmak and collaborators (2012). The authors found that four putative laccase-encoding genes including *lac1*, *lac2*, *lac3* and *arb2* were related to other fungal laccases. The data suggested that abolishing of laccase activity may not inhibit melanin production in *P. marneffei* because *P. marneffei* cells possess the ability to produce many types of melanin through several pathways such as DOPA melanin, DHN melanin and pyomelanin.

2.7.2 The contribution of laccase to pathogenesis

Laccases have been implicated as contributors to virulence in many fungal pathogens. Many studies supported that laccases can provide aggressive and defensive abilities to fungal cells. In *C. neoformans,* it is proposed that the laccase promoted virulence by inhibiting the oxidative burst in the phagosomal space of macrophages as a consequence of reducing Fe^{3+} to Fe^{2+}. Moreover, the putative location of laccase in *C. neoformans* is consistent with its interference of hydroxyl radical production by macrophages (Liu *et al*., 1999). The enzyme is tightly bound to the cell wall and contributes to the resistance to fungicides (Zhu *et al*., 2001). In addition, laccases may also promote virulence of *C. neoformans* by catalyzing the formation of melanin precursors. Melanized *C. neoformans* cells were more resistant to the action of antifungal agents and antifungal activity of macrophage (Liu *et al*., 1999). In addition, the melanin enhances the integrity of cell wall and increases the net negative charge of the cell wall and this latter interferes phagocytosis (Nosanchuk and Casadevall, 2006; Williamson *et al*., 2008). Melanin is also responsible for the neurotropism of *C. neoformans* and may protect the cells from oxidative stress, temperature extremes, iron reduction, and microbicidal peptides (Jacobson and Tinnell, 1993). In *Fonsecaea pedrosoi,* its melanin can inhibit nitric oxide production inside macrophage (Bocca *et al*., 2006). In addition to an other fungus, melanin prevents *S. schenckii* from being killed, enhances protection from UV solar irradiation, and during infection affects host defense mechanisms by reducing phagocytosis and scavenging reactive oxygen and nitrogen species. (Romero-Martinez *et al*., 2000). Based on DHN melanin localized on the surface of *A. fumigatus* conidia, it was shown to protect this fungus by increasing

cell wall integrity, masking immunogen, and inhibiting acidification of phagososomes of macrophage (Pihet *et al*., 2009; Chai *et al*., 2010).

In *P. marneffei,* Sapmak and collaborators (2012) demonstrated that losing of each gene did not affect growth ability of this fungus; however, deletion of *arb2* gene resulted in changing the grayish green to be light brown colony at 28^{o}C. A single laccase gene deletion did not affect the resistance to stress. Losing of four laccases could not abrogate melanin production but laccase activity existed in a lower level. Deletion of four laccase-encoding genes was more sensitive to oxidative stress (H_2O_2), cell wall stress (SDS), and antifungal agents such as itraconazole, fluconazole and clotrimazole. The exact role of laccase in pathogenicity of *P. marneffei* is still unclear and needed to be investigated.

2.8 Laboratory diagnosis

2.8.1 Staining and culture methods

Culture and staining methods are common for diagnosis *P. marneffei* infection. The clinical specimens included bone marrow aspirates, blood, lymph node biopsies, skin scrapings, sputum, pleural fluid, liver biopsies, cerebrospinal fluid, pharyngeal ulcer scrapings, urine, kidney, pericardium, stomach or intestine and stool samples were used in diagnosis of *P. marneffei* infection (Drouhet 1992; Supparatpinyo *et al*., 1994). Staining with Wright's stain of bone marrow aspirates and/or touch smear of skin biopsy or lymph node biopsy is a rapid diagnosis method (Supparatpinyo *et al*., 1992). The fungus can be seen in histopathological sections stained with Grocott methenamine silver (GMS) or periodic acid-Schiff stain (PAS). In contrast, *P. marneffei* yeast cells may result in the false impression that a capsule similar to *H. capsulatum* when staining with hematoxylin and eosin (H&E) (Nelson *et al*., 1999). Under a microscope, the fungus appears in fission arthroconidia or unicellular round to oval yeast cells, which may divide by cross wall formation in macrophages or histiocytes (Chiewchanvit *et al*., 1991).

Cultivation is the gold standard for diagnosis of *P. marneffei* infection. The bone marrow gave the highest yield for culture positive, approaching 100%, as followed by culture of the specimens obtained from skin biopsy and blood culture (Supparatpinyo *et al*., 1994). However, most of fungal isolated in routine laboratory screening are usually obtained from blood culture of HIV-infected patients. *P. marneffei* is identified by macroscopic and microscopic examination. However, a disadvantage of the culture method is time consuming (1 to 2 weeks).

2.8.2 Serodiagnosis

Several serodiagnostic tests have been developed for detection of specific antibodies against antigens of *P. marneffei* in clinical specimens. The indirect fluorescent antibody test was developed in which mycelia-form germinating conidia and yeast-like cells as antigens (Yuen *et al*., 1994). An immunodiffusion test was used to detect *P. marneffei* antigens and antibodies in sera from human infected with *P. marneffei* and of a latex agglutination test was also used to diagnose antigenemia (Kaufman *et al*., 1996). The protein antigens of the yeast form of *P. marneffei* produced during growth phases were found to be more immunoreactive than the proteins of the mold form (Vanittanakom *et al*., 1997). A Western blot results revealed the major proteins of 54 and 50 kDa, which were strongly reactive with sera of *P. marneffei*-infected AIDS patients. A recombinant antigenic mannoprotein of *P. marneffei* (Mp1p) that specific to *P. marneffei* was constructed (Cao *et al*., 1998). Then, the anti-Mp1p antibody was used in an enzyme-linked immunosorbent assay (ELISA)-based test for the serodiagnosis of the mannoprotein Mp1p in the sera of both immunocompetent and immunocompromised AIDS patients. Evaluation of the test revealed 100% specificity and approximately 80% sensitivity in HIV seropositive patients infected with *P. marneffei* (Cao *et al*., 1998). An ELISA for the detection of *P. marneffei* antigen in urine was developed by using fluorescein isothiocyanate-labelled purified rabbit hyperimmune immunoglobulin G (IgG) (Desakorn *et al*., 1999). Later, this polyclonal antibody was used in a dot blot ELISA and a latex agglutination (LA) test for the detection of *P. marneffei* antigen in urine (Desakorn *et al*., 2002). All tests were highly sensitive and specific. In addition, a monoclonal antibody (MAb)–based sandwich enzyme-linked immunosorbent assay was developed for the detection of *Penicillium*

antigen in clinical specimens from patients with *P. marneffei* infection (Panichakul *et al*., 2002). The mixture of two monoclonal antibodies (8B11 and 8C3) was used in an antigen capture ELISA for detection of *P. marneffei* antigens in sera of humans in areas where the organism is endemic. The result showed high sensitivity and specificity to the test (Chaiyaroj *et al*., 2003). Recently, our group has developed an inhibition enzyme-linked immunosorbent assay (inh-ELISA) incorporating the yeast phase specific MAb 4D1-recognized antigenic mannoproteins for the detection of *P. marneffei* infection (Prakit *et al*., 2014). Our data shows that detection of circulating antigens in *P. marneffei* infected patients is potentially significantly useful not only for diagnostic purposes but also as a tool to evaluate the clearance of fungal burden during treatment.

2.8.3 Molecular diagnosis

The polymerase chain reaction (PCR) assay has been used effectively for the specific detection of many fungi. The primer PM2 and PM4 were developed to amplify a 347 bp fragment of the internal transcribed spacer (ITS) region between 18S rDNA and 5.8S rDNA (LoBuglio and Taylor, 1995). Additional the primers RRF1 and RRH1 were used in PCR-Southern hybridization format for amplification of a 631 bp fragment of the 18S rDNA and then hybridized with a *P. marneffei*-specific 15-oligonucleotide probe (Vanittanakom *et al*., 1998). Moreover, single and nested PCR methods were then developed using newly designed specific primers, also based on the 18S rRNA gene sequence of *P. marneffei* (Vanittanakom *et al*., 2002). This assay was described to be sensitive for detection young fungal cultures (2-day-old filamentous colony, 2 mm in diameter). Further diagnostic PCR methods have also been described; a one-tube seminested PCR assay based on the 18S rRNA sequences was developed to identify *P. marneffei* DNA (Prariyachatigul *et al*., 2003). This assay was sensitive and could

identify *P. marneffei* DNA both from pure cultures and two clinical samples. However, the PCR asssay has been developed in research laboratory but is not available for routine clinical use.

2.9 Treatment

Based on an *in vitro* antifungal susceptibility test, *P. marneffei* showed high susceptibility of the fungus to miconazole, itraconazole, ketoconazole, and flucytosine (Supparatpinyo *et al*., 1993). The clinical and microbiological responses correlated with the most patterns of *in vitro* susceptibility to the azoles, whereas results with amphotericin B were more difficult to detect (Supparatpinyo *et al*., 1998). The initial treatment is to give intravenous amphotherin B at a dose of 0.6 mg/kg/day for 2 weeks, followed by 400 mg/day of itraconazole orally in two divided dose for the next 10 weeks. After initial treatment, the patients should be given 200 mg/day of itraconazole as secondary prophylaxis that has been effective in preventing relapses in a controlled clinical trial (Sirisanthana *et al*., 1998).