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Insights into the pathogenicity of *Penicillium marneffei*

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Penicillium marneffei is a significant pathogen of AIDS patients in Southeast Asia. This fungus is unique in that it is the only dimorphic member of the genus. Pathogenesis of *P. marneffei* requires the saprobic mold form to undergo a morphological change upon tissue invasion. The *in vivo* form of this fungus reproduces as a fission yeast that capably evades the host immune system. The processes that control these morphological changes, better termed as phase transition, can be replicated *in vitro* by incubation of the mold form at 37°C. The unidentified molecular mechanisms regulating phase transition in this fungus are now being uncovered using modern methodologies and novel strategies. A better comprehension of these underlying regulatory pathways will provide insight into eukaryotic cellular development as well as the potential factors responsible for infections caused by *P. marneffei* and other fungi. Such knowledge may lead to better chemotherapeutic interventions of fungal diseases.

Fungi belonging to the genus *Penicillium* are typically perceived as ubiquitous, monomorphic molds that play important roles in the environment, agriculture and industry [1]. With one exception, *Penicillium* species are infrequently associated with disease [2,3]. The relative avirulent nature of most species is evident in that only four cases of *Penicillium* infections, parochially termed penicilliosis (**Box 1** [4,5]), have been recorded among AIDS patients. One species, however, is conspicuously different from other members of this genus – that lone species being *Penicillium marneffei*.

P. marneffei has emerged as a highly significant pathogen and holds the distinction of being the only dimorphic member of the genus [5–8]. Infections due to *P. marneffei*, often referred to as 'penicilliosis marneffei', have been largely restricted to Southeast Asia. Almost all cases of penicilliosis due to this fungus have occurred in immunocompromised persons, the vast majority of whom have developed AIDS caused by HIV. Although individuals infected with *P. marneffei* have been diagnosed outside of the endemic region, virtually all have resided or traveled in the endemic region. In some of these cases, the disease did not appear until the afflicted individual developed immune dysfunction years after probable exposure to the fungus. This delay in the appearance of disease, in one instance up to 11 years after initial exposure, suggests that *P. marneffei* possesses the ability to evade a normally functioning immune system until *in vivo* conditions, for example, immunosuppression, are favorable for growth and dissemination throughout the host.

P. marneffei was first described in 1956 as an etiological agent that infected experimental bamboo rats being used to model rickettsiosis [6,9–11]. When isolated from the rats, cultures of the fungus incubated at room temperature grew as a mold bearing typical penicilliate structures of *Penicillium* species (**Figure 1**). However, only yeast forms were noted *in vivo* (**Figures 1 & 2**). This anomaly was resolved when the dimorphic nature of *P. marneffei* was demonstrated in the laboratory by incubating cultures at 37°C, whereupon the isolate developed colonies of single-celled yeasts that divided by fission.

Interestingly, with the exception of a laboratory-acquired infection caused by the original bamboo rat isolate, no human cases of penicilliosis due to *P. marneffei* were documented until 1973 [12]. Subsequent reports of *P. marneffei* infection were relatively rare and often misdiagnosed as histoplasmosis [13]. However, in the late 1980s, the entire dynamic changed in Southeast Asia, particularly in northern Thailand. The AIDS epidemic in this region began to expand rapidly. Concurrent reports of penicilliosis due to *P. marneffei* markedly increased as well. To date, more than 6000 documented cases have been observed in northern Thailand alone, almost all in HIV-infected individuals [7,8]. Infection by *P. marneffei* is now recognized as an AIDS-indicator disease. Moreover, *P. marneffei* infections have been recorded throughout Southeast Asia in a region circumscribed by Taiwan, India, China and Malaysia. Virtually all cases have involved HIV-infected persons or others with severe immune dysfunction. Few cases have been reported in apparently immunocompetent individuals.

Keywords: dimorphism, genomics, pathogenesis, penicilliosis, *Penicillium marneffei*, phase transition, proteomics, signal transduction, transcription factors, virulence factors

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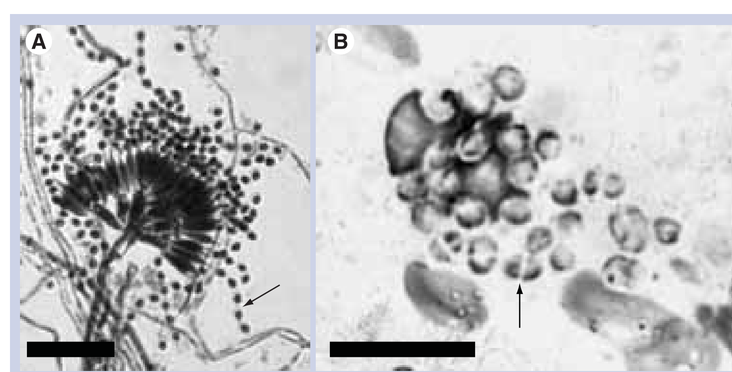
Box 1. Penicilliosis.

In accord with published recommendations regarding the nomenclature of fungal diseases [4,5], infections caused by species of *Penicillium* might be more appropriately termed 'hyalohyphomycoses'. This term defines a pathology in which colorless (hyaline) fungi grow filamentously in afflicted tissues. However, infections by *Penicillium marneffei* do not appropriately fit this pathologic description since this species exhibits a yeast phase *in vivo*. Therefore, the generic term 'penicilliosis' is used in the present text solely to define infections caused by any *Penicillium* species and is not meant to describe a particular tissue pathology. Where needed, a specific attribution to a particular species is employed, for example, 'penicilliosis due to *P. marneffei*'.

The ecological niche occupied by *P. marneffei* remains one of medical mycology's enigmatic puzzles. Infections by *P. marneffei* appear more rural than cosmopolitan and vary seasonally, with cases more common in the rainy season than in dry portions of the year [14], thereby suggesting an environmental source of infection. Yet, other than bamboo rats and their burrows, *P. marneffei* has never been cultured from nature, including soil, water, vegetation or air [7,8,15,16]. Moreover, bamboo rats seem to be incidental hosts rather than reservoirs of infection. A possible clue to resolving the mysterious ecology of *P. marneffei* was uncovered by high-resolution multilocus genotyping of clinical and rat isolates [17]. Genotypic clusters of isolates occupy discrete ecological zones arguing that the profound asexual nature of *P. marneffei* has led to the evolution of niche-adapted genotypes. This observation also helps explain the geographically restricted endemicity of this fungus.

The clinical features of *P. marneffei* infections have been extensively documented [6–8,13]. Infection is presumably initiated by the inhalation of conidia (spores) that are subsequently phagocytized by pulmonary histiocytes. Rather than being destroyed by the action of these immune cells, however, the fungus is able to survive and grow within this harsh intracellular environment. The conidia develop into very small yeasts (2–3 μm × 2–7 μm) and are approximately the same size as yeast cells of *Histoplasma capsulatum* (Figure 2). This may account for early cases being misdiagnosed as histoplasmosis. The distinguishing feature of *P. marneffei* yeast cells is that they divide by fission as opposed to the budding yeasts of *H. capsulatum*. Exactly how *P. marneffei* is able to evade destruction by the host is not entirely clear, although a number of factors that have been studied are discussed below. Conceivably, once established within the phagocyte, *P. marneffei* can be readily disseminated throughout the body and give rise to systemic infection upon depression of the host's immune status.

Figure 1. Comparison of the mold and yeast phases of *Penicillium marneffei*.



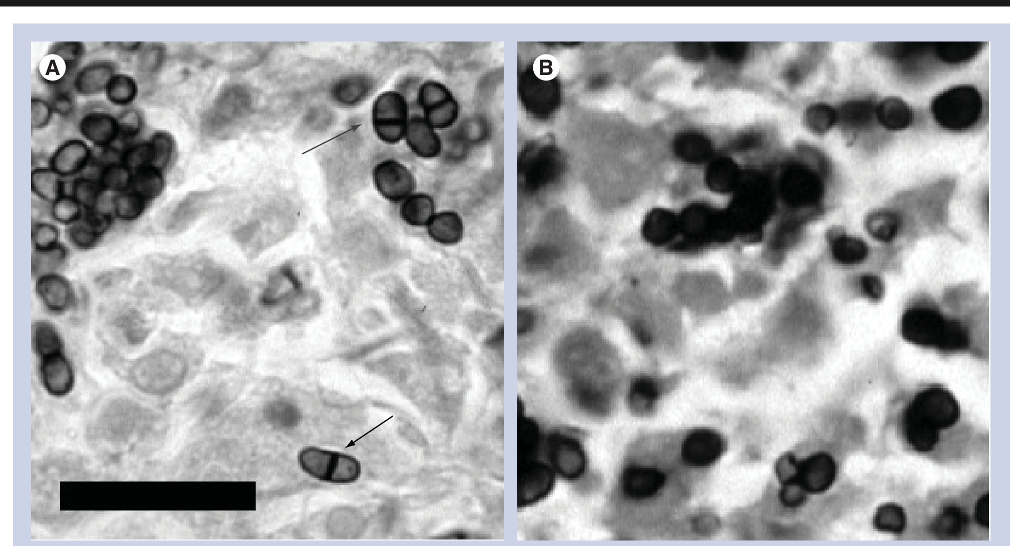
(A) Depicts a conidiophore comprised of metulae and phialides bearing chains of individual conidia (arrow) as observed by differential interference contrast optics. Upon infection, conidia undergo phase transition to form yeast cells that divide by fission (arrow in **(B)**; Wright stained blood smear). Scale bar: 10 μm.

Treatment of penicilliosis due to *P. marneffei* is essential. Left untreated, infections are typically fatal [6–8]. Treating cases during the 1980s and 1990s produced mixed results in that most isolates of *P. marneffei* were resistant to the antifungal effects of fluconazole and many were only moderately responsive to amphotericin B [18]. At the time, both of these drugs were considered primary antifungal agents for the treatment of most systemic fungal infections. Currently, many infected patients are first treated with amphotericin B, which is followed by prolonged prophylactic administration of itraconazole [19]. Documented studies, however, indicate that itraconazole use alone is very effective in the treatment of *P. marneffei* infections as is the newer antifungal agent, voriconazole [20–22]. To date, itraconazole or voriconazole resistance in *P. marneffei* infections has not been described. However, some incidents of clinical resistance to itraconazole have been reported in the closely related fungus *Aspergillus fumigatus* [23,24].

Dimorphism & phase transition in *Penicillium marneffei*

Dimorphism is broadly defined as "... the ability of a fungus to grow in at least two vegetative forms and to carry out any type of distinct vegetative-phase transition" [25]. In essence, dimorphism is the phenotypic expression of phase transition, which is comprised of the underlying processes that bring about stable, morphological changes. The most recognized type of dimorphism among fungi that cause systemic disease in mammals is the transition from growth as a saprobic mold to

Figure 2. Photomicrographs of *Penicillium marneffei* and *Histoplasma capsulatum* yeast cells *in vivo*.



The arrows in **(A)** denote yeast cells of *Penicillium marneffei* that are dividing by fission as opposed to the budding mode of reproduction exhibited by *Histoplasma capsulatum* **(B)**. The bar in **(A)** represents 10 μm and is applicable to both photomicrographs, which were taken from methenamine silver-stained tissues.

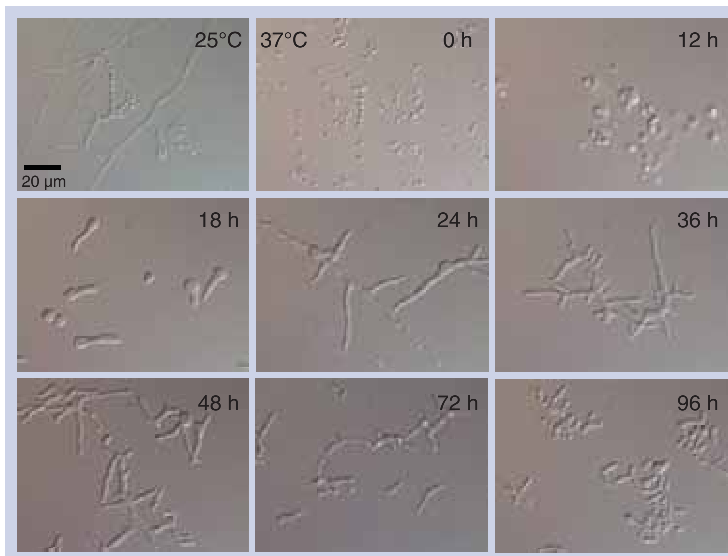
that of an *in vivo* yeast form [26,27]. Interestingly, six of these well-known pathogens, including *P. marneffei*, share one common feature: all express temperature-induced phase transitions.

Only in the past decade have studies focused on the molecular mechanisms of phase transition in *P. marneffei*. Early dimorphic studies of this fungus were limited to morphological examination of clinical isolates and the cellular events accompanying the mold-to-yeast transition process [28–30]. Subsequent investigations have refined these phenotypic observations [2,13,16,31,32].

In vitro studies in our laboratories, and in others, have documented the morphological features that characterize phase transition in *P. marneffei* **(Figure 3)**. Our experiments are often initiated using dormant, haploid conidia. Like other *Penicillium* species, the conidia of *P. marneffei* incubated at 25°C swell prior to germination. During germination, the haploid nucleus is duplicated as the germling grows apically, thereby producing true, septate hyphae (filaments) having multiple nuclei per cell. Continued incubation at 25°C on solid media results in asexual development (conidiation) by which the characteristic conidia-bearing structures of *Penicillium* species are formed **(Figure 1)**. Normally, conidiation does not occur in shaken liquid cultures of *P. marneffei* grown at 25°C. Instead, homogeneous cultures of hyphae are formed. Concurrently, these mold cultures secrete a diffusible, bright red pigment into the medium. Similarly, conidia incubated on solid or in liquid media at 37°C also germinate to form septate hyphae that are multinucleate. However, after 18–24 h of incubation, they are wider and much shorter in length. As these short hyphae age, they fragment into small, single-celled uninucleate reproductive entities, termed arthroconidia. The newly formed arthroconidia then grow as yeast-like cells that undergo brief elongation prior to septum formation and fission. Eventually, such growth establishes a nearly homogeneous culture of uninucleate yeast cells that are strikingly similar to those produced *in vivo* **(Figures 2 & 3)**.

Throughout development of the yeast phase at 37°C, the red pigment characteristic of the 25°C cultures is not produced. Significantly, reciprocal shifts in the incubation temperature entirely reverse the direction of the phase transition and pigment production in *P. marneffei*, specifically, uninucleate yeast cells transform into multinucleated hyphae that secrete a red pigment, and vice versa. These observations suggest that the tightly controlled thermal regulation of phase transition in *P. marneffei* is coupled with nuclear division in addition to secondary metabolism [31,32]. Moreover, these observations imply that the thermal regulatory mechanisms of dimorphism in *P. marneffei* might be similar to those of other fungal

Figure 3. Phase transition in *Penicillium marneffei*.



Incubation of conidia from the mold phase (upper left frame) in liquid media at 37°C initially induces isotropic growth followed by germination. At this temperature, the germlings develop apically as short filaments. However, after 24 h of incubation, the filaments begin to fragment into arthroconidia that subsequently divide by fission. Within 3 days, the culture is comprised mostly of yeast cells. All photomicrographs were generated using differential interference contrast optics. The bar in the upper left frame represents 20 µm and is applicable to all other frames.

pathogens. Recently, histidine kinase activity was demonstrated to govern dimorphism in *Blastomyces dermatitidis* and *H. capsulatum* via a thermal sensing mechanism [26,27]. A similar regulatory pathway remains to be discovered in *P. marneffei*.

Factors influencing pathogenesis in *Penicillium marneffei*

A review of the published literature pertaining to *P. marneffei* indicates that the majority of reports prior to 1990 described the clinical features, treatment and epidemiology of penicilliosis due to this fungus. Since then, more studies have focused on the biology of *P. marneffei,* particularly in areas pertaining to its dimorphic nature and interaction with the host defenses. Many of these studies used observational and biochemical approaches in an attempt to define those factors responsible for transition of the mold form to the yeast phase, given that this process is a requisite for pathogenesis. Using similar methods, other investigations addressed how *P. marneffei* interacts with host defenses, including its ability to invade and survive in the harsh intracellular environment of a phagocytic cell. The latter is viewed as a critical initial step in causing systemic disease.

Until recently, progress in understanding the molecular biology of *P. marneffei* has been limited. The multinucleate state of the mold phase has hampered genetic studies. Although haploid, conidia soon become multinucleate upon germination. Moreover, genetic analysis of meiotic products is not possible due to the absence of a sexual reproductive cycle in *P. marneffei*. Curiously, *P. marneffei* possesses genes shown to have roles in the sexual reproduction of other fungi [33,34], but is unable to mate for unknown reasons. However, the establishment of molecular methodologies, similar to those previously used to study filamentous fungi such as *Aspergillus* [32], has dramatically enhanced our insight into the factors that potentially influence pathogenesis in *P. marneffei*.

The following discussion describes some results from investigations using molecular strategies to specifically address the factors in *P. marneffei* involved in cellular development and phase transition, host–pathogen interactions and virulence. These studies mainly characterized genes having homologous functions in *Aspergillus* as well as other fungi. As noted later, these *P. marneffei* genes include those that regulate intracellular signaling, transcriptional regulation and cellular development **(Table 1)**. However, to date, no genes have been identified that specifically induce dimorphism in *P. marneffei.*

Transcriptional regulation

Given the similarity of asexual development between *P. marneffei* and *Aspergillus nidulans*, the function of the transcriptional regulatory gene, *abaA*, was investigated [35]. In both of these fungi this gene encodes an ATTS/TEA protein that is induced following action by the induction signals involved in conidiation. Once expressed, AbaA removes this developmental process from further influence of other inductive signals. In *P. marneffei*, AbaA appears to control some cell- cycle events during conidiation. Similarly, with regard to dimorphism in *P. marneffei*, the function of AbaA is restricted to governing those cell- cycle events that couple nuclear and cell division in developing yeast forms. Nonetheless, the absence of AbaA does not hinder phase transition since *abaA* deletion mutants continue to grow as yeasts that reproduce by fission. Initially, these yeasts are multinucleate, but over time they become uninucleate, thereby suggesting the existence of a separately regulated mechanism that fulfills the cell-cycle control functions of the nonfunctioning *abaA* gene.

Table 1. Known *Penicillium marneffei* genes associated with intracellular signaling, transcriptional regulation and cellular development.

Gene (GenBank Accession Number)	Identity	Function	Ref.
abaA (AF272838)	ATTS/TEA DNA-domain transcriptional regulator	Regulates cell-cycle events; involved in conidiation and hyphal-yeast development	[35]
cflA (AF330694)	Rho-like GTPase; ortholog of *Saccharomyces cerevisiae CDC42*	Regulation of conidial germination and polarized growth of yeast cells; in conjunction with *cflB*, controls polarized growth of hyphae	[44,45]
cflB (AF515698)	Rac-like protein	Regulates hyphal branching; interacts with *cflA* to control polarized growth of hyphae	[45,46]
gasA (AF448796)	G protein α-subunit (class I)	Negative regulator of asexual development; regulation of red pigment production at 25°C	[42]
gasB (AY301989)	G protein α-subunit (class II)	Unknown	[31]
gasC (AY170625)	G protein α-subunit (class III)	Regulator of germination; positive effector of red pigment production at 25°C; negative regulator of conidiation	[43]
rasA (AY232652)	Ras-like GTPase	Regulates conidial germination and polarized growth of both yeast and mold hyphal cells; inhibits asexual development	[45]
stlA (AF284062)	Ortholog of *Aspergillus nidulans steA*	Unknown function in *Penicillium marneffei*; complements sexual defect in *A. nidulans steA* mutant	[34]
stuA (AF436076)	APSES group of transcription factors	Required for development of sterigmata, metulae and phialides during conidiation	[36]
tbpA (AY945932)	TATA-binding protein	Essential for filamentous growth	[38]
tupA (AY082798)	Tup1/GROUCHO-related WD40 repeat transcription factor	Maintenance of hyphal growth	[37]

Another gene transcription regulator in *P. marneffei*, StuA, is a member of the APSES protein group [36]. APSES proteins regulate yeast–hyphal transitions in other fungi. However, despite the required role for StuA in the formation of metulae and phialides during conidiation in *P. marneffei*, this protein is not essential for dimorphism. The absence of a role for StuA in phase transition may be due to fundamental differences in opposing developmental processes. Conidiophore components (i.e., metulae and phialides) result from a blastic (budding) type of process, whereas the yeast phase of *P. marneffei* expresses a fission mode of growth. Hence, StuA and other APSES proteins are likely limited to controlling those morphogenic processes that involve blastic development.

Additional clues to the underlying molecular controls of phase transition in *P. marneffei* have come from studies involving the *tupA* gene [37]. *tupA* is a Tup1/GROUCHO-related WD40 repeat transcription factor. Homologs of this gene in other filamentous fungi regulate asexual development. In *P. marneffei*, TupA represses asexual development as well as yeast cell morphogenesis. Its primary function involves the maintenance of the hyphal form. Hence, *tupA* has no apparent role in phase transition.

The significance of the TATA-binding protein, encoded by *tbpA*, to growth and development in *P. marneffei* has also been examined [38]. TbpA is not only essential to filamentous growth and asexual development, but the level of *tbpA* expression is an important factor in the proper execution of these developmental programs. However, TbpA has virtually no role in yeast growth and development. Given that TATA-binding proteins are essential transcriptional regulatory elements for all cell types, another protein functionally equivalent to TbpA and having a key role in the development and maintenance of the yeast phase must exist in *P. marneffei*.

Signal transduction factors

Signal transduction pathways serve as important links between environmental stimuli and the genetic regulation of cellular development and fungal pathogenesis [39,40]. These stimuli include

signals that promote mating and differentiation of sexual reproductive structures. In the nonpathogenic fungus *A. nidulans*, *steA* is a critical regulator of the sexual cycle. A homolog of this gene, *stlA*, has been identified in *P. marneffei* [34]. In addition to *stlA*, virtually all known *A. nidulans* meiotic genes exist in *P. marneffei* [33,34]. These observations suggest that *P. marneffei* possesses a cryptic sexual cycle. Further support for this contention is based upon the ability of *stlA* to complement the sexual defect in *steA* mutants of *A. nidulans* [34]. However, deletion of *stlA* has no phenotypic effect in *P. marneffei*. Such mutants appear to undergo normal vegetative growth, asexual development and phase transition.

Another class of signaling proteins that have been well studied in filamentous fungi, including pathogens, includes the heterotrimeric guanine nucleotide-binding proteins (G-proteins) [41]. G-proteins link environmental stimuli received by surface molecules to cytoplasmic effector elements. Such signaling pathways are known to be involved in fungal morphogenesis and virulence. Among the three protein subunits comprising G proteins, it is the α-subunit that possesses GTPase activity. Furthermore, the α-subunit can be divided into three different phylogenetic classes.

The genes encoding all three classes of Gα-subunit proteins in *P. marneffei* have been cloned and characterized [31,42,43]. The proteins encoded by *gasA* (class I) and *gasC* (class III) have related functions; the former is a strong inhibitor of asexual development and GasC is a weak negative regulator of this process. Conversely, GasA is a weak promoter of red pigment production by *P. marneffei* at 25°C, but GasC is a stronger positive regulator of this secondary metabolic pathway. The overlapping functions of these two protein subunits suggest that they share various signaling components. In addition, GasC plays a major role in the regulation of conidial germination and may be linked to an as yet unidentified sensing mechanism that triggers this developmental process. However, GasA and GasC appear to have no significant role in the regulation of either hyphal or yeast growth, or in phase transition. The biological function of the third Gα-subunit encoded by *gasB* (class II) is unknown. *gasB* deletion mutants do not demonstrate any detectable effect upon growth and development, including phase transition.

In addition to *gasC*, two other genes, *rasA* and *cflA*, have roles in regulating conidial germination in *P. marneffei*. RasA, an ortholog of known Ras GTPases in other fungi, and CflA, an ortholog of the Rho GTPase Cdc42, coordinately regulate germination by controlling the correct temporal and spatial execution of this process [44,45]. Together, they also control polarized growth of both hyphal and yeast cells. However, RasA has additional functions acting as a negative regulator of asexual development and a positive growth response to environmental stresses. CflA has no role in these processes. A third gene, *cflB*, a RAC-like homolog, also positively influences polarized actin-dependent hyphal growth, particularly septation and branching [45,46]. In contrast to RasA, CflB stimulates asexual development by mediating the polarized growth of conidiophores, but has no role in yeast growth. Interestingly, neither *rasA*, *cflA*, nor *cflB* function is required for phase transition.

Differential gene expression

Although the cloning of orthologous genes from *P. marneffei* has greatly enhanced our understanding of asexual development in this fungus, the particular molecular mechanisms underlying phase transition remain unresolved. Attempts to address this problem have employed differential gene expression strategies. Using differential display techniques to identify genes specifically associated with mold or yeast growth, one encoding malate synthase was isolated [2]. Malate synthase and a second enzyme, isocitrate lyase, comprise the glyoxylate cycle. The glyoxylate cycle is an anapleurotic pathway that is known to play a role in both fungal and bacterial pathogenesis [47–50]. Recently, the gene encoding isocitrate lyase in *P. marneffei*, *acuD*, was cloned and characterized [51]. *acuD* expression is induced by AbaA as well as other unknown cell type (yeast phase) specific factors. The exact role of *acuD* in the virulence of *P. marneffei* awaits further study. In the pathogenic yeasts *Candida albicans* and *Cryptococcus neoformans*, isocitrate lyase appears to be important for survival within phagocytes, but is not required for progression of infection [50,52].

Subtractive hybridization was also used to identify 43 differentially expressed genes in the mold and yeast phases of *P. marneffei* [53]. Collectively, these homologues included genes involved in cell-cycle control, cell wall synthesis, transport, general metabolism, stress response and signal transduction. One of the yeast-specific sequences identified from *P. marneffei* is homologous to the small Ras-like GTPase gene (*Rsr1*) from *A. fumigatus*. In *Saccharomyces cerevisiae, RSR1* is known to affect cell polarity via a direct interaction with *CDC42* [54]. In *C. albicans*, the *Rsr1* ortholog

(*CaRSR1*) also functions in establishing correct polarity during morphogenesis [55,56]. Mutants in *CaRSR1* exhibit reduced virulence. Hence, with regard to phase transition and virulence in *P. marneffei*, the obvious experiment would be to investigate the interaction of *Rsr1* and *CDC42* (*CflA*) orthologs in this fungus. The results of such an experiment should be compared to those described above for the interaction between *RasA*, another small GTPase, and *CflA* [44,45].

Subtractive hybridization also identified a second yeast-phase gene that exhibited significant homology to a major surface glycoprotein found in *Pneumocystis carinii* [53]. This glycoprotein appears to play crucial roles in the pathogenesis of *P. carinii*, including facilitating the attachment of this microbe to host alveolar macrophages as well as complexing with host molecules to evade host immune responses [57]. The phase-specific expression of the *P. marneffei* ortholog suggests that it may have a role in virulence by promoting the attachment to host cells, much like previously described sialic acid-specific lectin [7,58].

Proteomic analysis of differential gene expression

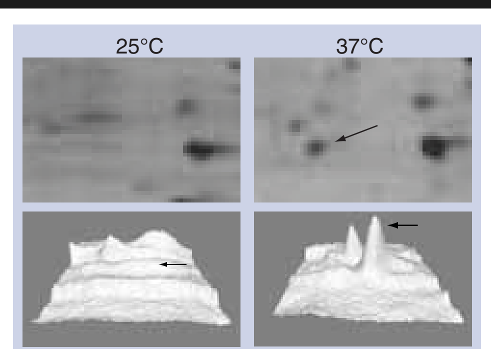
A different strategy to study phase transition in *P. marneffei* is to employ a proteomic approach. Peptide mass fingerprinting has been used to identify differentially expressed proteins [59,60]. One study, performed in conjunction with 2D difference gel electrophoresis, identified a number of yeast phase-specific proteins from fully differentiated cultures [59]. Of these proteins, most did not appear to be directly related to morphogenesis except for a hypothetical protein having homology to cyclophilin from *Magnaporthe grisea*. Despite the promise of these investigations, the identification of peptide mass fingerprinting is not as exact in identifying proteins compared with other methods that generate primary amino acid sequences. Moreover, aged cultures were employed, suggesting that the proteins identified in their investigation may be more likely involved in cellular maintenance functions, rather than in morphogenesis.

Using a similar proteomic strategy, one of our laboratories has examined whole-cell proteins extracted from the early stages of mold and yeast development in *P. marneffei*. Our rationale for this approach is that proteins involved in phase transition, and thereby having possible roles in pathogenicity, would be more likely identified in cells during the initial stages of differentiation. Specifically, we generated protein profiles by 2D gel electrophoresis, isolated proteins of interest, then performed peptide sequencing using capillary-liquid chromatography nanospray tandem mass spectroscopy. The sequence data was then used to identify the isolated proteins by searching for homologous sequences among known databases. Although a comprehensive survey has yet to be completed, we have identified three proteins from 24-h cultures incubated at 37°C that may have roles in development of the *P. marneffei* yeast phase. One protein, Cdc48p, is a component of the proteosome, but also has a role in the cell-cycle event of 'start' in the yeast *S. cerevisiae* [61,62]. A second protein, UDP-*N*-acetylglucosamine pyrophosphorylase, is an enzyme involved in chitin synthesis. Not only is chitin crucial to the integrity of the cell walls of fungi, but the enzymes that regulate chitin synthesis play significant roles in the dimorphism of fungal pathogens [63,64]. The third protein we identified is a small GTPase homologous to RanA proteins found in other fungi **(Figure 4)**. This protein primarily functions in the transport of materials across the nuclear membrane and has an unknown role in mitosis [65]. It is curious to note that the increased expression of the *P. marneffei* RanA protein correlates with the apparent co-ordination of cell division with nuclear division, which results in uninucleate yeast cells when this fungus is grown at 37°C [32].

Interaction of *Penicillium marneffei* with host defenses

One of the key difficulties in the pathogenesis of *P. marneffei* is its ability to initiate infection. As previously noted, infection with *P. marneffei* is presumed to originate via the inhalation of airborne conidia. These inhaled conidia are thought to be sufficiently small to reach the alveoli. Subsequent attachment of conidia to the bronchoalveolar epithelia has a hypothetical role to play in the establishment of the initial infection process before being ingested by phagocytes. The host extracellular matrix (ECM) proteins have been implicated in the attachment of a variety of pathogens to both host tissues and cells [66–69]. It has been demonstrated that *P. marneffei* conidia are able to bind to the ECM glycoproteins, fibronectin and laminin, via a sialic acid-specific lectin [7,58]. Conidia are also able to bind glycosaminoglycans such as chondroitin sulfate B and heparin [70]. In addition, as previously described, a yeast-phase gene was recently identified that exhibits significant homology to a major surface glycoprotein found in *P. carinii* known to

Figure 4. Differential expression of the *Penicillium marneffei* RanA protein.



Protein profiles of conidia incubated for 24 h at 25 or 37°C depicted the presence of a unique protein in the developing yeast phase (arrow in upper right frame). This protein was subsequently identified as RanA and presumably functions in the transport of materials across the nuclear membrane of *Penicillium marneffei* cells during mitosis. The lower two frames comprise 3D contour maps of corresponding regions in the profile that demonstrate the differences in the levels of RanA expression (arrows) at 25 and 37°C.

facilitate attachment to the host ECM [53]. Although the action of these cell-binding factors is presumed to play a role in the establishment of infection and subsequent disease progression, this has yet to be tested *in vivo*.

Another challenge to the establishment and progression of infection by *P. marneffei* is the host defense response. In healthy hosts, *P. marneffei* is targeted and cleared by the immune cells, especially T cells and macrophages [7,71]. It has been demonstrated that intracellular *P. marneffei* are damaged via the L-arginine-dependent nitric oxide pathway in macrophages stimulated with T-cell derived IFN-γ. In immunocompromised hosts, however, *P. marneffei* has been shown to survive and replicate as yeasts inside the phagosome. The mechanism of survival of *P. marneffei* under oxidative stress within the macrophage remains unclear. One possibility may be the expression of known acid phosphatase activity by *P. marneffei* [72]. Such activity may lead to a decrease in intracellular pH and improve the survival of the organism by inhibiting the phagocytic respiratory burst. Moreover, *P. marneffei* is equipped with several genes that may help to significantly reduce the antifungal activity of phagocytes, thereby acting as virulence factors. These genes, which are further described below, include those that encode a copper, zinc superoxide dismutase (*sodA*), catalase-peroxidase enzyme (*cpeA*) and heat shock protein 70 (*hsp70*) [73–75]. Confirmation of the role of these genes in pathogenesis awaits further experiments via the generation of mutants and the use of animal models.

Potential virulence factors

Superoxide dismutase (SOD), encoded by *sodA*, is an enzyme that converts superoxide radicals into hydrogen peroxide (H_2O_2) and oxygen. The enzyme has been implicated in intracellular pathogen survival and as a virulence factor in some pathogenic bacteria and fungi, to include *Mycobacterium tuberculosis* [76], *C. albicans* [77], *C. neoformans* [78] and *Paracoccidioides brasiliensis* [79]. In *P. marneffei,* the putative SodA polypeptide consists of 154 amino acids with similarity to fungal copper, zinc SODs [75]. The upregulation of the *sodA* transcript occurs during yeast growth of *P. marneffei* as well as during macrophage infection. Both observations are consistent with a possible role for SOD in the intracellular survival of the fungus.

Catalase-peroxidase is a unique bifunctional enzyme, capable of either reducing H_2O_2 with an external reductant (peroxidase activity) or converting it to H_2O and O_2 (catalase activity). The enzymes have been implicated as a virulence factor in *M. tuberculosis* and *A. fumigatus* [80,81]. In *P. marneffei*, the *cpeA* gene encodes this enzyme [74]. Interestingly, northern blot analysis and semi-quantitative reverse transcription-PCR show the increased synthesis of *cpeA* transcripts in the yeast phase and also during macrophage infection. Like SOD, catalase-peroxidase may be important *in vivo* as it would facilitate the intracellular survival of this fungus by providing a nontoxic environment within the macrophage phagosome.

Studies of fungal pathogenesis have included heat shock responses during phase transition as an adaptation response to a higher incubation temperature or to the presence of other noxious stimuli [82,83]. Recently, *hsp70*, the gene encoding heat shock protein (Hsp)70 was cloned and characterized from *P. marneffei* [73]. Expression of *hsp70* is upregulated during temperature-induced and heat shock conditions. Moreover, protein profiling of both mold and yeast phases of *P. marneffei* demonstrated the same Hsp70 expression pattern [Chandler JM, Cooper CR, Unpublished Data]. These results suggest that Hsp70 is produced upon temperature increase in order to prevent protein damage, thereby enabling the parasitic growth of *P. marneffei* in host cells.

Another possible host–pathogen factor that may play a role in virulence is pigment production. The red pigment of *P. marneffei*, which is synthesized only by the mold phase and is similar to that produced by the nonpathogenic species, *Penicillium herquei* [84], is not considered a virulence factor. However, melanins are known virulence factors for many pathogenic fungi [85]. Most fungal melanins are synthesized by either the 3,4-dihydroxy-L-phenylalanine (L-DOPA) or dihydroxynapthalene pathways. Collectively, these dark pigments appear to function in a variety of protective roles including the inhibition of killing by phagocytes. Like other fungal pathogens, yeast cells of *P. marneffei* have been shown to produce L-DOPA melanin *in vivo* [86]. Further experimentation will be needed to assess if melanin may be involved in the virulence of *P. marneffei*.

Conclusion

The biology of *P. marneffei* is not well understood, particularly its dimorphic nature and pathogenicity. However, advances in the application of modern molecular methodologies have established a number of genetic elements primarily involved in vegetative growth and asexual differentiation. These genes include those responsible for transcriptional regulation and signal transduction factors. Unfortunately, none of these genes appears to have a direct role in the induction of phase transition. Other strategies are now being employed to identify differentially expressed genes and include broad screening approaches employing genomic as well as proteomic methodologies. Access to a wholly sequenced *P. marneffei* genome, now in progress, will provide an essential tool for dissecting the underlying molecular mechanisms of phase transition.

Progress has been made in identifying potential virulence factors. These include yeast phase-specific or upregulated genes that encode enzymes known to combat oxidative host defense responses. Potential virulence factors related to host-cell attachment have also been identified. Melanin, a known virulence factor for many fungi, has been detected in tissues infected with *P. marneffei*. However, its role in the pathogenesis of this fungus remains to be assessed.

While the collective data does not provide a detailed description of the biology of *P. marneffei*, they do present some insight into the pathogenesis of this mycotic agent of disease. Moreover, it is important to note that the advent of molecular technologies and their application to the study of *P. marneffei* are relatively recent events. The results derived thus far have helped set the stage for further investigations that may not only elicit further insight into phase transition and virulence in *P. marneffei*, but also knowledge that may be exploited in the development of novel chemotherapeutic interventions for diseases caused by this and other fungi.

Future perspective

There are few laboratories actively engaged in investigating *P. marneffei* beyond its clinical significance. The endemic nature of this pathogen has not always attracted interest in funding basic biological investigations. Moreover, the ethnocentric attitude of individuals, governments and funding agencies outside the area of endemicity has belittled the potential worldwide significance of this fungal pathogen. Nonetheless, there is hope that *P. marneffei* will be recognized beyond its endemic region as a very significant public health threat, particularly in this era of increased frequency of host immunosuppression.

Although the investigative results described above have provided a number of insights regarding the factors influencing pathogenesis in *P. marneffei*, more needs to be discovered. Like past observations, new discoveries will depend upon the further development and application of molecular techniques to identify morphogenic mechanisms and potential virulence factors. This will involve the creation of well-characterized mutants for testing in previously described murine models of infection [87,88]. It is curious to note the absence of testing currently available *P. marneffei* mutants for virulence in mice.

Areas of interest that should continue to be addressed include the characterization of factors involved in signal transduction and transcriptional regulation. Additional small GTPases belonging to the Ras superfamily should be identified and the network of activities they regulate needs to be defined, particularly with regard to development of the yeast phase. The role of kinases in cellular development calls for further exploration. The recent identification of histidine kinase and its role in phase transition should prompt immediate studies of this enzyme in *P. marneffei*. In addition, evidence that *P. marneffei abaA, tupA* and *tbpA* mutants do not have a role in phase transition suggests the existence of yeast phase-specific transcriptional regulators. Strategies to identify such regulatory proteins need to be developed.

Finally, it is critical that a publicly available genome sequence of *P. marneffei* becomes available. Currently, this issue is being aggressively addressed [Andrianopoulos A, Pers. Comm.]. Once the genome is available, the avenues of discovery will be broadened. For example, present proteomic approaches that rely solely upon discerning homologies with non-*P. marneffei* sequences will become more accurate and complete. This will facilitate the identification of specific DNA sequences that may be important in virulence. Subsequently, potential development of unique antifungal strategies will be forthcoming not only for *P. marneffei* infections, but for other fungal diseases as well. Such advances might include the creation of a vaccine that could be employed in endemic areas as a preventative measure for both indigenous populations and visitors to this part of the world.

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Executive summary

Penicillium marneffei *as a pathogen*

- A broad region in Southeast Asia encompasses the endemic area for infections (penicilliosis) caused by the fungus *Penicillium marneffei*.
- *P. marneffei* is the only member of its genus known to be dimorphic. Dimorphism is requisite for pathogenesis.
- Over the past 20 years, the marked increase in the number of penicilliosis cases correlates with the concurrent rise in immunosuppression due to AIDS. The continuing epidemic of HIV infection suggests that there will be a concomitant increase in the frequency of disease due to *P. marneffei*.
- Infections by *P. marneffei* are fatal if left untreated. The successful treatment of patients requires prolonged therapy.

Dimorphism & phase transition

- Transition of the mold phase of *P. marneffei* to a yeast form *in vivo* defines the dimorphic nature of this fungus, but the underlying molecular mechanisms are unknown.
- The phase transition expressed by *P. marneffei* is unique among the major dimorphic fungi pathogenic for mammals. An understanding of the regulatory mechanisms involved will provide a better insight into those factors that govern pathogenicity of this fungus.

Transcriptional regulators & signal transduction factors

- Several genes encoding transcriptional regulators have been characterized in *P. marneffei*. They function to coordinate cell-cycle events and growth. However, these regulatory proteins play little, if any, role in phase transition or maintenance of the yeast form.
- A number of genetically encoded signal transduction factors that regulate morphogenesis in *P. marneffei* have been identified. These factors exhibit GTPase activity and have functions homologous to those previously identified in nonpathogenic fungi. However, none appear to directly induce phase transition.

Genomic & proteomic analyses of differential gene expression

- Analyses of differential gene expression between the mold and yeast phases of *P. marneffei* have been conducted. Genomic-based methods have identified several genes that may play a role in pathogenicity.
- Additional genes that may play a role in phase transition have been discovered using the proteomic strategy of protein profiling.
- Both the genomic and proteomic approaches would be more accurate if a complete genome sequence of *P. marneffei* were available.

Host–parasite interactions & virulence factors

- The conidia of *P. marneffei* are able to initiate infection through binding to the surface of the host cells. The conidia employ specific binding elements that promote phagocytosis. Once in the phagocyte, conidia produce proteinaceous factors that help ameliorate host responses.
- By exploiting differences in gene expression between the mold and yeast phases of *P. marneffei*, several genes encoding potential virulence factors have been identified. The expression of these genes is upregulated or specific to the yeast phase. The functions of these proteins would potentially negate the host response to intracellular invasion of the fungus.

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