# VI. DISCUSSION

## 6.1.Phagocytosis and killing assay of *Penicillium marneffei* and *Penicillium citrinum* using mouse macrophage J774.1 cells

*Penicillium marneffei*, a pathogenic dimorphic fungus, can cause disseminated disease in people with impaired cell-mediated immunity, especially those infected with human immunodeficiency virus (HIV). The types of exposure and route of entry of this fungus, leading to human infection, are still unclear. By analogy with other opportunistic fungal pathogens, it seems quite likely that conidia may be inhaled from a contaminated environment and subsequently disseminate from the lungs when immunosuppression occurs in the host's condition (Cooper and McGinnis, 1997; Vanittanakom *et al*., 2006). During infection, phagocytic cells are likely to be the primary line of the host defense against this fungus. In phagocytes, the fungus can survive and proliferate in the phagosome. They also demonstrated that histiocytes of tissues infected with *P. marneffei* contained yeast like cells of *P. marneffei*. As the lesion progressed, the intracellular fungal cells were released following cellular necrosis and subsequent abscess formation. Free fungal cells or phagocytes containing fungal cells can disseminate throughout the body. In this study, the J774.1 murine macrophage-like cell line, which was found to be appropriate for *in vitro* studies of *P. marneffei*, was used (Cogliati *et al*., 1997). Phagocytosis and the killing activity of mouse macrophage J774.1 against conidia of pathogenic *P. marneffei* were examined in comparison with non-pathogenic *Penicillium citrinum in vitro*. The collective results of this study, suggested that the macrophages play a crucial role in the immune response to the fungi. Differences in the percentage of phagocytosis of both pathogenic and nonpathogenic *Penicillium* isolates used in this study by J774.1 were not statistically significant. However, the phagocytic indices of *P. marneffei* were higher than those of *P. citrinum* after incubation at 60 min. This result revealed that *P. marneffei* was more infective to macrophage J774.1 cells than *P. citrinum*. In other words, macrophage cells are the possible target of *P. marneffei* infection. In addition,

the conidia of *P. marneffei* seemed to be more resistant to being killed by macrophages than the non-pathogenic fungus, *P. citrinum*. These results suggest that *P. marneffei* conidia are able to inactivate macrophage defense. Phagocytosis of microbes by macrophages normally results in microbe elimination through the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and the action of lysosomal enzymes following phagolysosomal fusion (Missall *et al*., 2004; Nathan and Shiloh, 2000). Thus, survival within the phagocyte environment of *P. marneffei* may depend on some virulence mechanisms that counter or inactivate macrophage defenses, and diverse mechanisms likely exist for the dimorphic transition (Cogliati *et al*., 1997). In contrast, the conidia of non-pathogenic *P. citrinum* could not escape from these defense mechanisms and were readily killed by macrophage cells. In many respects, macrophage interactions with *P. marneffei* are quite similar to that seen with *Histoplasma capsulatum*. Yeast cells from both species are bound and phagocytosed in the absence of opsonin and both stimulate a respiratory burst (Bullock and Wright, 1987; Newman, 1999; Newman *et al*., 1990). Similarly, the pathophysiology and clinical presentation of histoplasmosis and penicilliosis marneffei share overlapping features including the parasitism of macrophages (Bullock, 1993; Cooper and McGinnis, 1997; Mootsikapan and Srikulbutr, 2006). However, clearly there are differences, most notably in the mechanisms by which the two fungi gain access to the macrophage. *H. capsulatum* entry into macrophages is divalent cation dependent and uses CD18 complex (Bullock and Wright, 1987; Newman *et al*., 1990), whereas *P. marneffei* is divalent cation independent and gain access through a lectin wheat germ agglutinin (WGA) inhibitable process (Rongrungruang and Levitz, 1999). However, many unanswered questions remain, including the definition of the specific receptor(s) responsible for binding and the conditions necessary to activate the macrophage to inhibit and kill *P. marneffei*. Moreover, the study of interactions between macrophage and *P. marneffei* could serve to increase general understanding of the mechanisms of intracellular parasitism. The mechanism of intracellular infection and survival inside the macrophage of *P. marneffei* will be investigated further.

### 6.2. Characterization of putative virulent genes and their possible roles in *P. marneffei* pathogenicity

In order to establish a successful infection, *P. marneffei* cells that colonize within the dynamic substrate of a human host must have the ability to adapt to and modify gene expression in response to changes in the host environment. Over the years, several genes of *P. marneffei* have been identified, including catalase peroxidase (*cpeA*), isocitrate lyase (*acuD*) and heat shock protein 70 (*hsp70*). Besides the importance in phase transition of *P. marneffei*, the functions of these genes are also involved in host defense response. For instance, catalase peroxidase may play a role in preventing the damage of $H_2O_2$ inside macrophage and heat shock protein can protect fungal cells from the temperature shift, especially in the host cells. In addition, the response of fungus to host environments, such as nutritional deprivation, could induce expression of the potential virulence genes for fungal survival. However their roles in virulence have not been investigated. To better understand its pathogenicity, we isolated and characterized more putative virulent genes that are involved in stress response and adaptation such as, superoxide dismutase, catalase, GAPDH and calmodulin encoding genes from *P. marneffei*. The degenerate primers were designed to amplify certain gene fragments being used as the probes for cDNA and gDNA library screening. The copper, zinc superoxide dismutase (Cu, Zn SOD) encoding cDNA of *P. marneffei* was isolated and subsequently designated as *sodA*. The deduced amino acid sequence of this gene exhibited a high level of identity to other fungal Cu, Zn SODs, including from the ascomycetous fungi, *Aspergillus fumigatus*, *Aspergillus oryzae* and *Paracoccidioides brasiliensis*, as well as to SODs from mammals, e.g. *Mus musculus* and *Homo sapiens* (data not shown). Moreover, the multiple sequence alignment showed many conserved sequence positions among all species. One, the catalytic site, encompasses the six histidine residues that coordinated binding of the copper and zinc atoms. Other conserved residues surrounding the active site include two cysteine residues which form a putative disulfide bridge that is invariantly found in all eukaryotic Cu, Zn SODs. Cu, Zn SOD located at or just below the cell surface could have a hypothetical role in protecting the fungal cell against extracellularly generated superoxide. This postulated role would, of course, be most effectively performed by extracellular Cu, Zn SOD, which might or might not be

associated with the fungal cell wall/capsule. It is known from various studies that the addition of extraneous SOD to *in vitro* killing assays involving fungal pathogens may abrogate fungicidal activity (Brummer and Stevens, 1989; Desai *et al.*, 1995; Potoka *et al.*, 1998). Potentially, Cu, Zn SOD produced by the invading fungus might function in the same way and thus act as a virulence determinant. In this study, we demonstrated the differential expression of *sodA* during phase transition from the conidia to mycelia and yeast phase by using semi-quantitative RT-PCR with exponential amplification and subsequently confirmed by Northern blot analysis. The results from both methods revealed the downregulation of *sodA* transcript in the mycelia phase and upregulation in the yeast phase. It is likely possible that *sodA* might play an important role in the phase transition of *P. marneffei*, and may be important *in vivo*, as it would facilitate the intracellular survival of this fungus by providing a non-toxic environment in the macrophage phagolysosomes. Consequently, we demonstrated the differential *sodA* expression during macrophage infection. Within the phagocytic cell lines, the expression of *sodA* increases significantly in comparison with the conidia control (Figures 31, 36, Table 3). As previously documented, *P. marneffei* resides in macrophages during many stages of experimental and human infections (Chan and Chow, 1990) and resistance to macrophage-mediated killing may be important for virulence in this fungus. Thus, our findings strongly suggest that Cu, Zn SOD is involved in the virulence of *P. marneffei*. Our study also provides important clues as to how the antioxidant enzymes of fungal pathogens function in the infection process. To confirmation this hypothesis, further experiments by generating genetically defined *sodA*-deficient mutants and the use of animal models will be needed for this important fungal pathogen.

Other interesting genes that may affect metabolism and nutrient availability were found. The deduced amino acid sequence derived from *P. marneffei gapdh* showed high identity to the corresponding sequence of *Ajellomyces capsulatus* in the closely related Eurotiomycetes. The analysis of GAPDH sequences has been shown to be useful to determine evolutionary relationships between different organisms (Ridder and Osiewacz, 1992). Interestingly, the presence of well-defined clades comprising Eurotiomycetes, Dothideomycetes, and Sordariomycetes could be correlated to the

patterns of introns in the cognate genes. Our data also confirmed that the position of introns in *gapdh* genes corresponded to systematic relatedness. Protein homology and intron positions of *gapdh*s could probably be analyzed to give information of phylogenetic relationships in Ascomycota. Although *gapdh* has been considered to be a constitutively expressed gene in most organisms, increasing amount of evidence supports the contention that the regulation of this gene depends on several cellular conditions. Barbosa *et al*. demonstrated that the expression of GAPDH and its transcript is more abundant in the parasitic yeast phase of *P. brasiliensis* (Barbosa *et al.*, 2004; 2006). These observations raise the intriguing question of whether the fungal GAPDH may also perform other important non-glycolytic functions. In our study, the transcript of *gapdh* was constitutively expressed in conidia, mycelia, and yeast phase and during macrophage infection of *P. marneffei* by RT-PCR analysis. On the contrary, the differential expressed of *gapdh* could be observed by Northern blot analysis: the transcript of this gene was more expressed in mycelium and conidia more than in yeast phase. During macrophage infection, this gene seems to be repressed upon internalization by macrophage since 2 h (Figure 39, Table 3).

In this study, we failed to isolate full-length catalase and calmodulin encoding genes from cDNA library. However, approximately 1,100 bp sequences of catalase gene could be isolated from gDNA library of *P. marneffei* (Yuen *et al*., 2003). The homology search of the sequences showed 67% identity to spore-specific catalase (*CatA*) of *Aspergillus fumigatus* (XM742595). The functional analysis of these sequences revealed the specific domains of catalase (Figure 20). From the gene expression analysis by using RT-PCR, the expression of *P. marneffei cat* seemed to be specific for conidial form of *P. marneffei*. The cat transcript was barely expressed in mycelial phases and undetectable in the yeast phase. Consequently, we could not isolate this gene from cDNA library which was constructed from yeast phase of *P. marneffei*. In *H. capsulatum*, expression of *CATA* is high in mycelium but extremely low in yeast cells. Interestingly, after challenge of yeast cells with $H_2O_2$, a rapid response was seen in *CATA* gene expression (Johnson *et al.*, 2003). Likewise, *A. fumigatus* CatAp is the only catalase present in resting conidia and is not found in hyphae. CatAp could protect the spores against the deleterious effects of $H_2O_2$ *in vitro* but it does not play a role in protecting conidia against the oxidative burst of

macrophages (Paris *et al*., 2003). In *P. marneffei*, catalases need to be further characterized and investigated for their protective function against the stress environments.

The other gene of interest, calmodulin of *P. marneffei* (*cam*) was partially characterized. The positive clone containing calmodulin could be isolated from neither cDNA nor gDNA library. However, the sequences of 550 bp from degenerate primer amplification was characterized and showed 87% identity to calmodulin (*cmd*) gene of *Talaromyces flavus*, *Penicillium pinophilum* and *Penicillium aculeatum*. The functional analysis of these sequences revealed the EF-hand calcium binding motif, the specific domain of calmodulin gene (Figure 19). As a calcium-binding protein, calmodulin is a primary transducer of intracellular calcium signals (Schulman, 1993). In other fungal systems, calmodulin has been implicated in the control of virulence, morphogenesis, and responses to stress (Brown *et al*., 2007; de Carvalho *et al*., 2003; Joseph and Means, 2002; el-Rady and Shearer, 1996; Rasmussen *et al*., 1990). In the plant pathogenic fungi *Magnaporthe grisea*, *cam* gene expression was inhibited by self-inhibitors at an early stage in the process of conidial germination and differentiation (Liu and Kolattukudy, 1999). In the present study, low transcript of *cam* was found to be restricted in only conidia of *P. marneffei*. This results indicate that calmodulin of *P. marneffei* is probably involved in the early stage of dormant conidial germination. However, the role of calmodulin in the regulation of cellular responses to environmental changes of *P. marneffei* needs further investigation.

### 6.3. Differential expression of putative virulence genes in different phases and during macrophage infection

The expression of putative virulent genes was examined by semi-quantitative RT-PCR and Northern blot analysis. The expression patterns of *sodA* and *cpeA* in different phases and during macrophage infection were in agreement by both methods. However, differences of *acuD, hsp70* and *gapdh* expression in phases and during macrophage infection were not statistic significant by RT-PCR while the distinction of differential expression pattern could be observed in Northern blot analysis. Although RT-PCR is the most sensitive method for detecting low-abundance mRNA, often obtained from limited samples, there are substantial problems

associated with its true sensitivity, reproducibility and specificity, as a quantitative method, it suffers from the problems inherent in PCR. The discrepancy in our study might be explained in several ways, including primers interfering and sensitivity of RT-PCR. Thus, the slightly differential gene expression might be undetectable by RT-PCR. However, this approach could be applied to examine the expression of *sodA* and *cpeA* in both phases and during macrophage infection. Thus, we have validated differential gene expression pattern recorded in the array by semi-quantitative RT-PCR and Northern blot analysis (Table 3).

The expression patterns of *cpeA*, *acuD* and *hsp70* in phases transition of *P. marneffei* were examined previously (Cánovas and Andrianopoulos, 2006; Cooper and Haycocks, 2000; Kummasook *et al*., 2007; Pongpom *et al*., 2005). In this study, our obtained data were in agreement of previous describes. For the novel characterized genes, *sodA*, downregulation could be observed in the mycelial phase and the upregulation in the yeast phase. In contrast, *gapdh* expression was higher in mycelia and conidia than yeast phase. This result suggested that the *sodA* as well as *cpeA* and *acuD* might play an important role in the phase transition of *P. marneffei*. Additionally, the accumulation of *hsp70* and *gapdh* transcripts could be found in conidial dormant form of *P. marneffei*, but their possible roles in fungal virulence remain unknown.

Several studies reveal that facultative intracellular bacteria and fungi alter their gene expression programs in response to the macrophage environment (Chatterjee *et al.*, 2006; Fan *et al*., 2005; Lorenz *et al*., 2004) The understanding of this process should present key elements on how *P. marneffei* manage to survive and replicate intracellularly and eventually lead to discovery of candidate genes for pathogenicity. In the present study, we examined the expression of potential virulent genes of *P. marneffei* in response to macrophages environment. The experiment was begun with collective total RNA from the *P. marneffei* conidia inside macrophage cells after co-incubation at 2, 4 and 8 h. The appropriate times were considered from data of phagocytosis and killing assay. In an early 2 h of infection, the maximum phagocytosis occurred and almost conidia were phagocytized. After 4 and 8 h of late infection, an increasing number of *P. marneffei* conidia was internalized and killed by macrophage cells; in the meanwhile the morphology of *P. marneffei* conidia was not

changed after infection. After internalization, macrophages expose pathogens to a group of toxic antimicrobial molecules, including reactive oxygen intermediates (ROI) generated by the phagocyte NADPH oxidase system, and reactive nitrogen intermediates (RNI) generated by the inducible nitric oxide synthase (iNOS). In this sense, the significant production of $O2^-$ and $H_2O_2$ by *P. marneffei* infected macrophages corroborates the idea that an efficient adaptive system for detoxification of NADPH oxidase-dependent oxidative burst products may be operating in this fungi in order to promote its survival within phagosomes. In fact, our data revealed oxidative stress related genes (*sodA, cpeA* and *hsp70*) that were up-regulated in phagocytized *P. marneffe*i. The significantly induced gene in our analysis, *sodA* encodes for a putative Cu, Zn SOD, which is an enzyme involved in the elimination of superoxide anions. The importance of Cu, Zn SOD in fungal virulence and viability has been recently addressed. *Candida albicans* cells lacking *sod1,* which encodes the cytoplasmatic localized SOD, are unable to survive against the fungicidal attack of a macrophage cell line and have attenuated virulence in a mouse model of infection (Hwang *et al*., 2002). Similar results were obtained with *Cryptococcus neoformans* (Cox *et al.*, 2003) implicating SOD in protection against host-derived oxygen radicals. In *P. brasiliensis*, Cu, Zn SOD encoding gene or *sod3,* was induced after internalization by macrophage and also in yeast cells treated with $H_2O_2$ *in vitro* (Tavares *et al*., 2007).

Another important oxidative stress response gene of *P. marneffei*, the *cpeA* gene, was induced after exposure to oxidative radicals produced by macrophages. Transcript of *cpeA* was extremely upregulated in an early response to macrophage environment. Pongpom *et al.* (2005) reported previously that the catalase peroxidase mRNA level was elevated in the yeast but not in the mycelial phase. Recently, Xi *et al.* (2007) demonstrated an increase in the protein level of catalase peroxidase in the yeast phase comparing with low level in the mycelial phase. Besides, the function of gene in phase transition, in this study, *cpeA* of *P. marneffei* may have an important role in detoxification of stress response within macrophage cell. Further, an Hsp70 encoding gene which is a molecular chaperone that promotes the correct folding of several proteins was also induced during macrophage infection at 8 h. In addition to its function as a chaperone, Hsp70 is involved in cellular response to oxidative stress

that plays a critical role in normal cellular function and in recovery and survival after heat shock (Feder and Krebs, 1997; Mayer and Bukau, 1998). As the parasitic phase is closely linked with the higher temperature of the mammalian host, the developmental class factors will likely include heat shock proteins (HSPs) and other temperature-related stress responses. During the mycelia-to-yeast transition upon temperature shift, *H. capsulatum* HSP70 becomes significantly elevated and then returns to constitutive levels, implying that HSP70 may be required only for the initial adaptation to higher-temperature growth (Caruso *et al.*, 1987). In contrast, a *P. brasiliensis* HSP70 member is highly produced only after establishment of the yeast phase (Silva *et al.*, 1999). The decreased virulence of the less thermotolerant Downs strain of *H. capsulatum* suggests that adaptation to higher-temperature environments is important *in vivo*. The production of Hsp70 may contribute to the protection of cells from damage and to repair cells exposed to stress, which may occur during infection. Thus, maintenance of *hsp70* expression level in *P. marneffei* exposed to the oxidative radicals produced by macrophages may have a similar protective role.

The macrophage phagosome is believed to be a poor source of glucose and amino acids (Fan *et al*., 2005; Lorenz *et al*., 2004). Such nutrient deprivation inside the macrophage induces a similar adaptative response of intracellular bacterial and fungal pathogens. Shortly following phagocytosis, *Listeria monocytogenes* and *C. albicans* have a strong reduction in the expression of genes involved in glycolysis (Chatterjee *et al*., 2006; Lorenz *et al*., 2004). We show here that early phagocytized *P. marneffei* also sense and respond to the glucose depleted environment of the macrophage phagosome. The gene encoding GAPDH enzyme in the glycolysis pathway was down-regulated in internalized *P. marneffei*, and the similar results were found in *P. brasiliensis* (Tavares *et al*., 2007). Interestingly, we could observe an up-regulation of isocitrate lyase gene (*acuD*), which encodes one of the glyoxylate cycle key enzymes. The induction of *acuD* observed following phagocytosis suggests a role of this metabolic pathway in *P. marneffei* adaptation inside macrophages, strongly reinforcing nutritional deprivation inside the phagosome. Since this fungus is a facultative intracellular pathogen and glycolytic substrates are supposed to be absent or spare in the phagolysosome environment (Schnappinger *et al*., 2003), glyoxylate

cycle may be required for the utilization of C2 compounds derived from fatty acids in energy production. The role of this pathway in the survival of microorganisms within macrophages has been shown in pathogens, such as *M. tuberculosis, C. albicans* (Barelle *et al*., 2006; Graham and Clark-Curtiss, 1999; Lorenz and Fink, 2001; Schnappinger *et al*., 2003). In *P. brasiliensis*, induction of this cycle in response to macrophage microenvironments was shown to be coordinated with the upregulation of the gluconeogenic phosphoenolpyruvate carboxykinase gene (pck) (Derengowski *et al*., 2008). Induction of the glyoxylate cycle upon phagocytosis has been described as an important pathogen adaptation to the glucose-poor environment within macrophages since it facilitates the assimilation of two-carbon compounds (Lorenz *et al*., 2004). This may be an indication that the organism has identified simple carbon sources in this environment and is activating the pathways necessary to utilize them. An intriguing possibility is that substrates derived from the breakdown of fatty acids via β-oxidation, which results in acetyl-CoA, whose utilization would require the glyoxylate cycle. If fatty acid breakdown, whether of host lipids or lipids from the microorganism itself, occurs within the phagolysosome, it might provide a carbon source accessible to the pathogen while it is inside the macrophage. These data suggested that upon internalization by macrophage, *P. marneffei* responses to phagocytosis by concomitant with the induction of alternate carbon metabolism, there is a dramatic downregulation of *P. marneffei gapdh* gene in glycolysis pathway. Thus, this interaction consists of *P. marneffei* resuming an induction of the glyoxylate cycle to be a response to nutrient deprivation inside the macrophages, resulting in an upregulation of *P. marneffei acuD* that encodes isocitrate lyase enzyme. More generally, the discovery of the role of the glyoxylate cycle in microbial virulence should refocus attention on the critical importance of basic metabolic pathways in the development of disease. If a pathogen is unable to synthesize the precursor nucleic and amino acids required for growth, it is unlikely to proliferate or persist. For this reason, elucidation of a pathogen's nutritional requirements in vivo as well as the mechanism by which microbes acquire these nutrients once inside a host are critical in understanding virulence and disease.

Collectively, in an early stage of infection, *P. marneffei* conidia could induce the oxidative stress response presumably due to the burst of reactive oxygen and nitrogen

species from the macrophages. Then, intense response to phagocytosis by concomitant with the induction of alternate carbon metabolism, there is a dramatic downregulation of *P. marneffei gapdh* gene in glycolysis pathway. Thus, a late phase of this interaction consists of *P. marneffei* resuming an induction of the glyoxylate cycle to be a response to nutrient deprivation inside the macrophages, resulting in an upregulation of *P. marneffei acuD* (Figure 41).

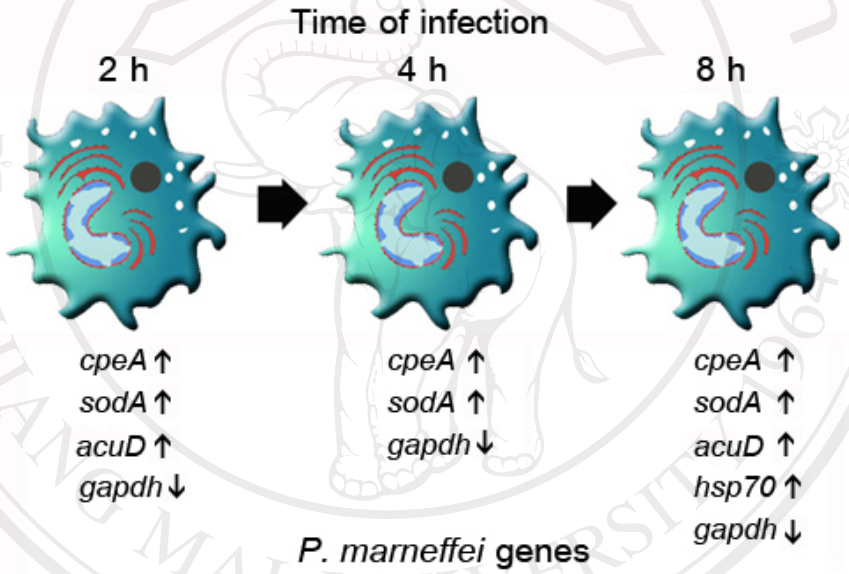


**Figure 41. Proposed model of differential *P. marneffei* genes expression during macrophage infection.** Results for the following genes are shown: *P .marneffei.* Arrows indicate gene induction (↑) or gene repression (↓). Differential expression of stress related genes, *cpeA* (catalase-peroxidase), *sodA* (superoxide dismutase), *acuD* (isocitrate lyase), *gapdh* (glyceraldehyde-3-phosphate dehydrogenase) and *hsp70* (heat shock protein 70).

**6.4. Future prospects**

It is likely that during *Penicillium marneffei* infection in the host cells, it encounters various environmental stress to which the fungus adapts by regulating expression of specific genes and, as the parasite, it can survive within macrophage that leads to fatal infection in immunosuppressed hosts, such as patients with AIDS. Identifying differentially expressed genes in a given model system is a powerful tool for investigating how organisms change in the response to environmental conditions. Discovering which genes are involved in such response can lead research down a number of paths, from identifying signaling mechanism to defining novel drug targets. Our study provides the gene expression analysis of *P. marneffei* response to the intracellular environment of murine macrophages. The differentially expressed genes identified in this report are potential targets for molecular studies of possible virulence factors. Further gene knock out experiment together with an experimental infection model will be needed to assess which genes are involved in the virulence of *P. marneffei.*

Genome projects for *P. marneffei* are nearing completed at this time. Comparative genome analysis has the potential to identify gene products required for specific pathogenic lifestyles or shared pathogenesis characteristics. The completion of genome sequencing projects and use of genomics-based analyses will rapidly expand the set of candidate genes important for virulence. In contrast to the abovementioned reverse-genetic strategies for defining virulence factors, forward-genetic screens are a powerful approach to identify virulence associated genes directly through reduced virulence phenotypes. *Agrobacterium*-mediated transformation for DNA transfers has been developed. When combined with a fungal selectable marker such as hygromycin resistance, this methodology essentially makes possible random insertional mutagenesis through the transfer of T-DNA sequences into the fungal chromosome. Additionally, RNAi holds great promise as an alternative for gene replacement methodologies in dimorphic fungal pathogenesis studies. This natural process relies in the ability of the cell to target and destroy foreign genetic material may represent an attractive alternative for gene analysis in *P. marneffei*.