COMPARATIVE PROTOEMIC ANALYSIS OF PHASE-SWITCH IN THE DIMORPHIC FUNGUS, *Penicillium marneffei*

by

Karen E. Stangl

Submitted in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the

Biology

Program

YOUNGSTOWN STATE UNIVERSITY

December, 2008

ETD Program

Digitally signed by ETD Program
DN: cn=ETD Program,
o=Youngstown State University,
ou=School of Graduate Studies
and Research,
email=etdadmin@cc.ysu.edu,
c=US
Date: 2009.01.27 11:53:00 -05'00'

Comparative Proteomic Analysis of Phase-Switch in
the Dimorphic Fungus, *Penicillium marneffei*

Karen E. Stangl

I hereby release this thesis to the public. I understand that this thesis will be made available from the OhioLINK ETD Center and the Maag Library Circulation Desk for public access. I also authorize the University or other individuals to make copies of this thesis as needed for scholarly research.

Signature:

_____

Karen E. Stangl Date

Approvals:

_____

Dr. Chester R. Cooper, Jr., Thesis Advisor Date

_____

Dr. Gary R. Walker, Committee Member Date

_____

Dr. Jonathan J. Caguiat, Committee Member Date

_____

Dr. David K. Asch, Committee Member Date

_____

Peter J. Kasvinsky, Dean of School of Graduate Studies & Research Date

ABSTRACT

Fungal dimorphism is an interesting process by which some multicellular fungi can become unicellular pathogens. The increased incidence of infections by *Penicillium marneffei*, itself a dimorphic pathogen, have made this fungus the third leading cause of death due to AIDS-related illnesses in Thailand. The genetic basis of its temperature-dependent dimorphic-switch trigger has yet to be identified.

This study analyzed the protein profile of *P. marneffei* during phase-switch experiments in which four cultures were initially grown at both the mycelial (25°C) and yeast (37°C) temperatures for 12 hours. Subsequently, two of these cultures were switched to the other temperature for the remaining 12 hours (25°C to 37°C, and 37°C to 25°C). The proteins were isolated from each 24-hour culture and separated by two-dimensional gel electrophoresis. Differentially expressed protein spots were excised for mass spectrometry analysis to determine their amino acid sequence.

Three proteins stood out with respect to their high levels of expression in the 37°C (yeast) phase and 25°C-37°C switch phase. One, Hsp30 (*Penicillium marneffei*), is a molecular chaperone linked to the heat-shock response, and has been previously identified as a potential human antigen. The second, a synaptobrevin/VAMP-like protein (*Debaryomyces hansenii* CBS767), is likely to be highly involved in intracellular vesicle trafficking during the morphological change. And the third, an aldo-keto reductase (*Phaeosphaeria nodorum* SN15), has been indicated in the heat-shock response in other dimorphic fungi. These collective results open possible doors for cloning differentially expressed genes, the development of a pharmaceutical agent to slow infection, or elucidate the gene(s) involved in the dimorphic switch.

ACKNOWLEDGEMENTS

This research paper could not have been possible without
the assistance of the following individuals:

The Cushwa Family
For their provision of research funding through the Cushwa Commercial-Shearing Engineering Fellowship program at Youngstown State University.

Dr. Chester R. Cooper, Jr., Dr. Gary R. Walker and Dr. Thomas D. Kim
The original "Three Amigos" of the Proteomics Research Group, who invited me first to spend the summer of 2004 learning the basics of their new proteomics program, and who then believed in me enough to invite me back for the MS program.

Dr. David Asch and Dr. Jonathan Caguiat
For lending their expertise and editing skills so I can create a publication worthy of their time and signatures, and of the caliber to represent the PRG.

Ms. Erin Treece
For inspiring me to apply myself to this program and going through it with me- even though I can't believe you left me here for my home state.

Ms. Julie Chandler and Ms. Heather Trenary
For teaching me the ropes of proteomics –thank you for being the trailblazers.

My Parents: Barbara and Ralph Stangl
A simple 'Thank You' will never be enough. Although I've worked hard to get here, I'll never forget how hard *you've* worked to get *me* here. I will forever aspire to match your patience, generosity and work ethic.

Mr. Adam Lee
You put a roof over my head, food in my stomach, and a hand over my mouth when necessary. Thank you. I'd hate to think where I'd be without you.

Mr. Aksarakorn Kummasook
I look forward to personally witnessing your Ph.D. defense in Thailand, and I'm very proud to call you my friend – and maybe even my colleague someday. Thank you for sharing your office, your time and your mind with me.

Fellow Graduate Students, YSU Staff and Youngstown-Area Friends
I'm glad we could be one another's sounding boards, cheerleaders and safety nets- all things necessary for graduate school survival. Best wishes always, and unending thanks.

TABLE OF CONTENTS

Abstract	**iii**
Acknowledgements	**iv**
Table of Contents	**v**
List of Tables and Figures	**vi**

CHAPTERS

I.	**Introduction**	**1**
II.	**Hypothesis and Research Objectives**	**10**
III.	**Materials and Methods**	**11**
IV.	**Results**	**18**
V.	**Discussion**	**30**
VI.	**Appendix**	**38**
VII.	**Literature Cited**	**40**

LIST OF TABLES AND FIGURES

Figure 1. **23**
Images of the cells from each phase under 100x magnification.

Figure 2. **24-25**
2-DGE protein profiles of the four phases, with spots of interest marked.

Table 1. **26-29**
Results of MS analysis of protein spots chosed from 2-DGE images.

Table 2. **30**
Summary of protein presence in the four experimental phases.

INTRODUCTION

Typically, the mycelium of a pathogenic fungus enables its invasion of another organism, invoking the "penetrating power" of hyphae to violate insects, plants, and often the human skin (Deacon, 2006). But for dimorphic fungi, reliance on hyphae for parasitizing organisms is not always the case. Once inside a host, the spore of a dimorphic fungus, such as *Penicillium marneffei*, moves throughout body tissues by changing into a single, yeast-like cell. This morphological differentiation has been found to be integral to the progress of systemic host infection in a number of fungi (Rooney *et. al*., 2002; Kerridge, 1993; Andrianopoulous, 2002). Fungal dimorphism is a global concern, as endemic species of dimorphic fungi, such as *Paracoccidioides brasiliensis* (Brazil), *Histoplasma capsulatum* (Mississippi River Valley), *Blastomyces dermatitidis* (South Eastern, South Western and North Western United States), *Coccidioides immitis* (South Western United States), *Sporothrix schenckii* (Central India, among other regions), and *P. marneffei* (South East Asia), cause numerous cases of systemic infections human hosts worldwide.

The dimorphic change is a result of a cellular response to an environmental stress, such as a change in temperature or nutrient supply. It can also be a survival adaptation, as in the case of *P. marneffei*, in that its temperature-dependent dimorphism corresponds to a morphological change from mold to yeast at human body temperature. The key to perhaps understanding fungal pathogenicity from a medical mycology perspective may be revealing the proteins involved in the dimorphic switch of organisms like *P. marneffei*. Andrianopoulos *et. al*., (2006) note that although there are several different dimorphic fungal organisms that infect humans, most of them are predominantly yeasts that switch

to the hyphal form via chemical signaling. By comparison, "*P. marneffei* is predominantly hyphal and switches between a hyphal and yeast form," in response to a physical signal of to temperature as its switch or trigger.

Fungal Dimorphism

Because temperature alone has been found to be the morphological switch trigger for *P. marneffei*, it is not a coincidence that its mycelial morphology (room temperature or 25°C) changes to yeast form when subjected to human body temperature (37°C). Knudeken *et. al*., (1999) note that the formation of the yeast cell or hyphal cell occurs "within a short time during " the first 12 hours of development. This was also observed by Andrianopoulos (2002) who noticed differences in cellular branching for cultures originating from the two temperatures. Cao *et al.* (2007) observed optimum mycelium growth between 17-28°C, and a switch to yeast cell formation between as early as 32°C.

The original, early differentiation of *P. marneffei* conidia into either mold or yeast cells seems to begin the same. However, there are subtle structural differences: the mold state produces a germ tube within the first 12 hours that subapically can produce more branches, whereas the 37°C culture produces more highly branched hyphal cells within the first 12 hours (Andrianopoulos, 2002). Chandler *et. al.*, (2008) and Cooper and McGinnis (1997) have identified these beginning yeast cells as "determinate length hyphal cells", since at this early stage in development, they do not yet resemble the more common, spherical morphology of true yeasts. Cooper and McGinnis (1997) have identified the first 6-12 hours of growth of *P. marneffei* at 37°C as the time when differential development of the yeast phase begins to take place, and this finding is the basis of the growth condition variables used in this study

It has been found that there are also specific differences in the directionality of growth between the two phases. Kudeken (1999) mentions, "90% of the wall extension occurs in the hyphal tip…whereas, in budding yeasts, only 70% occurs at the apex of the bud," and that this distribution is consistent until the bud's growth initiates growth over 90% of its entire surface. Hence, the first 24 hours of growth are integral in determining fungal morphology, and an important time/place to begin investigating dimorphism. Beyond the recognition of the temperature-induced repolarization of cell wall and/or membrane constituents for fungal growth, there is little known about the specifics of the biochemical processes involved in the dimorphism of *P. marneffei*.

Studying *Aspergillus nidulans*, one of the more closely related organisms to *P. marneffei*, (Borneman *et. al*., 2000), has revealed some important details about the events that take place during the change in growth phases. After a specialized foot cell in the mycelium of *A. nidulans* depolarizes into a "globose vesicle," the apical extension and septation common to mycelial reproduction "give[s] way to a budding mode that yield[s] asexual, uninucleate cells" via sterigmata (Borneman *et. al*., 2000). The asexual replication of *P. marneffei* at 25°C is similar to this process, but hyphae exposed to 37°C temperatures develop a process where cell septation and nuclear division occur at the same time. This results in spore production via hyphal fragmentation, a process called arthroconidiation. From there, the fungal cells take on a smaller size, double their septa and disperse via fission in yeast-like fashion instead of hyphal form (Borneman *et. al*., 2000). The commonality of transformations from yeast and mold phases between organisms makes understanding the proteins involved in *P. marneffei's* switch even more important.

Pathogenicity and Clinical Manifestations

Studies on the biochemical processes that make pathogenicity possible for the infectious, single-celled form of *P. marneffei* are rare in comparison to the epidemiological reports of its occurrence in immunocompromised patients (Wong *et. al*., 2001). What is known about the infection mechanism of *P. marneffei* can be summed up by Kerridge (1993): "All fungal pathogens, with the exception of *Candida albicans*, infect via the lung." From there, the human immune response to fungal pathogens like *P. marneffei* conidia is fought largely by polymorphonuclear leukocytes (PMN's). Virulence of unopsonized *P. marneffei* may be enhanced by its ability to survive inside mononuclear phagocytes (Vanittanakom *et. al*., 2006). This also supports the finding that *in vivo* foci of *P. marneffei* cell colonies tend to be the spleen and lymph nodes, as engulfed *P. marneffei* cells are carried there by leukocytes (Van Cutsem, 1993).

The means of infection described above demonstrate why those who are immunosuppressed, or, more often, those who are infected with HIV and display symptoms of AIDS are extremely susceptible to fungal infections. With dangerously low levels of CD4+ T cells, immunocompromised individuals are not able to summon the immune response necessary for combating systemic *P. marneffei* fungal infection (Vanittanakom *et. al*., 2006; Viviani *et. al*., 1993).

Epidemiology and Treatment

Localized to Southeast Asia, the natural habitat of *P. marneffei* has not yet been identified (Vanittanakom *et. al*., 2006), but has been suspected to be a soil fungus (Pryce-Miller *et. al.*, 2008; Gugnani *et. al*., 2007; Ustianowski *et. al*., 2008). Apparently, at least four species of bamboo rats have been characterized as natural reservoirs for *P.*

marneffei, in several different geographical areas (Gugnani *et. al.*, 2004). Bamboo rats native to Vietnam and Northern Thailand were noted as the first carriers of *P. marneffei* infections in 1956. No other living hosts besides the bamboo rat and human have been identified thus far. "It is unclear whether penicilliosis marneffei occurs as a consequence of zoonotic or sapronotic transmission, and what the ecological reservoir(s) are," (Vanittanakom *et. al*., 2006). Symptoms of human infection include fever and weight loss, accompanied by liver, spleen and lymph node swelling or infections (Nguyen *et. al*., 2006). Visual presentation appears as papules on the skin, typically around the facial area or on mucosal surfaces. Analysis of the CD4+ T cells of patients, especially those infected with HIV, shows a significantly low count (Nguyen *et. al.*, 2006; Ustianowski *et. al.*, 2008; Wantabe *et. al.*, 2008) .

Penicillium marneffei infection, an AIDS-related indicator disease, is the third leading cause of death due to AIDS-related illness in Thailand (Cooper and Haycocks, 2000; Ustianowski *et. al.*, 2008). As the planet moved toward globalization throughout the late 1990's and into the early 2000's, so did *P. marneffei*. India's first reported cases of penicilliosis in HIV patients surfaced in 1999 (Michael *et. al*., 2004), while a U.S. case of a man who had been in a refugee camp in Thailand was later recorded (Nguyen *et. al*., 2006).

Overall, the collapse of the human immune system during HIV infection allows *P. marneffei* to persist and proliferate throughout the body as intracellular yeasts localized mostly to bone marrow, lymph nodes, the liver, and cutaneous lesions (Ustianowski *et. al.*, 2008). Common fungicidal treatments to ward off *P. marneffei* infections vary by individual, but can include amphotericin B in the short-term (2-3 weeks) to resolve

symptoms, and itraconazole for the long term (6-10 weeks at a high dose, followed by a lower dose for the life of the patient) (Ngyuen *et. al*., 2006; Ustianowski *et. al*., 2008). This two-fold attack as a treatment became necessary when studies like that of Supparatpinyo *et. al.*, (2003), reported that 30% of patients displayed a relapse of infection within six months after initial treatment.

Preventive treatment of penicilliosis infections is difficult to initiate since little is known about the ecological niche of this fungus. There have been relatively few in-depth studies published to date that investigate the presence and means of detection of *P. marneffei* in the wild. In their quest to determine if *P. marneffei* is a saprotrophic fungus, Pryce-Miller *et al.*, (2008) determined that it is indeed found in the soil in endemic regions in specific niches that demonstrate competitive inhibition with other soil-based microbes such as *Talaromyces stipitatus*. Other studies have focused on determining if dimorphic fungi such as *P. marneffei* are present in the burrows of bamboo rats as far west as India (Gugnani *et. al.*, 2007), finding none.

Proteomics

Assuming the two morphological phases of *P. marneffei* can be grown under identical conditions, a "blueprint" of their biochemical mechanisms would reveal which proteins are involved in directing differentiation and development. The global analysis of proteins in a case like this is the working definition of proteomics (Twyman, 2004): the study of the collection of proteins in an organism under certain conditions or at a specific time. Two-dimensional gel profiles of proteins are often referred to as maps, constellations, or snapshots of intricate cellular processes representing a certain set conditions. A good analogy for proteomic studies is that they are like a 'parts list' – or

inventory of proteins present in the cellular 'factory'. Once those proteins become identified, a blueprint of the factory's processes can begin to be formed as scientists discover the interconnectedness of the biochemical pathways involved in the metabolism of the organism during specified growth conditions.

My study involves the proteomic analysis of 24-hour growths of both the mold (25°C), and yeast (37°C) forms of *Penicillium marneffei*, as well as samples that were first grown at one temperature (25 or 37°C) for 12 hours and then switched to finish out the 24-hour growth period at the other temperature. Because much of the differentiation takes place within the first few hours of growth, I believe that this system will allow for recognition of proteins that are either unique to each morphology, or seem to be over or under produced in the differing conditions. This can be visualized by using 2-dimensional gel electrophoresis (2-DGE) of protein isolates of cells from each of the growth conditions. By comparing standardized protein maps of each of the 4 samples, interesting protein spots can be excised and sent for mass spectrometry analysis to determine a probable sequence for each protein chosen.

By consulting the databases of protein sequences in bioinformatics, conclusions can be drawn about the type, family, structure and function of a protein. A successful choice of spots for excision will corroborate with sequence results from previous studies, validate this method, and give insight into the proteins involved in the dimorphic switch of *P. marneffei.*

The use of bioinformatics databases proves to be a helpful tool not only in discovering proteins and their functions, but also phylogenetic relationships between organisms. Because much of the *P. marneffei* genome is yet unmapped, many of the

bioinformatics results will correlate to that cataloged for other species, both closely and not very closely at all related. But since there is evidence for the evolution of protein family-member isotypes between similarly complex species, these results are still applicable for investigations focusing on *P. marneffei*.

Implications of This Study

Once the proteins involved in the differentiation of *P. marneffei* to yeast cells are identified, they can become potential pharmaceutical targets. A method implied by Kaufman (1993) suggests that anamorph selection for creation of antigens is vital to the future of diagnostic screenings and etiological agent identification for fungal infections. This is a crucial step in the treatment of mycoses, as many of the original cases of penicilliosis, for example, gave way to mortalities based on misdiagnosis (Viviani, 1993). Ngyuen (2006) brings to light a case study of penicilliosis in a patient in the US who had other infections that masked a *P. marneffei* infection and reiterates the importance of early diagnosis and "lifelong prophylaxis… to avoid recurrences." An ultimate goal would be to be able to remove *P. marneffei* as the third leading cause of death due to AIDS-related illnesses in South East Asia, either by controlling its human mortality with pharmaceutical treatment, or by learning how to prevent infection in immunocompromised hosts.

The relatively high level of sequence conservation between species is another important factor in discovering dimorphic switch processes in *P. marneffei* and other dimorphic fungi. Since human infection with pathogenic, dimorphic fungi is a global concern, the more the scientific community can learn about the process in one organism,

the more conclusions can be drawn about similar processes in even distantly related dimorphic fungi.

Also, as the genomes and proteomes of dimorphic fungi begin to be published and subsequently annotated, sequences and protein characterization from one organism can be used to predict outcomes of similarly sequenced genes or isolated proteins.

The hurdles toward discovering the exact biochemical processes involved in differential development in dimorphic organisms is just as difficult as the fight an organism's immune system undergoes to overcome a fungal illnesses. It is not yet fully understood how organisms develop pathogenetic adaptations. Insight into the proteins involved in dimorphic switching mechanisms in *Penicillium marneffei* will shed light on these elusive pathways for other fungal pathogens as well and ensure proper diagnosis and treatment courses for patients with penicilliosis or infections from dimorphic fungi.

HYPOTHESIS

Although a complete survey of the proteome of *P. marneffei* is beyond the scope of this master's thesis, I propose the following hypothesis:

> *The two-dimensional gel electrophoresis (2-DGE) gel patterns of 12-hour cultures of P. marneffei that have been switched between 25°C and 37°C are likely to reveal proteins related to the dimorphic switch when compared to 24-hour growth samples from each temperature.*

RESEARCH OBJECTIVES

1. Consistently replicate two-dimensional protein gels of 24-hour mold and yeast samples as well as 25-37°C and 37-25°C switches by creating triplicate 2-DGE gels of each variable.

2. Identify and isolate 'candidate' spots for mass spectrometry analysis/protein sequencing by observing differences in intensity or location of focused proteins for each growth condition.

3. Analyze the sequence data obtained from the mass spectrometry analysis of chosen protein spots, and draw inferences about the role of each reliable protein hit in the dimorphic switch of *P. marneffei*.**.**

MATERIALS AND METHODS

The recipes for reagents used in this experiment can be found in Appendix I, and are listed in order of use.

Fungal Strain Growth and Experimental Culture Setup

Penicillium marneffei F4 (CBS 119456) strain conidia were collected from stock grown for 8 days at 25°C on potato dextrose agar (PDA) (Difco brand, Becton Dickinson and Co., Sparks, MD) in 150 cm^2 culture flasks (Corning, Acton, MA). This strain was clinically isolated from a patient at Maharaj Nakorn Chiang Mai Hospital and provided by Dr. Nongnuch Vanittanakom (Chiang Mai University, Thailand) and a culture stock was kept in our lab. This same clinical isolate has been used by Pongpom *et. al.* (2005), and Chandler *et. al.* (2008), among other studies. After 10 ml of ddH_2O was added to the culture flask, the conidia were collected by gentle scraping of the culture surface with a cell scraper (Fisherbrand, Fisher Scientific, KY). Conidia were separated from agar and hyphal fragments in the ddH_2O solution by an autoclave-sterilized filtration unit made up of 1 inch of glass wool (Corning, Acton, MA) packed between two screw-threaded screen caps (BioRad). This filter unit was secured to the top of a sterile, 50 ml conical tube (Corning, Acton, MA), with the cap placed on top and sealed with ParafilmR (Pechiny, WI) before centrifugation in a swinging bucket rotor for 30 seconds at 150-200g, 4°C. This allowed the conidia in the ddH_2O to pass through the filter and into the conical tube during the spin, while the hyphae and agar fragments remained in the filter. A cell count using a hemocytometer and the $C_1V_1=C_2V_2$ equation was used to determine the number of conidia harvested per ml of ddH_2O during scraping, and the amount of the suspension necessary to acquire a 1 X 10^7 conidia/ml concentration in 50 ml of Sabouraud Dextrose

Broth (SDB), (Difco brand, Becton Dickinson and Co., Sparks, MD). This amount was then divided into two 15 ml conical tubes (Corning, Corning, NY) based on amount of SDB to be inoculated at each starting temperature; in this case, 100 ml (50 ml in each of two 500 ml Erlenmeyer flasks) at 25°C and 150ml (50 ml in each of three 500 ml Erlenmeyer flasks) at 37°C were used. After discarding the supernatant, the pellets were resuspended in 3-5 ml of pre-warmed SDB (either 25°C or 37°C) before addition to the final stock of pre-warmed SDB for incubation. After 12 hours at each starting temperature, one of the flasks that began at 25°C was switched to 37°C, and two of the flasks beginning at 37°C were moved to 25°C for the remaining 12 hours to finish out the 24-hr incubation period.

Before their collection from their 24-hour incubation in SDB, a few drops of the broth containing the cells representing each phase or phase-transition sample were examined under a microscope to i) eliminate the possibility of contamination of proteins from other microorganisms in the final protein pellet from each sample and ii) assess if the expected cellular phenotype was attained. Also, about 250 ml of each culture was preserved in a microcentrifuge tube with the addition of an equal amount of 4% formaldehyde. The cells were then deposited on a slide in 5 µl amounts and sealed with lacquer under a cover slip for a closer microscopic analysis of morphological changes. Images were taken with Olympus IX51 microscope at 40X and 100x power using AdvancedSPOT software on Normanski settings .

Cell Collection

Each flask of 50 ml of culture was removed at 24 hours of incubation and immediately placed into pre-chilled 50 ml Sorvall tubes (Sorvall, Thermo Scientific, MA)

on ice and centrifuged at 17,600 x g for 15 min at 4°C. The SDB supernatant was discarded, and the cell pellets were washed twice by suspension in 30-40 ml, ice-cold TE buffer and centrifugation for 10-15 min at 17,600 x g for 10 min, 4°C. The isolated cells were then transferred to sterile, o-ring, screw-capped microcentrifuge tubes (FisherBrand, Fisher Scientific, KY) and centrifuged at 6,000 x g for 10 min at 4°C to remove any remaining supernatant. Ideally, pellets resulted in 400 mg masses. Each sample was frozen at -80°C for up to 3 weeks before homogenization and protein extraction.

Protein Extraction and Quantification

To each 400 mg tube of ice-thawed cells, an equal volume of acid-washed, glass beads (0.5 mm diameter; Biospec, Bartlesville, OK), and 800 μl of lysis buffer were added. For smaller or larger masses of cell samples, the lysis buffer was scaled accordingly. Cells were homogenized in a Mini-BeadBeater (Biospec) set to 5,000 rpm for 30 seconds, followed by 30 seconds on ice for a total of 4 minutes of vigorous shaking. After centrifugation (6,000 x g, 10 min, 4°C), the supernatant of the cellular homogenate was transferred to a pre-weighed, sterile, 1.5 ml microcentrifuge tube. The proteins contained in this supernatant were precipitated into a crude protein pellet by the following three steps: 1) the addition of 20% v/v ice-cold trichloroacetic acid; and 3) incubation on ice for 20 minutes; 2) centrifugation at 6,000 x g for 20 min at 4°C and three 500 μl acetone washes (3 min centrifugation at 850 x g). The resulting pellet was allowed to air dry for 1-2 hours in a sterile, laminar-flow hood, or dried in a vacuum-centrifuge for 7-12 min. Resuspension in modified sample buffer (MSB) was mass-dependent for each pellet, and scaled as follows: a 1-15 mg pellet in 500 μl MSB, a 16-30

mg pellet in 750 μl MSB, and pellets 31 mg or more into 1000 μl of MSB. Samples could then be stored at -80°C or used immediately for the following protein assay.

The quantitative protein analysis was carried out by modified Bradford Assay (Bradford, M. 1976), using the following reagent amounts for each sample: 80 μl of ddH_2O, 10 μl of 0.1 M HCl, 10 μl 2DE buffer, 5 or 10 μl of protein sample or a serial dilution of bovine serum albumin for a standard curve, and 4.0 ml of Bradford dye. Absorbances were read in a spectrophotometer set to 595 nm, and the concentration (μg/ml) of each protein sample was calculated when the standard curve R^2 value was $\geq$ 0.970.

Isoelectric focusing and 2-DGE

The first dimension of electrophoresis was achieved with active rehydration of 17 cm immobilized pH gradient (IPG) strips in the range of pH 5-8. The strips were loaded with 300 μl of rehydration buffer (what is in the rehydration buffer?) and 250 μg of protein (for matchset images) or 325-400 μg of protein (for excision), and run for 20,000 V-hr at 20°C in a Protean IEF cell (BioRad). This was followed by gentle agitation in equilibration buffers I and II, respectively, and a rinse in 1 x TGS buffer to prepare the strips for the second dimension of electrophoresis.

The 12% 20 X 20 cm polyacrylamide gels used for the second dimension were cast 1-2 days prior to use, and stored at 4°C. Gel plates were brought to room temperature in ½ X TGS buffer before the strip was loaded onto the gel between the plates and secured with overlay agarose. The gels were loaded into a Protean Plus Dodeca Cell (BioRad) filled with running buffer (1 X TGS) and held under constant 200 mV for between 6-8 ½ hours. The gels were then carefully released from their plates and

either stored in 500 ml 5% acetic acid solution in plastic containers (for matchset gels), or fixed for one hour in gel fixing solution before overnight staining in SYPRO Ruby protein gel stain (Bio-Rad). The gels were carefully rinsed with ddH_2O prior to imaging.

Imaging, analysis and spot excision

Digital images of the gels were taken in a Molecular Imager ChemiDoc XRS system (BioRad) under UV illumination at 365 nm for three independent cultures to create one match set per experimental condition using the PDQuest 2-DGE Analysis Software (BioRad). A master from each of the match sets was then used for comparison between experimental conditions to identify differences in levels of protein expression. To prepare gels for spot excision, they were placed in fixing solution for one hour, incubated in washing solution overnight, stained with fresh SYPRO Ruby for at least three hours and rinsed in ddH_2O before imaging. A 2500 µl sterile pipette tip was used to excise the spots by hand over UV illumination. The gel-embedded protein spot was stored in 5% acetic acid solution in a sterile microcentrifuge tube during submission to The Ohio State University Mass Spectrometry and Proteomics Facility (Columbus, OH; Http://www.Ccic.ohio-state.edu/MS/proteomics.htm) for sequencing.

Mass Spectrometric Preparation and Analysis

This part of the protocol took place as cited by Chandler *et. al.*, (2008). According to The Ohio State University Mass Spectrometry and Proteomics Facility, the protein samples were first digested with sequencing grade trypsin (Promega, Madison, WI), or sequencing grade chymotrypsin (Roche, Indianapolis, IN) following the recommended protocols in the Montage In-Gel Digestion Kit (Millipore, Bedford, MA). Briefly, samples were trimmed as close as possible to minimize background

polyacrylamide material, and then washed for one hour in a 50% methanol/ 50% acetic acid solution twice before dehydration with acetonitrile. The samples were then rehydrated in a DTT solution for 30 minutes prior to the addition of iodoacetamide and a 30 min incubation in the dark. These chemicals were washed from the samples with acetonitrile and ammonium bicarbonate for 5 minutes each, respectively, and vacuum dried before a final rehydration for 10 minutes in 50 μl of 50 mM ammonium bicarbonate containing 20 μg/ml of either sequencing grade modified trypsin or chymotrypsin before an additional 20 μl of 50 mM ammonium bicarbonate was added. The incubation then continued overnight at room temperature, and the peptides were extracted several times from the polyacrylamide using an acetonitrile/formic acid solution, pooled and concentrated under vacuum to a final volume of approximately 25 μl.

A Thermo Finnigan LTQ mass spectrometer was used to perform capillary-liquid chromatography-nanospray tandem MS with the nanospray source in positive ion mode. The LC system was an UltiMate Plus system (Dionex, Sunnyvale, CA) with a Famous autosampler and Switchos column switcher. Solvent A was water containing 50 mM acetic acid and solvent B was acetonitrile. Five microliters of each sample was first injected on to the trapping column, and then washed with 50 mM of acetic acid. The injector port was switched to 'inject' and the peptides were eluted off of he trap onto the column. A 5 cm 75 μm ID ProteoPep II C18 column (New Objective, Woburn, MA) packed directly in the nanospray tip was used for chromatographic separations. Peptides were eluted directly off the column into the LTQ system using a gradient of 2-80% B over 50 minutes, with a flow rate of 300 nl/min. A total run time was 60 minutes. The scan sequence of the mass spectrometer was programmed for a full scan, a zoom scan to

determine the charge of the peptide, and a MS/MS scan of the most abundant peak in the spectrum. Dynamic exclusion was used to exclude multiple MS/MS of the same peptide.

Data processing was performed following recommended guidelines (Carr et al., 2004). At the Ohio State University Mass Spectrometry and Proteomics Facility, sequence information from the MS/MS data was processed by converting the raw data files into a merged file (.mgf) using MGF creator (merge.pl, a Perl script) with first scan number, last scan number, number of intermediate scans, minimum number of grouped scans and minimum number of ions set to blank, blank, 1, 0 and 8, respectively. The resulting .mgf files were searched using Mascot Daemon (version 2.2.1; Matrix Science, Boston, MA) against the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov) databases (NCBI nr). These databases contained in excess of 6 x 10^6 sequences comprised of more than 2 x 10^9 residues. Selected files were subjected to a more narrow search using the NCBI nr database limited to the taxon Fungi (>3 x 10^5 sequences). These databases were employed because a completely sequenced and annotated genome of *P. marneffei* has yet to be publicly released. The mass accuracy of the precursor ions were set to 2.0 Da given that the data was acquired on an ion trap mass analyzer and the fragment mass accuracy was set to 0.5 Da. Considered modifications (variable) were methionine oxidation and carbamidomethyl cysteine. Two missed cleavages for the enzyme were permitted. Peptides with a score less than 20 were filtered and proteins were identified having a significance threshold of $p < 0.05$. Protein identifications were checked manually and proteins with a Mascot score of 50 or higher with a minimum of two unique peptides from one protein having a –*b* or –*y* ion sequence tag of five residues or better were accepted.

RESULTS

The cultures in this experiment were started from conidia harvested from 8-day, 25°C growth of *P. marneffei* on PDA. It is important to begin all phases from the same starting condition, conidia in this case, to maintain consistency in morphological differentiation. Also, the main route of infection of hosts is most likely inhalation of conidia from the environment, so it is useful to begin at the conidial stage when observing any change in morphology and protein composition during the phase switch.

The images of the 100x magnification of each cellular differentiation phase can be seen in Figure 1 a-d. The most notable differences in morphology remain between the mold and yeast cultures, while the phase-switch cultures have characteristics of both the pathogenic and non-pathogenic forms of the fungus.

It is interesting to note the differences in hyphal development over the stages that included time spent at 25°C – there was a notable difference in the length and branching of hyphae during early development. While the yeast cells exhibit a germ tube and the mycelial stage shows thin, branching filaments, the cultures that endured a temperature shift during incubation differed in their branch development and formation. The 25°C - 37°C samples showed a highly branched pattern, with thicker filaments that contained bulges, making it appear "bumpy". The 37°C - 25°C sample culture took on a morphology that seemed a true 'mix' of both the normal 37°C and 25°C cultures, with its filaments just slightly less branched and bulged than the 25°C - 37°C sample. These images are a reminder of the lack of spherical, yeast-like morphology in the 37°C culture at this stage in development. Instead, it seems that every conidium begins even the

slightest bit to develop as a mycelium before septating, budding and developing the more round, yeast-like shaped cells.

As stated previously, 2-DGE gels of protein profiles were created in triplicate for each 24 hour growth condition as follows: 24 hours each at 25°C and 37°C for mold and yeast phases, respectively and the switch phases beginning with 12 hours at either 25°C or 37°C, and switched to 37°C or 25°C, respectively for the remaining 12 hours. Each replicate was initiated from a separate 8-day culture of *P. marneffei* conidia, to ensure reproducibility. The images in Figure 2 presented below are oriented with pH 5-8 from left to right, and the protein mass decreasing from top to bottom.

Imaging and Analysis

The first attempt at selecting and sending spots for MS analysis was unsuccessful, because spots were chosen only by inspecting differences in the overload gels. Because of their higher protein content- to ensure enough protein was present in the excision to yield a good sequence result – some of the spots and streaks were not representative of the images of the normal, 250 μg protein-loaded gels. In this case, spots that were chosen from the overloads because they were seemingly interesting were not visible in the same intensity level, or location in the normal load gels. Therefore, any MS results were not indicative of true phase differences in protein expression and focusing and could not be used for analysis. Subsequently, another triplicate of gels and images were produced, followed by a more careful comparison of spots in both the normal load and overload gels before spot selection and sequence analysis was requested.

The 2-DGE images in Figure 1a-d represent those used to create match sets for spot selection. Spots outlined with a triangle indicate a presence unique to that phase or

an up-regulation/higher intensity of staining in comparison to spots in the same location in other gels, while spots outlined with a rectangle indicate a down-regulated/lowered staining intensity, or weaker presence in comparison. The spots outlined with a circle represent the 'landmark' spots chosen from each gel and serve as a reference point between the four images since the spot appears in the same location and generally is of the same intensity in each of the for phases. Another interesting difference associated with spots chosen for MS analysis is their presence or absence in clusters or groups. In some phases, for example, two spots appear close to one another in a certain area of the gel, whereas in another phase, one of the spots 'disappears' and the other may still be present, or have changed in intensity.

An immediate observation of the overall spot correlation between the phases is that the protein spots represented by either the 24-hour mold sample or those of the samples that ended the last 12 hours at the 25°C temperature tend to be of lower intensity/down-regulated than those at the 24-hour 37°C temperature, or ending the last 12 hours at 37°C.

As overload gels were used for spot extraction, their images are not represented because they appear with more vertical and horizontal streaking due to the higher protein load impressed during focusing and electrophoresis. The spots that are chosen from the overload gels were verified for presence, location and intensity on the regular-load gel images before final selection, to eliminate the possibility of choosing spots that are not representative of true differences in intensity or presence between the phases.

Table 1 lists the results of the MS analysis and the bioinformatics search of the selected protein spots of interest. It was difficult to estimate the molecular weight and pI

for each of the proteins selected; some did not match well with the MS-determined values. A correlation could not be determined for two of the spots, 11 and 16, since the results for spot 16 returned results of contamination with keratin. Also, one of the 'landmarks', spot 17 also returned keratin contamination results, however, the other three landmarks correlated well to one another and the expected results of a RACK-like protein, as noted in Chandler *et. al*. (2008).

With focus on the first 12 -24 hours of development, most of the interesting spots chosen in this experiment came from the pathogenic, 24-hour yeast phase, or the cultures that were switched after 12 hours from one temperature to another. Also, fewer spots were chosen from the 24-hour mold gel because they were absent from the profile in comparison to those from the other phase.

Table 2 below summarizes occurrence of three proteins with phase-related presence. Two of the four results returned for the 37-25°C sample are inconclusive for at mainly because the spots chosen were of very low intensity, which did not yield a MASCOT score worth considering valid (scores must be greater than 100). All of the numbers in parentheses were corresponding spots that did not yield reliable results from the MS analysis. The 'Species' column indicates the organism with the closest matching sequence to the fragments from MS for each protein.

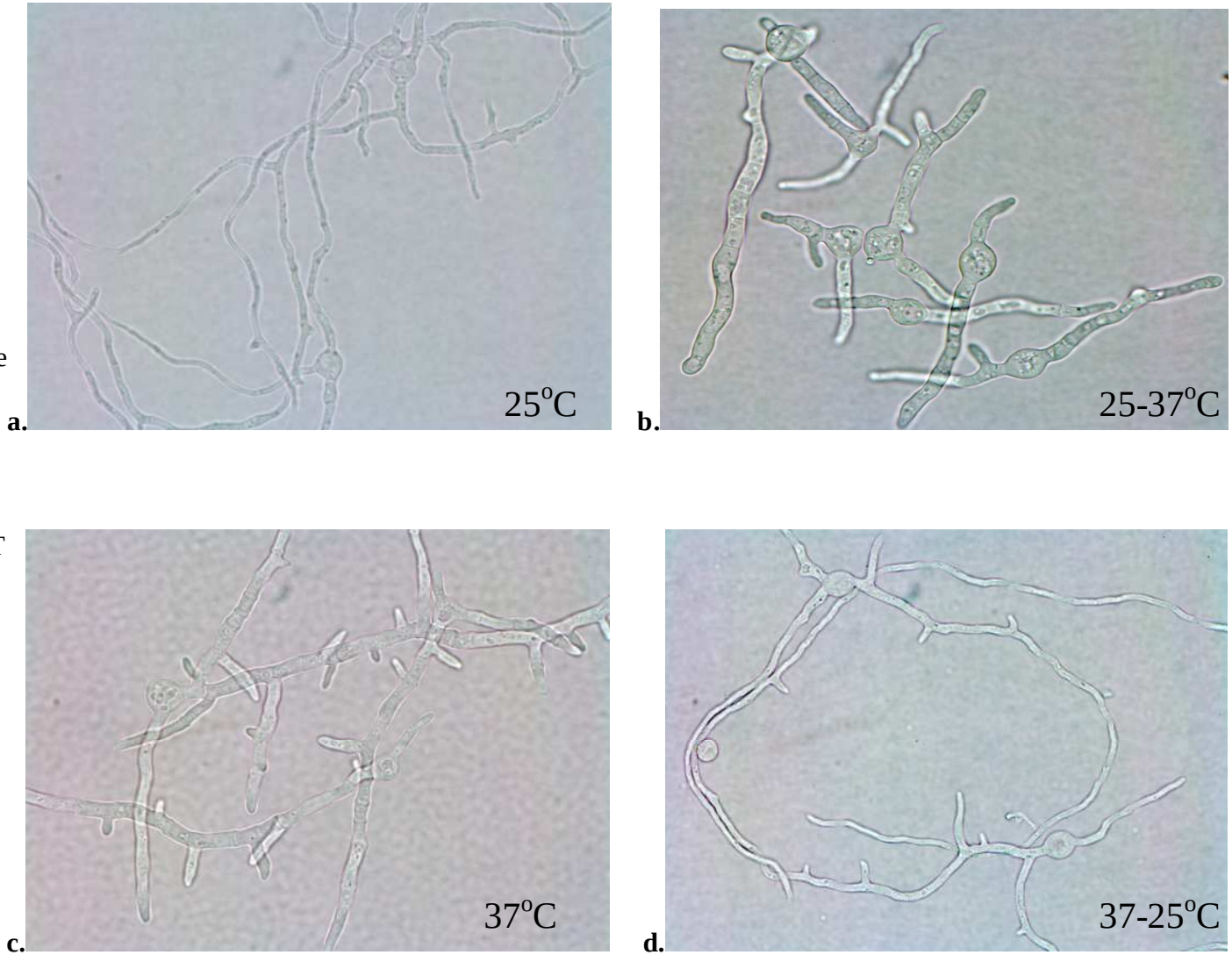

Figure 1. Images of cells from each phase at 100x magnification as taken with Olympus IX51 microscope and AdvancedSPOT software using Normanski settings.

Figure 2. F4 strain: (a) 24-hour, 25°C mold culture, (b) 12-hour 25°C switched to 37°C for 12 hours, (c), 12 hour 37°C-switched to 25°C for 12 hours and (d) 24-hour, 37°C yeast culture. Spots are colored and numbered to correlate to results in Table 1. The spots outlined with a circle represent the 'landmark' spots from each gel to serve as a reference for location. Spots in triangles represent the presence and up-regulation, or increased intensity with respect to the other images, while those in rectangular shapes indicate a down-regulated, or weaker presence.

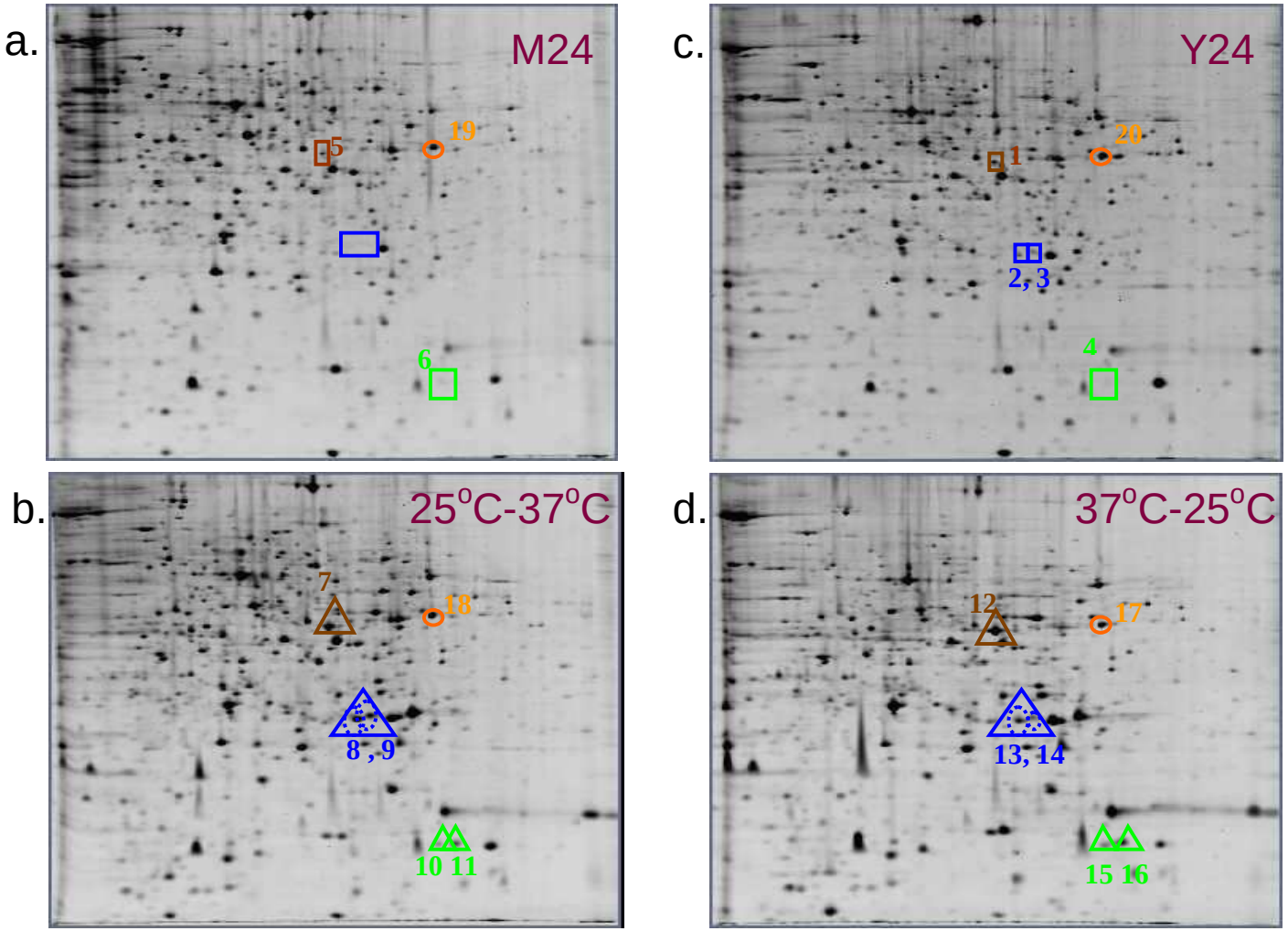
a.
M24
5
19
6
c.
Y24
1
20
2, 3
4
b.
25°C-37°C
7
18
8 , 9
10 11
d.
37°C-25°C
12
17
13, 14
15 16

Figure 2. Results of MS analysis of protein spots chosen from 2-DGE images.

Spot #/ Sample	UP or DOWN regulated *	Protein name	NCBI accession #	MS pI/ MW	Estimated pI/MW[a]	Species	MASCOT Score[b]	# peptides matched/ detected[c]	Protein Sequence	%SC[d]
1/ 37-25	Down	ATP phosphoribosyl-transferase	EAW19474	5.70/ 32.56	6.4/47	Neosartorya fischeri NRRL 181	212	4/8	R.LQQATLDLLSGSDIQFR.R R.ETRLDIALVK.N R.VDLGITGR.D R.YVLCQYNIPR.S	14
2/ 37-25	down, very weak	Ras-like GTP-binding protein; RAN-A	ABP87598		6.5/26	Penicillium marneffei	retrieved from blast info with low, but mildly acceptable e value			
3/ 37-25	down, very weak	glucose-6-phosphate isomerase	EAQ64230	5.98/ 60.02	6.6/26	Marinomonas sp. MED121	67	2/3	K.TLDSSTQALIK.A K.TLDSSTQALIKAFKLK.Q	2
4/ 37-25	Down, Very weak	putative DNA-directed RNA polymerase II subunit RPB11a	EAW19410	6.30/ 18.77	7/14	Neosartorya fischeri NRRL 181	84	2/2	K.FELRVQTDGEITPK.D R.VQTDGEITPK.D	8
5/ M24	down, very weak	aldehyde reductase	EAL60496	5.95/ 33.86	6.4/47	Dictyostelium discoideum AX4	71	2/2	K.REDVFITSK.L R.EDVFITSK.L	3
6/ M24	Down Very weak	hypothetical protein DEHA0D08822g	Q6BSL0	6.44/ 23.11	7/14	Debaryomyces hansenii CBS767	124	2/4	K.KYQDPSQADSIMK.V K.YQDPSQADSIMK.V	
7/ 25-37	up	hypothetical protein SNOG_02188	EAT90400	6.28/ 36.67	6.4/47	Phaeosphaeria nodorum SN15	109	3/8	R.HLDLAK.V K.REDVFITSK.L R.EDVFITSK.L	4
8/ 25-37	up	heat shock protein 30	ABF82266	6.17/ 20.74	6.5/26	Penicillium marneffei	452	6/20	R.LLDDYDLHR.S R.DGQTPASSSISSFAPR.F R.ESKDAYHLDGELPGIAQK.D K.DAYHLDGELPGIAQK.D R.SFNFPSR.V K.NGVLTVTVPK.A	40
9/ 25-37	up	heat shock protein 30	ABF82266	6.17/ 20.74	6.6/26	Penicillium marneffei	356	6/11	R.LLDDYDLHR.S R.ESKDAYHLDGELPGIAQK.D K.DAYHLDGELPGIAQK.D K.DVEIEFSDPQTLTIK.G R.SFNFPSR.V K.NGVLTVTVPK.A	31

Spot #/ Sample	UP or DOWN regulated *	Protein name	NCBI accession #	MS pI/ MW	Estimated pI/MW[a]	Species	MASCOT Score[b]	# peptides matched/ detected[c]	Protein Sequence	%SC[d]
10/ 25-37	sl. Up	hypothetical protein DEHA0D08822g	XP_458810	6.44/ 23.11	7/14	Debaryomyces hansenii CBS767	105	2/2	K.KYQDPSQADSIMK.V K.YQDPSQADSIMK.V	6
11/ 25-37	up	unnamed protein product	BAE60514	7.59/ 17.44	7.2/13	Aspergillus oryzae	62	1/3	R.QSGSTYSLVAVVPK.D	8
12/ Y24	up	hypothetical protein SNOG_02188	EAT90400	6.28/ 36.67	6.4/47	Phaeosphaeria nodorum SN15	112	3/5	R.HLDLAK.V K.REDVFITSK.L R.EDVFITSK.L	4
13/ Y24	sl. up	heat shock protein 30	ABF82266	6.17	6.5/26	Penicillium marneffei	361	6/17	R.SGDFAPLFR.L R.LLDDYDLHR.S K.DAYHLDGELPGIAQK.D K.DVEIEFSDPQTLTIK.G R.SFNFPSR.V K.NGVLTVTVPK.A	34
14/ Y24	weak	heat shock protein 30	ABF82266	6.17	6.6/26	Penicillium marneffei	399	8/16	R.SGDFAPLFR.L R.LLDDYDLHR.S R.DGQTPASSSISSFAPR.F K.DAYHLDGELPGIAQK.D K.DVEIEFSDPQTLTIK.G R.SVGEFHR.S R.SFNFPSR.V K.NGVLTVTVPK.A	47
15/ Y24	present, sl. up-regulated	hypothetical protein DEHA0D08822g	XP_458810	6.44	7/14	Debaryomyces hansenii CBS767	115	2/4	K.KYQDPSQADSIMK.V K.YQDPSQADSIMK.V	6
16/ Y24	present	*no valid results*			7.2/13					
17/ Y24	Landmark (E1, RACK)	*no valid results*			7/38					

Spot #/ Sample	UP or DOWN regulated *	Protein name	NCBI accession #	MS pI/ MW	Estimated pI/MW[a]	Species	MASCOT Score[b]	# peptides matched/ detected[c]	Protein Sequence	%SC[d]
18/ 25-37	Landmark (E1, RACK)	RACK1-like protein	AAN40696	6.59	7/38	Paracoccidioides brasiliensis	409	8/20	R.GTLEGHNGWVTSLATSLENPNMLLSASR.D R.LWELATGNTTR.T K.LWNTLGDCK.F K.FTITDK.G R.FSPNPQNPVIVSAGWDK.L K.DGTTMLWDLNESK.H K.SKVDELKPEYVEK.G K.VDELKPEYVEK.G	30
19/ M24	Landmark (E1, RACK)	RACK1-like protein	AAN40696	6.59	7/38	Paracoccidioides brasiliensis	520	9/19	R.LWELATGNTTR.T K.LWNTLGDCK.F K.FTITDK.G R.FSPNPQNPVIVSAGWDK.L R.IQTDHIGHTGYINTVTISPDGSLCASGGK.D K.DGTTMLWDLNESK.H K.HLYSLQAGDEIHALVFSPNR.Y K.SKVDELKPEYVEK.G K.VDELKPEYVEK.G	37
20/ 37-25	Landmark (E1, RACK)	RACK1-like protein	AAN40696	6.59	7/38	Paracoccidioides brasiliensis	419	8/15	R.GTLEGHNGWVTSLATSLENPNMLLSASR.D R.LWELATGNTTR.T, K.FTITDK.G, R.FSPNPQNPVIVSAGWDK.L, K.DGTTMLWDLNESK.H, K.HLYSLQAGDEIHALVFSPNR.Y, K.SKVDELKPEYVEK.G, K.VDELKPEYVEK.G	34

* - based on visualization of spot intensity in comparison with the images of the other phases.
[a] – based on horizontal and vertical location after 2-dimensional electrophoresis separation.
[b] – from bioinformatics database search protocol; scores $\geq$100 increase in reliability directly.
[c] – from bioinformatics database search results; describes how many different fragments and their frequency of homology.
[d] – %SC, percent sequence coverage of the detected fragment to the homologous sequence from the database search.

Table 2. Summary of protein presence in the four experimental phases.

	M24	**25°C-37°C**	**37°C-25°C**	**Y24**	**average MASCOT score**	**Species**
Synaptobrevin (4),6,10,(11),15	+	++	inconclusive	++	114	*D. hansenii*
Aldo-keto reductase (1) 7, 5, 12	+	++	inconclusive	++	97	*P. nodorum, D. discoideum*
Hsp30 (2,3),8,9,13,14	-	++	+	+	392	*P. marneffei*
Rack-1 (17)-20	P	P	P	P	449	*P. brasiliensis*

LEGEND:
+ visually present
++ up-regulated visual presence
- not present
P present in all at approximately the same intensity, used as a landmark

DISCUSSION

As previously stated, the protein spots in the 2-dimensional gels representing each phase-switch condition were chosen for MS analysis based in intensity and location as visually compared to one another. Although some of the proteins that corresponded to one another based on their location returned different NCBI accession numbers, organism originations or slightly different molecular weights or pI values, research into their function showed that some could indeed perform related functions. These somewhat scattered sequence sources can be the result of the fact that much of the genome of *Penicillium marneffei* has not yet been uploaded into sequence databases as of yet.

Of the spots chosen from the mold (25°C) and yeast (37°C) forms and the 25°C – 37°C many of the corresponding spots 'matched' one another in function, but differed in intensity depending on their phase. This is true for spots 6, 10, and 15, for example, which all represent a synaptobrevin protein from *Debaryomyces hansenii* CBS767. This spot appears down-regulated in the mold form compared to the yeast and 25°C – 37°C switch sample. Synaptrobrevins are small integral membrane proteins of secretory vesicles, and are a part of the SNARE proteins that are involved in membrane-bound vesicle trafficking within the cell (Chen and Scheller, 2001). These proteins may be important to the beginning stages of development in both the single-celled yeast and mycelial form, as germ tube formation and apical expansion, respectively, are active processes in growth. In both processes, vesicles containing chemical messengers and the building blocks for cellular expansion are released from their respective organelles with v-SNAREs (vesicle SNARE) and distributed to the target areas where t-SNAREs, or target SNAREs, like an address identifies where a letter is to be sent by post mail.

Understanding polarized cellular development such as this is of particular interest for dimorphic and pathogenic fungi as they grow and develop in both their host organism and their ecological niche. In the case of *P. marneffei*, the detection of an environmental temperature change initiates a cellular signaling cascade that will drive its genomic expression down one path (mycelial) or the other (yeast). The delivery of molecular components to the correct part of the cell to carry out the change in morphology is integral to this process.

Since timing is a key factor to the onset of infection for a virulent fungal spore, the efficiency and speed of cellular reproduction is also a priority. A study by Taheri-Talesh *et. al*., (2008) focused on the role of SYNA, a synaptobrevin homologue in the apical tip growth of *Aspergillus nidulans* (a close relative of *P. marneffei*). SYNA, and exocytic protein, was tagged with a fluorescent marker and tracked to the Spitzenkorper, plasma membrane, and cytoplasm. As it is used to target vesicles to the tip region, its structural components are suspected to be recycled again during endocytosis. They also describe other work that indicates that budding yeasts use a set of SNAREs that are mammalian homologues of synaptobrevin.

In this study, the indication that synaptobrevin-like proteins are down-regulated or less intense in the mycelial cultures than in the yeast or 25°C – 37°C switched sample is somewhat contradictory to the results from the *A. nidulans* study mentioned above. However, it could be that more of these proteins are involved in yeast budding or germ-tube formation than in hyphal, apical growth, thereby having more of a presence in the 2-dimensional gels representing the yeast or temperature-switch phases.

Another protein that reappeared in the sequence results for three of the four chosen corresponding spots was an aldo-keto reductase for spots 5, 7 and 12, representatives of the mold, 25-37°C phase switch, and yeast samples, respectively. Like in the previous protein-grouping example, it was the mold phase where this spot was down-regulated to a very weak presence in comparison to the other two experimental conditions. In actuality, spot number 5 (perceived as down-regulated and very weak from its mold form) was suggested to be an aldehyde reductase by alignment with a sequence from *Dictyostelium discoideum*, while spots 7 and 12 (which appeared up-regulated and more intense) were more directly correlated to an aldo-keto reductase from *Phaeosphaeria nodorum* SN15. Both reactions, however, involve reversible dehydration by a reductase, or oxidoreductase enzyme. An up-regulation of production of this enzyme could be important to adjust cellular metabolism at a raised environmental temperature, which is often described as 'stressing' the mycelial cells.

These observations fit with a previous report that stress conditions, such as a change in environmental temperature – or heat shock conditions - increase the presence of reductase-type enzymes in yeast cells of other dimorphic fungi such as *Coccidioides posadasii* (Delgado *et. al*., 2004). The authors note that in order to shift from a non-pathogenic morphological form to an infectious state, several different biochemical processes that make up what is known as a "heat shock response" must take place. These response processes often provide alternative pathways for carbohydrate metabolism or reduction of metabolites that may build up over time while the cell adapts to the temperature increase. In the case of *C. posadasii* – another dimorphic fungus - the discovery of an up-regulation of the gene *ALDR1* that codes for an aldehyde reductase is

specific to the pathogenic form – and a possible target for antifungal medication treatment in infected patients (Delgado *et. al*., 2004).

Three of the four spots chosen as landmarks – known spots in the same location and intensity in each gel – returned corroborating results as RACK1-like protein, containing repeats of a WD40 domain. RACK is an acronym for receptor-activated protein-C kinase (Yarwood *et. al.*, 1999). Its presence in all four phases is indicative of its importance - both as a "scaffold" or path-connector for cAMP signaling, and as a receptor for activated C-kinase (Yarwood *et. al.*, 1999). Not to be understated is the importance of cAMP in multiple signaling pathways, as it has been connected to the ability of *Paracoccidioides brasiliensis* to switch from mold to pathogenic yeast form (Chen *et. al.*, 2007). It follows, then that there appears only one RACK-1 like spot in the protein profile of the 24-hour mold sample, while there are more than one very near that same area in the profiles representing the three other growth conditions in this experiment. The location and consistent high-intensity of the RACK-1 like protein spot was also confirmed by Chandler *et. al.*, (2008). Its reliability in their studies for presence in both mould and yeast phases also resulted in its isolation and cloning from the *P. marneffei* genome by those authors.

Spots 8, 9, 13 and 14 all corresponded to reliable hits for a heat shock protein 30 (hsp30) from *P. marneffei*, one of the sequences already uploaded into the bioinformatics databases. However, only two of these spots were thought to correlate with one another in pairs. In Figure 2, the blue rectangle near the center of each gel image that contains two spots contains spots 8 and 9 (Fig. 2d, 25-37°C) and spots 13 and 14 (Fig. 2c, 37°C). It was originally thought that spots 8 and 13 would reveal a sequence for one protein,

while results for spots 9 and 14 would correlate for a different protein. The high-scoring protein sequence results, however, indicate that all four spots represent sequence fragments from hsp30. Therefore, one must overcome the assumption that a single spot correlates to a single protein, and begin to realize that a "single protein" can be represented by more than one "spot" in the two-dimensional profile. This could be due to the separation of structural or functional components of one protein, or posttranslational modifications to some quantity of the protein as its production is increased during a particular phase. The latter could be the case here, since the spots are in close proximity to one another in each profile, are completely absent from the mold phase, and yield different sequence results from the samples taken from the under-expressed spots in the 37°C -25°C sample. Two different polypeptides of the same size may form complexes, which may not completely dissociate during IEF. Thus, the two proteins may end up in the same spot.

It also follows that the two phase-change samples presented higher intensities of this spot over the mycelial stage, since an increase in temperature, or a sustained higher incubation temperature would require a larger concentration of heat shock proteins – or molecular chaperones – to be present to guide the newly formed heat shock response molecules into proper folding and structure. Vanittanakom *et. al.*, (2008) isolated and investigated the role of hsp30 in *P. marneffei*. In their study, the presence and level of expression of hsp30 increased significantly at a 37°C incubation temperature representing the yeast phase, and was nearly undetectable at the 25°C incubation temperature of the mould phase. The authors also were the first to infer the antigenic properties of the expression of hsp30, insinuating its role in the immune response of the *P. marneffei*-

infected host. These results corroborate the study by Vanittanakom, *et. al.* above – the total absence of an hsp30-protein spot in the mold phase, and a significant presence in the yeast and switched phases.

Since heat shock proteins (hsp) are molecular chaperones – guiding the folding of proteins during synthesis, it is not coincidental that more of these proteins will be necessary during a change in cellular morphology. Even when first characterized by Jakob *et. al.*, (1993), hsp's have been identified to be up-regulated growth and differentiation stages, and play an important role in reducing the aggregation of improperly folded proteins during synthesis, including the prevention of the formation of polymers during heat-shock conditions.

This research is unique in its phase-switch induction and subsequent isolation of proteins for sequence analysis and identification. Previously published work by Xi *et. al.* (2007), also attempted to elucidate differentially expressed proteins in *P. marneffei* by using two-dimensional gel electrophoresis similar to this study. These authors used fluorescent protein tagging and mixed the proteins on one gel, instead of the phase-switch design presented in this work. Their study lead to identification of 26 proteins out of the same gel by mass fingerprinting mass spectrometry, whereas the MS data presented here were based on capillary-liquid chromatography-nanospray tandem mass spectrometry of spots chosen from visually comparing separate protein profiles of each phase or phase-switch. Proteins from the heat-shock family, as well as a catalase, a glyoxylate bypass, and transcription and energy/metabolism related proteins seemed to stand out as different between the two phases, as well as several hypothetical proteins from sequence data from related species (Xi *et. al.*, 2007). Corroborations can be found between this study and

that of Xi *et. al.* (2007) in the apparent up-regulation of heat shock proteins and those in a carbohydrate bypass system (aldo-keto reductase, perhaps), even while considering the lack of annotation of the *P. marneffei* genome online, along with the subtle differences in culturing, two-dimensional analysis, and mass spectrometric sequence attainment techniques.

Further Research Considerations

Bioinformatic database searches of the protein sequences obtained from mass spectrometry analysis in this study may lead to identification of a particular gene responsible for the production of the protein of interest. With the development of primers for that specific gene, amplification by the polymerase chain reaction (PCR) can be carried out to clone and further analyze the genetic involvement of the biochemical process. Insight into the function of this gene and its relation to the role of fungal dimorphism will allow for the production of antibodies for clinical screening, a target for drug design, and the possibility of removing the threat of penicilliosis as a cause of mortality for infected or immunocompromised individuals.

It would also be interesting to do another level of comparison with the 12-hour 2-DGE profiles for both the mold and yeast phases. This step was initially a part of the methods for this study, but there was not enough incubator space in the laboratory to accommodate the number of flasks of SDB cultures necessary to get a cell pellet of acceptable mass for protein isolation.

Another perspective that could be considered is to culture the 37°C samples in brain-heart infusion (BHI) medium instead of using SDB. It has been found that this medium contains the 1% peptone necessary for optimal yeast growth, and has been used

by several other studies (Liu *et. al.*, 2007; Xi *et. al.*,2007; Kummasook *et. al.*, 2007) to compare protein profiles of mold and yeast phases of the same organism.

Moreover, this work opens up doors for future research in the areas of isolation and cloning of the differentially expressed genes and their protein products, as well as broader or narrower time frames for phase-switch protein isolation and characterization through two-dimensional gel electrophoresis techniques. This research is important not only to *P. marneffei*, but for other dimorphic fungal species, as researchers are just beginning to piece together the biochemical mechanisms involved in triggering, controlling and directing cellular processes during the switch between pathogenic and non-pathogenic forms. One option is to look at the differential expression of genes between the mold and yeast phase by suppression subtractive hybridization, as Liu *et. al.*, (2007) did. This technique could be focused on the gene-sources of the proteins identified in this study, since it focuses on the creation of subtracted cDNA libraries to identify "differentially expressed genes in response to an environmental stimulus" (Liu *et. al.*, 2007) – such as forced temperature change (and therefore morphological phase change) during early, mid- and late-stage development.

APPENDIX

Reagent Recipes, In Order of Use

TE Buffer, 1L:
10 ml 1M TRIS (pH 8.0) and 2 ml 0.5M EDTA (pH 8.0).
May be autoclaved.

Lysis Buffer:
20 mM Tris-HCl
pH 7.6
10 mM NaCl
0.5mM sodium deoxycholate
40 µl/ml of protease inhibitor cocktail (pic)

Modified Sample Buffer (MSB):
2 M thiourea
7 M urea
4% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate(CHAPS)
1% dithiothreitol (DTT)
2% [v/v] carrier ampholytes, pH 3–10

2DE Buffer:
8.4 M urea
2.4 M thiourea
5% CHAPS
25 mM spermine base
50 mM DTT

Bradford Dye:
0.01% Coomassie Brilliant Blue G-250
8.5% [v/v] phosphoric acid

Rehydration Buffer:
8 M urea
1% CHAPS
15 mM DTT
0.2% [v/v] BioLytes
0.001% bromophenol blue

Equilibration Buffer I:
6 M urea
2% sodium dodecyl sulfate (SDS)
0.375 M Tris-HCl pH 8.8
20% [v/v] glycerol
130 mM DTT

Equilibration Buffer II:
6 M urea
2% SDS
0.375 M Tris-HCl pH 8.8
20% [v/v] glycerol
135 mM iodoacetamide

TRIS-Glycine-SDS (TGS) Buffer:
25 mM Tris base
192 mM glycine
0.1% SDS

Polyacrylamide Gel, 12% *1000 ml*:
430 ml ddH_2O
300 ml 40% acrylamide
250 ml 1.5M TRIS, pH 8.8
10 ml 10% SDS
10 ml 10% ammonium persulfate ($(NH_4)_2S_2O_8$)
0.4 ml TEMED

Overlay Agarose:
0.5% low melt agarose in TGS buffer with 0.001% bromophenol blue

Gel Fixing Solution:
40% methanol and 10%acetic acid, v/v

Gel Fixing Solution for MS Analysis:
50% ethanol and 10% acetic acid, v/v

Gel Washing Solution for MS Analysis:
50% methanol and 10% acetic acid, v/v

Gel Storage Solution for MS Analysis:
5% [v/v] acetic acid

Literature Cited

Andrianopoulos, A. 2002. Control of morphogenesis in the human fungal pathogen *Penicillium marneffei*. International Journal of Medical Microbiology. **292:**331-347.

Andrianopoulos, A. and S. Zuber. 2006. Signaling Pathways in the Dimorphic Human Fungal Pathogen *Penicillium marneffei*, p. 441-454. *In* Heitman *et. al*., (eds.), Molecular Principles of Fungal Pathogenesis. ASM Press, Washington, D.C.

BioRad ReadyStrip™ IPG Strip Instruction Manual.

Borneman, A., M. Hynes and A. Andrianopoulos. 2000. The *abaA* homologue of *Penicillium marneffei* participates in two developmental programmes: conidiation and dimorphic growth. Molecular Microbiology. **38**(5):1034-104.

Bradford, M. 1976. A Rapid Sensitive Method for the Quantitation of microgram Quantities f Protein Utilizing the Principle of Protein-Dye Binding. Analytical Biochemistry. **72**:248-254.

Cao, C., R. Li, Z. Wan, W. Liu, X. Wang, J. Qiao, D. Wang, G. Bulmer, G and R. Calderone. 2007. The effects of temperature, pH and salinity on the growth and dimorphism of *Penicillium marneffei*. Medical Mycology. **45:**401-407.

Chandler, J. M., E. R. Treece, H. R. Trenary, J. L. Brenneman, T. J. Flickner, J. L. Frommelt, Z. M. Oo, M. M. Patterson, W. T. Rundle, O. V. Valle, T. D. Kim, G. R. Walker and C. R. Cooper Jr. 2008. Protein Profiling of the dimorphic, pathogenic fungus, *Penicillium marneffei*. Proteome Science. **(6)17:**1-18.

Chen, D., T. K. Janganan, G. Chen, E. R. Marques, M. R. Kress, G. H. Goldman, A. R. Walmsley and M. I. Borges-Walmsley. 2007. The cAMP pathway is important for controlling the morphological switch to the pathogenic yeast form of *Paracoccidioides brasiliensis*. Molecular Microbiology. **65(3):**761-779.

Chen, Y. A. and R. H. Scheller. 2001. Snare-Mediated Membrane Fusion. Nature Reviews of Molecular and Cellular Biology. **2:**98-106.

Cooper, C. R., Jr., and N. Haycocks. 2000. *Penicillium marneffei*: An Insurgent Species among the Penicillia. Journal of Eukaryotic Microbiology. **47:**24-28.

Cooper, C. R. Jr. and M. McGinnis. 1997. Pathology of *Penicillium marneffei*: An Emerging Acquired Immunodeficiency Syndrome-Related Pathogen. Archives of Pathological Laboratory Medicine. **121:**798-804.

Deacon, J. 2006. Fungal Biology, pgs 1-141, 159-182, 322-337. Blackwell Publishing, Malden, Massachusetts.

Delgado, N., C. Y. Hung, E. Tarcha, M. J. Gardner, and G. T. Cole. 2004. Profiling gene expression in *Coccidioides posadasii*. Medical Mycology. **42:**59-71.

Gugnani, H. C., A. Paliwal-Joshi, H. Rahman, A. A. Padhye, T. S. K. Singh, T. K. Das, B. Khanal, R. Bajaj, S. Rao and R. Chkhani. 2007. Occurrence of pathogenic fungi in soil burrows of rats and other sites in bamboo plantations in India and Nepal. Mycoses. **50:**507-511.

Gugnani, H., M. C. Fisher, A. Paliwal-Joshi, N. Vanittanakom, I. Singh and P. S. Yadav. 2004. Role of *Cannomys badius* as a Natural Animal Host of *Penicillium marneffei* in India. Journal of Clinical Microbiology. **42(11):**5070-5075.

Jakob, U., M. Gaestel, K. Engel and J. Buchner. 1993. Small Heat Shock Proteins Are Molecular Chaperones. The Journal of Biological Chemistry. **268(3):**1517-1520.

Kaufman, L. 1993. Significance of Morphologic Form in Producing Immunodiagnostic Antigens for Dimorphic Fungal Pathogens, p. 319-331. *In* Vanden Bossche, H. *et. al.* (eds.), Dimorphic Fungi in Biology and Medicine. Plenum Press, NY.

Kerridge, D. 1993. Fungal Dimorphism: A Sideways Look, p. 3-10. *In* Vanden Bossche, H. *et. al.*, (eds.), Dimorphic Fungi in Biology and Medicine. Plenum Press, new York, NY.

Kudeken, N., K. Kawakami and A. Saito. 1999. Cytokine-induced fungicidal activity of human polymorphonuclear leukocytes against *Penicillium marneffei*. FEMS Immunology and Medical Microbiology. **26:**115-124.

Kummasook, A., P. Pongpom and N. Vanittanakom. 2007. Cloning, characterization and differential expression of an *hsp70* gene from the pathogenic dimorphic fungus, *Penicillium marneffei*. DNA Sequence. **18(5):**385-394.

Liu, H., L. Xi, J. Zhang, X. Li, X. Liu, C. Lu and J. Sun. 2007. Identifying differentially expressed genes in the dimorphic fungus *Penicillium marneffei* by suppression subtractive hybridization. FEMS Microbiological Letters. **270:**97-103.

Michael, J. S., O. C. Abraham, D. Mathai and M. S. Mathews. 2004. Varied clinical manifestations of *Penicillium marneffei* in patients with human immunodeficiency virus: a report from south India. Mycoses. **48:**120-121.

Nguyen, K., S. Taylor, A. Wagner, A. Ali and R. Rapini. 2006. A case of *Penicillium marneffei* in a US hospital. Journal of the American Academy of Dermatology Letters. **54(4):**730-732.

PDQuest 2-D analysis Software User Guide. BioRad.

Pongpom, P., C. R. Cooper, Jr. and N. Vanittanakom. 2005. Isolation and Characterization of a catalase-peroxidase gene from the pathogenic fungus, *Penicillium marneffei*. Medical Mycology. **43:**403-411.

Pryce-Miller, E., D. Aanensen, N. Vanittanakom and M. C. Fischer. 2008. Environmental detection of *Penicillium marneffei* and growth in soil mocrocosms in competition with *Talaromyces stipitatus*. Fungal Ecology. **I:**49-56.

Rooney, P. J. and B. S. Klein. 2002. Linking fungal morphogenesis with virulence. Cellular Microbiology. **4(3):**127-137.

Supparatpinyo, K., K. E. Nelson, W. G. Merz, B. J. Breslin, Cooper , C, R, Jr., C. Kamwan and T. Sirisanthana. 1993. Response to antifungal therapy by human immunodeficiency virus-infected patients with disseminated *Penicillium marneffei* infections and in vitro susceptibilities of isolates from clinical specimens. Antimicrobial Agents & Chemotherapy. **37:**2407-2411.

Taheri-Talesh, N., T. Horio, L. Araujo-Bazán, X. Dou, E. A. Espeso, M. A. Peñalva, S. A. Osmani and B. R. Oakley. 2008. The Tip Growth Apparatus of *Aspergillus nidulans*. Molecular Biology of the Cell. **19:**1439-1449.

Twyman, R. M. 2004. Principles of Proteomics, p. 1-102, 217-235. BIOS Scientific Publishers, New York, NY.

Ungpakorn, R. 2005. Mycoses in Thailand: Current Concerns. Japanese Journal of Medical Mycology. **46:**81-86.

Ustianowski, A.P., T. P. M. Sieu and J. N. Day. 2008. *Penicillium marneffei* infection in HIV. Current Opinion in Infectious Diseases. **21:**31-36.

Van Cutsem, J., F. Van Gerven and J. Fransen. 1993. Animal Models of Mycoses Caused by Fungi that Produce Yeast- and Hyphal Forms, p. 341-360, 341-344, 354-355. *In* Vanden Bossche, H. *et. al.*, (eds.), Dimorphic Fungi in Biology and Medicine. Plenum Press, New York, NY.

Vanittanakom, N., C. R. Cooper, Jr., M. C. Fisher and T. Sirisanthana. 2006. *Penicillium marneffei* Infection and Recent Advances in the Epidemiology and Molecular Biology Aspects. Clinical Microbiology Reviews. **19:**95-110.

Vanittanakom, N., M. Pongpom, J. Praparattanapam, C. R. Cooper, Jr. and T. Sirisanthana. 2008. Isolation and expression of heat shock protein 30 gene from *Penicillium marneffei*. Medical Mycology. (in press).

Viviani, M. A., J. O. Hill and D. M. Dixon. 1993. *Penicillium marneffei*: Dimorphism and Treatment, p. 413-422. *In* Vanden Bossche, H. *et. al.*, (eds.), Dimorphic Fungi in Biology and Medicine Plenum Press, New York, NY.

Wantabe, H., N. Asoh, S. Kobayashi, K. Wantabe, K. Oishi, W. Kositsakulchai, T. Sanchai, B. Khantawa, P. Tharavichitkul, T. Sirisanthana and T. Nagatake. 2008. Clinical and microbiological characteristics of community-accquired pneumonia among human immunodeficiency virus-infected patients in northern Thailand. Journal of Infection and Chemotherapy. **14:**105-109.

Xi, L., X. Xu, W. Liu, X. Li, Y. Liu, M. Li, J. Zhang and M. Li. 2007. Differentially expressed proteins of pathogenic *Penicillium marneffei* in yeast and mycelial phase. Journal of Medical Microbiology. **56:**298-304.

Yarwood, S. J., M. J. Steele, G. Scotland, M. D. Houslay and G. B. Bolger. 1999. The RACK1 signaling scaffold protein selectively interacts with the cAMP-specific phosphodiesterase PDE4D5 isoform. The Journal of Biological Chemistry**.** **274:**14909-14917.